Table 2. Recovery tests of organophosphorus pesticides from fruits and vegetables by acetonitrile extraction

Pesticide tested	fortified (μg/g)	Average recovery, % (RSD)			
		apple extract	cucumber extract	tomato extract	strawberry extract
Chlorpyrifos	0.01	85.7 (4.48)	85.7 (4.09)	82.1 (1.3)	94.5 (7.3)
Diazinon	0.01	85.1(2.64)	83.5 (6.72)	91.3 (3.84)	95.5 (3.9)
Fenitrothion	0.01	98.8 (15.3)	88.4 (6.79)	89.9 (1.47)	89.3 (7.1)
Isoxathion	0.01	102 (22.9)	92.5 (22)	84.3 (5.42)	99.5 (12.2)
Benthiocarb	0.1	87.1 (3.58)	89.2 (4.02)	88.5 (5.05)	92.6 (6.9)
Carbaryl	0.1	90.5 (6.75)	88.2 (5.9)	89.2 (7.51)	95.3 (10.2)
Fenocarb	0.1	91.8 (3.5)	90.5 (8.01)	84.8 (4.05)	98.5 (9.8)
Isoprocarb	0.1	86.2(10.2)	85.1 (3.09)	89.4 (5.02)	90.8 (6.9)

RSD = Relative standard deviation.

Table 3. Chlorine consumption and residual chlorine within fruit and vegetable extracts (acetonitrile) at 25°C for 15 min

Chlorine dose to the extract (mg/l)	Chlorine-consumed and remained in the extracts (as HOCl, mg/l)							
	Apple		Cucumber		Tomato		Strawberry	
	consumed	remained	consumed	remained	consumed	remained	consumed	remained
5	3.4	0	5	0	5	0	5	0
12.5	10.5	1.5	12.5	0	12.5	0	10	0
25	21.2	3.8	24.5	0.5	23.4	1.6	25	0
50	46.7	3.3	47.6	2.4	46.5	3.5	50	0
100	93	7	91.4	8.6	90	10	100	0
200	183.1	16.9	177	23	179.5	20.5	200	0
350	321.1	28.9	315	35	315	35	349	1
500	456.9	43.1	439	61	443	57	495	5
750	679.7	70.3	664	86	651	99	731	19
1000	900	100	890	110	885	115	963	37
2000	1831	169	1770	230	1795	205	1940	60

Chlorine consumptions of acetonitrile extract containing different matrices and ChE inhibition assays

We have confirmed recovery rates for pesticides from fruit and vegetable samples by acetonitrile extraction and clean-up procedure (Table 2). It is also important to discern how the distribution of oxidation products varies with experimental conditions, such as hypochlorite oxidation and ChE-inhibiting assays. Our previous papers (Onodera *et al.*, 1992; Kanno *et al.*, 2012) revealed that excess hypochlorite in water at pH 7.0 oxidised the P=S pesticides stoichiometrically within 5 min into their P=O analogues. The resulting products were comparatively stable in water at low concentrations of active chlorine (less than 100 mg/l) in an acidic solution of pH 6.0. In addition, greater ChE-inhibiting activities (I_{50} = 0.004-0.1 μM) were also found for the oxidative compounds formed by the hypochlorite oxidations of diazinon, ethion, isoxathion, parathion, cumafos, phorate, azinfos-methyl, and runnel in water.

Table 3 summarises the chlorine consumptions by the acetonitrile extracts containing fruit and vegetable matrices and the residual chlorine concentrations in these extracts after exposure to various chlorine doses at 25°C for 15 min. As shown in the table, the chlorine consumptions depended strongly on the species of fruit or vegetable examined and the concentrations of active chlorine exposed. At the chlorine dose of 100 mg/l (in the form of HOCl), extracts of apple-, cucumber-, tomato-, and strawberry consumed 93, 91.4, 90, and 100% of the total amount of chlorine added, respectively, and they showed a very low concentration of residual chlorine (less than 10 mg/l). The largest consumption of active chlorine and lowest remaining residual chlorine were observed for strawberry extract, even at a high chlorine dose of 500 mg/l. These chlorine consumptions in Table 3 reflect the reductive powers of fruit and vegetable extracts containing several reducing reagents, such as sugars, hydrocarbons, metal ions and Vitamin C. It is known that the commercial strawberry, tomato, cucumber, and apple contain 80, 20, 13, and 3 mg/100 g of vitamin C, respectively (Standard Tables of FOOD Composition in Japan, 2010). On the other hand, Kim *et al.* (2000) reported that treatment of nine organophosphorus pesticides with excess bromine in organic solvent (pure acetonitrile) is a rapid and efficient method of achieving their oxidation prior to the ChE-inhibition assays. However, the previous study lacked information on the oxidation conditions in the presence of different matrices in fruit and vegetable extracts and the chemical fate of oxons in non-aqueous solutions.

Table 4 presents the inhibition 50% (I_{50}) and 20% (I_{20}) values towards ChE of the horse serum observed for chlorpyrifos, diazinon, fenitrothion, and isoxathion in fruit and vegetable extracts before and after solid phase clean-up using ENV™-Carb column and following oxidation with hypochlorite with a chlorine dose of 1,000 mg/l (as active Cl) and at 25°C for 15 min. The I_{50} and I_{20} values of individual chlorinated acetonitrile extracts calculated from these ChE-inhibiting curves were in agreement with those observed for aqueous pesticide solutions after treatment with hypochlorite, as shown in Fig. 2. The GC-MS determinations also indicated that exposure to high chlorine doses oxidised the P=S pesticides within a short time into their P=O analogues (excepted for the isozathion products), and the resulting products were comparative-

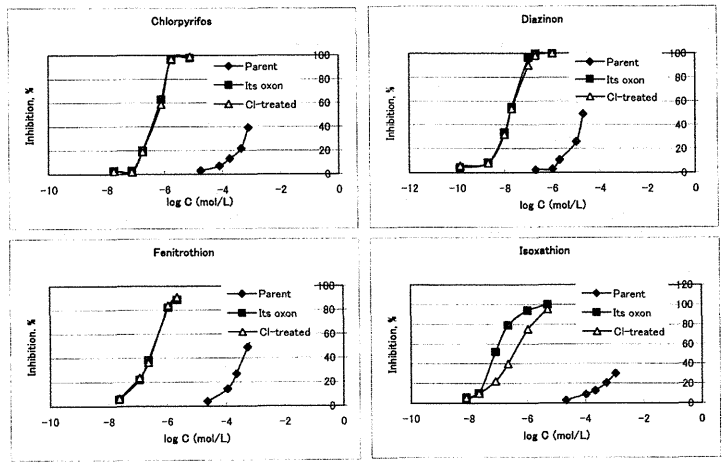

Fig. 2. Plot of ChE-inhibition activity vs. concentration of organophosphorus pesticides before and after hypochlorite oxidation in phosphate buffer solution. Each pesticide solution (pH 7.0) was treated with excess hypochlorite (final concentration, 100 mg/l) at 25°C for 15 min. The mixture of the enzyme solution and pesticide solution was incubated at 37°C for 30 min, after which the anti-ChE activity was assessed. The mean of the inhibition (%) obtained in three test runs was plotted.

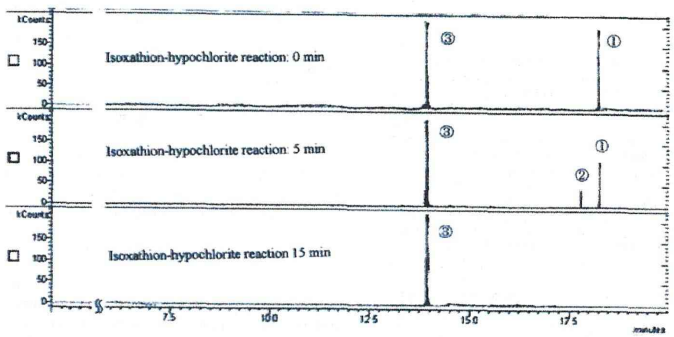

Fig. 3. Example of gas chromatograms (total ion currents) of the ethyl acetate extracts from the isoxathion-hypochlorite reactions in phosphate buffer solution of pH 7.0 and hypochlorite concentration of 100 mg/l. ① = isoxathion; ② = isoxathion-oxon; and ③ = phenathrene-d10 as surrogate compound.

Table 4. The inhibition 50% (I_{50}) and 20% (I_{20}) values of organophosphorus pesticides in acetonitrile extracts of fruit and vegetable samples, before and after clean-upped by the Standard method in Japan and following the hypochlorite-oxidation.

Pesticides tested (fortified)	I_{50}/I_{20} values (ug/g)*1 of chlorinated acetonitrile extracts							
	Apple		Cucumber		Tomato		Strawverry	
	original	clean-upped	original	clean-upped	original	clean-upped	original	clean-upped
Chlorpyrifos (0.2 μg/g)	0.14/0.04	0.15/0.04	0.15/0.04	0.14/0.04	0.13/0.04	0.14/0.04	0.13/0.04	0.13/0.04
Diazinon (0.1 μg/g)	0.005/0.001	0.004/0.001	0.004/0.001	0.005/0.001	0.004/0.001	0.005/0.001	0.006/0.001	0.005/0.001
Fenitrothion (0.2 μg/g)	0.13/0.04	0.13/0.004	0.14/0.04	0.14/0.04	0.14/0.04	0.15/0.04	0.16/0.04	0.16/0.04
Isoxathion (0.2 μg/g)	0.03/0.01	0.03/0.01	0.03/0.01	0.04/0.01	0.03/0.01	0.03/0.01	0.03/0.01	0.03/0.01

*1 These values indicate the average of three test runs, calculated from each of their ChE-inhibition curves.

ly stable in the chlorinated fruit and vegetable extracts. Therefore, the different matrices in the fruit and vegetable extracts did not interfere with the hypochlorite oxidations of organophosphates and their ChE-inhibiting assays.

ChE inhibition assays for organophosphorus pesticide combinations

A number of organophosphate mixtures, and pesticide and carbamate mixtures such as a combination of isoxathion-DDVP, acephate-NAC, and so on are often utilised to control insects and pests worldwide. Where a mixture of pesticides is present, the inhibition of AChE represents the total cholinesterase activity. However, it is not clear whether a mixture of pesticides causes a greater inhibition effect than the sum of its individual effects, (i.e., a synergistic effect). Fig. 4 shows the influence of DDVP as

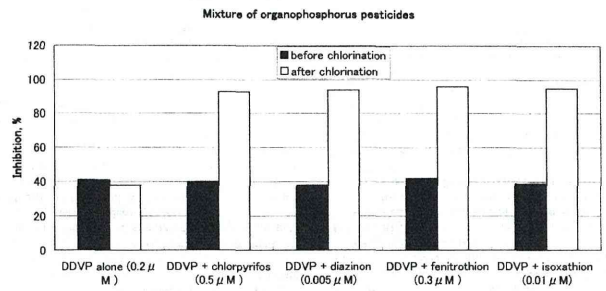

Fig. 4. Influence of coexisting DDVP on the formation of organophosphorus compounds (P=O analogues) during organothiophosphorus (P=S) pesticides–hypochlorite reactions. Each P=S pesticide and DDVP mixture, having the ability to inhibit 40% of horse serum cholinesterase before chlorination, was treated with hypochlorite (100 mg active Cl/l) for 15 min at 25°C for subsequent ChE assay.

a typical P=O pesticide on the P=S compound-hypochlorite reactions and following the ChE inhibition assay. A certain amount of each P=S compound exhibiting I_{50} in the ChE activity, was examined using the DDVP solution (0.2 μM). The presence of DDVP did not interfere with P=O compound formation in either the hypochlorite oxidation or the subsequent enzymatic assays. In addition, the inhibitory effects of a combination of DDVP and several P=S pesticides were found to be additive, although some pesticides are known to be potentiators or antagonists of the toxicity of other organophosphates *in vitro* (Elhalwagy and Zaki, 2009). Previously, Richardson *et al.* (2001) and Tahara *et al.* (2005) evaluated the *in vitro* interaction between two or three combinations of organophosphates on ChE activity. When two or three mixtures were added simultaneously to brain tissue *in vitro*, they had an additive effect.

On the basis of the above-mentioned fundamental studies, we calculated the degree of enhancement of anti-ChE activities (I_{50}) and detection limits (I_{20}) for each solution of chlorinated pesticides (1,000 mg/l of active chlorine at 25°C for 15 min, followed by the incubation with ChE of horse serum at 37°C for 30 min). Higher anti-ChE activity (I_{50} = 0.006 and 0.03 μg/g) was found for the P=S compounds, such as diazinon and isoxathion, when these pesticides were treated with hypochlorite in fruit and vegetable extracts. Moderate anti-ChE activity (I_{50} = 0.15 and 0.13 μg/g) was also observed for the P=S compounds, such as chloropyrifos and fenitrothion, after chlorination. These I_{50} values and the detection limits (I_{20}) presented in Table 4 are not able to achieve the sensitivity of traditional chromatographic methods (detection limits: lower than 0.001 μg/g) . However, the combination method of ChE assay and hypochlorite oxidation in acetonitrile extracts may be useful as a screening tool which could allow the screening of hundreds of samples in a short period of time and a lower cost. In addition, anti-ChE activity can serve as a "toxicological index", a measure of the toxicity of sample.

Application to real fruit and vegetable samples

The combination method of ChE assay and hypochlorite oxidation was used to monitor the inhibitors present in environmental samples, according to previous papers (Onodera *et al.*, 1992; Kawakami *et al.*, 2008; Kanno *et al.*, 2012). The organic concentrates obtained from fruit and vegetable samples that had been spiked with organophosphorus pesticides, followed by hypochlorite oxidation (1,000 mg active Cl/l), were tested at three doses for their ChE-inhibiting activity. Dose–response relationships between sample volume and ChE-inhibiting activity were clearly apparent (Table 5). Substantial ChE-inhibiting activity was observed in the chlorinated fruit and vegetable extracts, compared with the lower level of activity observed in the non-chlorinated concentrates. Thus, the ChE inhibitors were definitely recovered from the sam-

Table 5. Determination of organophosphorus pesticide in fruirt and vegetable samples before and after spiked with pesticide by ChE-inhibition assays, after extrsction with acetoritrile and hypochlorite oxidation.

Pesticide	fortified		Apple		Cucumber		Tomato		Strawberry	
tested	ng/g		inhibition %	S.D.	inhibition %	S.D.	inhibition %	S.D.	inhibition %	S.D.
Diazinon	0	(original)	ND*		ND		ND		ND	
	3.13		25.4	5.02	28.8	7.1	25.8	2.01	23.8	11.1
	6.25		46.2	8.57	45.1	11.6	43.5	7.15	44.3	5.8
	31.3		85.4	8.97	76.2	10.6	82.5	3.64	79.9	12.6
Fenitrotion	0	(original)	ND		ND		ND		ND	
	31.2		11.8	3.3	6.44	2.58	2.1	1.8	5.5	5.8
	62.5		23.2	4.16	11.5	9.05	18.5	1.58	15.3	3.55
	313		71.6	5.45	49.2	1.59	62.4	3.32	62.1	8.09
Chloropyrifos	0	(original)	ND		ND		ND		ND	
	156		34.8	6.38	47.5	18.6	45.8	29.7	33.6	10.8
	313		59.8	7.67	106	12.3	53.5	34.2	60.1	5.9
	625		89.4	8.12	115	13.5	85	7.16	85.8	9.25
Isoxathion	0	(original)	ND		ND		ND		ND	
	6.25		16.7	2.29	13.3	16.2	24.2	12	18.9	14.5
	31.3		53.6	6.33	53.6	9.75	51.6	6.98	49.9	7.4
	62.5		88.3	9.25	84.8	5.9	63.9	23.5	86.8	11.8

* ND = Not detected.

ples by acetritrile extraction and underwent the transformation of P=S pesticides into P=O compounds by hypochlorite oxidation. Several insecticides such as chlorpyrifos, DDVP, diazinon, fenitrotion, and isoxathion were not detected at all or were detected in trace amounts in the raw fruits and vegetables collected from the supermarket.

ACKNOWLEDGMENTS

The authors wish to thank all members of the Environmental Science Laboratory, Faculty of Pharmaceutical Sciences, Tokyo University of Science, for their help and support during this study.

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—Regular Article—

有害物質含有家庭用品規制法で規制されている繊維製品中のトリス（2,3-ジブロムプロピル）ホスフェイト分析法の改定に向けた検討

味村真弓,*,[a] 中島晴信,[a,†] 吉田　仁,[a] 吉田俊明,[a] 河上強志,[b] 伊佐間和郎[b]

Study for the Revision of Analytical Method for Tris (2,3-dibromopropyl) phosphate with Restriction in Textiles

Mayumi Mimura,*,[a] Harunobu Nakashima,[a,†] Jin Yoshida,[a] Toshiaki Yoshida,[a] Tsuyoshi Kawakami,[b] and Kazuo Isama[b]

[a]*Osaka Prefectural Institute of Public Health; 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan: and* [b]*National Institute of Health Sciences; 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.*

(Received June 4, 2013; Accepted October 1, 2013)

The official analytical method for tris (2,3-dibromopropyl) phosphate (TDBPP), which is banned from use in textile products by the "Act on Control of Household Products Containing Harmful Substances", requires revision. This study examined an analytical method for TDBPP by GC/MS using a capillary column. Thermal decomposition of TDBPP was observed by GC/MS measurement using capillary column, unlike in the case of gas chromatography/flame photometric detector (GC/FPD) measurement based on a direct injection method using a capillary megabore column. A quadratic curve, $Y=2572X^{1.416}$, was obtained for the calibration curve of GC/FPD in the concentration range 2.0–100 μg/mL. The detection limit was 1.0 μg/mL under S/N=3. The reproducibility for repetitive injections was satisfactory. A pretreatment method was established using methanol extraction, followed by liquid-liquid partition and purification with a florisil cartridge column. The recovery rate of this method was ～100%. TDBPP was not detected in any of the five commercial products that this study analyzed. To understand the cause of TDBPP decomposition during GC/MS (electron ionization; EI) measurement using capillary column, GC/MS (chemical ionization; CI), GC/FPD, and gas chromatography/flame ionization detector (GC/FID) measurements were conducted. It was suggested that TDBPP might thermally decompose both during GC injection, especially through a splitless injection method, and in the column or ion sources. To attempt GC/MS measurement, an injection part comprising quartz liner was used and the column length was halved (15 m); thus, only one peak could be obtained.

Key words——tris (2,3-dibromopropyl) phosphate; organophosphate flame retardant; GC/MS; gas chromatography/flame photometric detector; textile; household product

緒　　言

トリス（2,3-ジブロムプロピル）ホスフェイト（TDBPP）は，セルローズ（繊維素），トリアセテート及びポリエステル生地の難燃剤として使用されてきたが，[1] ラット及びマウスによる動物実験で発がん作用を示すことが明らかになった。[2-4] そこで，TDBPPは，欧州の数ヵ国，米国，日本などにおいて使用禁止となった．さらに，International Agency for Research on Cancer（IARC，国際がん研究機関）は，1987年にTDBPPを発がん物質分類のグループ2A（ヒトに対しておそらく発がん性を示す：probably carcinogenic to humans）に分類した。[5]

日本では，1978年に「有害物質を含有する家庭用品の規制に関する法律」（家庭用品法）により，繊維製品にTDBPPを使用することが禁止され，公定法も定められた．しかし，その分析法は，日本薬局方原案作成要領[6]で原則使用しないこととされている有害性のあるベンゼンを用いた方法で精製し，分離能の低いパックドカラムを用いて，リン化合物のみに選択性がある炎光光度型検出器（リン用干渉フィルター）（flame photometric detector; FPD）付きガスクロマトグラフ（GC/FPD）で測定する方法である。[7] 家庭用品法では，公定法の結果に基づいて製品回収などの行政措置を行うことが原則となっている．「不適」事例報告があった1980年代前半，公定法で検査を実施し，TDBPPとほぼ同じ保持時間を持つ物質が検出されたためGC/MSを用いて確認同定したところ，TDBPPとは異なる物質であることが判明し，さらに，その物質以外に疑似物質が存在することも報告されている。[8] TDBPPの検査は，都道府県や政令市で，現在も毎年継続して行われている。[9] 近年，「不適」事例報告はないものの，防炎加工の必要性が高まり，新たなリン系難燃剤が使用される可能性があるため，より選択性の高い分析法の開発が求められている．そこで，ベンゼン等有害な試薬を使用しない精製法や，より選択性及び精度の高いキャピラリーカラムを使用したGC/MSを導入するための分析法を検討することとした．その分析法の検討過程で，TDBPP標準品が測定時に分解する現象が観察された．そこで，分解の影響が少ない直接注入のメガボアカラムを用いたGC/FPDで分析法を検討し，良好な回収率を得る前処理法を確立した．さらに，キャピラリーカラムを用いたGC測定時にTDBPPが分解する現象について原因究明を行い，解決策を検討したので報告する．

The authors declare no conflict of interest.

[a]大阪府立公衆衛生研究所，[b]国立医薬品食品衛生研究所
現所属：[†]国立医薬品食品衛生研究所（〒158-8501 東京都世田谷区上用賀 1-18-1）
*e-mail: mimura@iph.pref.osaka.jp

方　　法

1. 試料　市販の防炎加工繊維製品5製品（5部位）を試験試料とした．

2. 試薬　トリス（2,3-ジブロムプロピル）ホスフェイト（TDBPP）の標準試薬は複数のメーカーから購入した．1つは，和光純薬工業製の家庭用品試験用標準試薬（MW：697.61，含量90%以上）を用いた．そのほかに，Sigma-Aldrichから購入したものを用いたが，そのうち，SUPELCO製(MW：697.67，純度95.5%）についてLot番号の異なる2種類（A：Lot LB75285及びB：Lot LB83032V）を購入し，さらにFluka製（MW：697.61，純度98.3%）も入手した．

メタノールは和光純薬工業製LC/MS用，酢酸エチル，シクロヘキサン，*n*-ヘキサン及びアセトンは和光純薬工業製残留農薬分析用，*n*-ノナンは和光純薬工業製特級を用いた．フロリジルカートリッジカラムはWaters製のSep-Pack® plus Florisil® (910 mg/1.4 mL）を用いた．

3. 装置及び測定条件

3-1. 炎光光度型検出器（リン用干渉フィルター）付きガスクロマトグラフ（GC/FPD）（メガボアカラム測定）　GC装置は，Hewlett Packard製5890 Series II GCにFPD検出器を装着した装置を用いた．メガボアカラムは，DB-1（0.53 mmφ×15 m，膜厚1.5 μm，J&W Scientific製）を用いた．キャリアーガス流量は，He 14.5 mL/minに設定した．カラム温度は，100℃ (2 min)−20℃/min−290℃ (7.5 min）にプログラミングし，注入口及び検出器温度は290℃に設定した．注入方法は直接注入法，注入量は1 μLとした．

3-2. GC/FPD（キャピラリーカラム測定）　GC装置はメガボアカラム測定と同じ装置を用いた．キャピラリーカラムは，DB-5（0.25 mmφ×30 m，膜厚0.25 μm，J&W Scientific製）を用いた．キャリアーガス流量は，He 1 mL/minに設定した．カラム温度は，100℃ (2 min)−20℃/min−290℃ (10 min）にプログラミングし，注入口及び検出器温度は290℃に設定した．注入方法はスプリットレスで，注入量は1 μLとした．

3-3. GC/MS（electron ionization; EI測定）

3-3-1. 測定条件(1)　GC/MS装置は，Hewlett Packard製5890 Series II GCにMSD検出器（HP-5971）を装着した装置を用い，自動注入装置は，Hewlett Packard製7673型を用いた．キャピラリーカラムは，Inert Cap 5 MS/NP（0.25 mmφ×30 m，膜厚0.25 μm，GLサイエンス製）を用いた．キャリアーガス流量は，He 1 mL/minに設定した．カラム温度は，100℃ (2 min)−20℃/min−290℃ (6 min）にプログラミングし，注入口及びインターフェイス温度は290℃に設定した．注入方法はスプリットレスで，注入量は1 μLとした．イオン化法はEI法で，イオン化電圧は70 eVとした．スキャンモード（SCAN: m/z=50–550）及びselected ion monitoring（SIM）モードで測定した．

3-3-2. 測定条件(2)　GC/MS装置は，Hewlett Packard製6890 N GCに，日本電子製MSD検出器（JEOL JMS-Q1000GCK9）を装着した装置を用いた．キャピラリーカラムは，HP-5（0.25 mmφ×15 m，膜厚0.25 μm, J&W Scientific製）を用いた．キャリアーガス流量は，He 2 mL/minに設定した．カラム温度は，100℃ (2 min)−20℃/min−290℃

(6 min) にプログラミングし，注入口温度は 290℃に，インターフェイス温度は 250℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．イオン化法は EI 法で，イオン化電圧は 70 eV，イオン源温度は 160℃とした．スキャンモード（SCAN: m/z=50–750）及び SIM モードで測定した．

3-4. GC/MS（chemical ionization; CI 測定）

3-4-1. 測定条件(1) GC/MS 装置は，Hewlett Packard 製 6890GC に MSD 検出器（HP-5973）を装着した装置を用いた．キャピラリーカラムは，HP-5MSI（0.25 mmφ×30 m，膜厚 0.25 μm，J&W Scientific 製）を用いた．キャリアーガス流量は，He 1 mL/min に設定した．カラム温度は，100℃ (2 min)–20℃/min–290℃ (6 min) にプログラミングし，注入口は 290℃，インターフェイス温度は 250℃，イオン源は 160℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．イオン化法は CI 法で，反応ガスはメタンを用いた．m/z =50–750 の範囲でスキャン測定した．

3-4-2. 測定条件(2) GC/MS 装置は，Hewlett Packard 製 6890N GC に，日本電子製 MSD 検出器（JEOL JMS-Q1000GCK9）を装着した装置を用いた．キャピラリーカラムは，DB-5MS（0.25 mmφ ×30 m，膜厚 0.25 μm，J&W Scientific 製）を用いた．キャリアーガス流量は，He 1 mL/min に設定した．カラム温度は，100℃ (2 min)–20℃/min–290℃ (6 min) にプログラミングし，注入口は 290℃，インターフェイス温度は 250℃，イオン源は 160℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．イオン化法は CI 法で，反応ガスはイソブタンを用いた．m/z=50–1000 の範囲でスキャン測定した．

3-5. 水素炎イオン化検出器付きガスクロマトグラフ（GC-FID） GC 装置は，Hewlett Packard 製 5890 Series II GC に flame ionization detector (FID) 検出器を装着した装置を用いた．キャピラリーカラムは，DB-5（0.25 mmφ×30 m，膜厚 0.25 μm，J&W Scientific 製）を用いた．キャリアーガス流量は，He 1 mL/min に設定した．カラム温度は，100℃ (2 min)–20℃/min–290℃ (10 min) にプログラミングし，注入口及び検出器温度は 290℃に設定した．注入方法はスプリットレスで，注入量は 1 μL とした．

3-6. ESI/MS 高分解能 MS 装置は，フーリエ変換-リニアイオントラップ型質量分析計 LTQ Orbitrap XL（Thermo Fisher Scientific 製）を用いた．試料はスプレーチップを用いて infusion 法で導入し，イオン化法には electron spray ionization (ESI) 法を用いた．試料溶液は，Positive モードでは MeOH/0.2％ギ酸水溶液（1：1）を，Negative モードでは MeOH/水（1：1）を用いて調製し，流速は 0.2 μL/min に設定した．スプレー電圧は 1.8 kV に，キャピラリー温度は 200℃とした．キャピラリー電圧は Positive mode は 35 V，Negative mode は－49.0 V とし，分解能は 10 万に設定した．ポリチロシン三量体由来のイオンをロックマス用とした（Positive mode: m/z=508.20783，Negative mode: m/z=506.19327）．精密質量の理論値及び測定値との質量差の計算には，Thermofisher Scientific の Xcalibur ver. 2.1/Qual Browser ソフトを使用した．

4. 試験溶液の調製 細切した試料 1 g をナス型フラスコに秤量し，メタノール溶液 50 mL を加え，30 分間 70℃で還流抽出した．抽出液をガラスろ過器でろ過し，200 mL のナス型フラスコに採取した．20 mL のメタノールで抽出に用いたガラス器具及び試料を洗浄して，洗液とろ液をあわせた．抽出液を 10 mL に濃縮し，50 mL の遠沈管に移した．精製水 10 mL，シクロヘキサン 10 mL を加え 3 分間激しく振とうした後，3000 rpm で 10 分間遠心分離を行い，シクロヘキサン層を分取した．さらに，シクロヘキサン 10 mL を加えて振とう後，シクロヘキサン層をあわせる操作を 2 回行った．抽出液を無水硫酸ナトリウムで脱水した．ろ液をロータリーエバポレーターで 2 mL に濃縮し，あらかじめヘキサン 10 mL で調製したフロリジルカートリッジカラムに負荷した．カラムをヘキサン 20 mL で洗浄した後，30％エタノール含有ヘキサン 30 mL で TDBPP を溶出させた．溶出液をナス型フラスコに採り，ロータリーエバポレーターで減圧濃縮し，アルゴン気流下で溶媒を留去した．残渣をヘキサン 1 mL に溶解し，GC/MS, GC/FPD 及び GC/FID 測定試料とした．

5. 検量線の作成 TDBPP 標準品（SUPELCO 製 B）1000 mg をアセトン 100 mL で溶解し，標準

原液（10000 μg/mL）を調製した．その溶液をヘキサンで希釈し，0.5, 1.0, 2.0, 10, 20, 40, 60, 80, 100 μg/mL となるように検量線用標準溶液を調製し，メガボアカラムを用いた GC/FPD 測定による検量線を作成した．

結果及び考察

1. 検出法の検討 詳細は後述するが，GC 注入口などでの TDBPP（Fig. 1）の分解現象が観察され，GC/MS による定量は困難なことが分かった．そこで，熱分解の影響が少ないメガボアカラムを用いた直接注入法で GC/FPD 測定を実施したところ，TDBPP の分解物はほとんど検出されなかった（Fig. 2）．今までに報告されているメガボアカラムを用いた測定[8,10,11]でも，分解現象について報告されておらず，現公定法で指定されているパックドカラムでも分解現象は観察されなかったと推測される．そこで，公定法に近いメガボアカラム（DB-1）を用いた直接注入 GC/FPD で定量法を検討した．

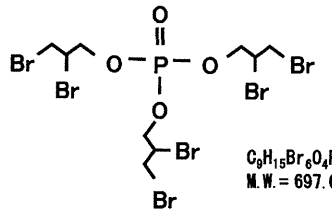

Fig. 1. Chemical Structure of TDBPP

2. 検量線（メガボアカラムによる GC/FPD 測定） GC/FPD 測定による検量線は，2.0–100 μg/mL の範囲で，$Y=2572X^{1.416}$ の 2 次曲線を示す検量線が得られた．検出限界は，S/N=3 として 1.0 μg/mL であった．繰り返し注入の再現性も良好であった．

3. 液–液分配による極性物質の除去 繊維製品からの抽出法は，現公定法に従ってメタノールによる還流抽出法で行うこととした．まず，液–液分配法で，メタノール抽出液から極性物質を除去する方法を検討した．50, 250 μg の TDBPP をメタノール溶液（抽出液）2.5 mL とし，そこに水 2.5 mL 及び有機溶媒 2.5 mL（ヘキサン，シクロヘキサン又は酢酸エチル–ヘキサン混液）を加えて，TDBPP を有機溶媒層に再抽出可能かを検討した．TDBPP は，ヘキサン層に 48–55％，シクロヘキサン層に 62–79％，酢酸エチル–ヘキサン混液（2：3）層に 69–82％，酢酸エチル–ヘキサン混液（1：4）層に 63–84％が移行した．後述するが，酢酸エチル–ヘキサン混液では，フロリジルカラム操作時に回収率の低下が観察されたため，シクロヘキサンを用いることとした．すなわち，メタノール抽出液に同量の水とシクロヘキサンを加え，TDBPP をシクロヘキサン層に再抽出し，共存極性物質を除去した．

4. フロリジルカラムによる無極性（脂溶性）物

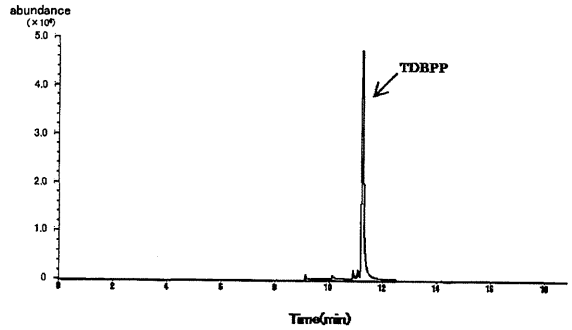

Fig. 2. FPD Gas Chromatogram of TDBPP Using DB-1 Capillary Mega Bore Column

質の除去　繊維製品からは，界面活性剤や脂溶性の共存物質も抽出されてくる．水-シクロヘキサンによる液-液分配後も，これら成分がともにシクロヘキサン層に移行する．そこで，フロリジルカラムによる脂溶性物質の除去法を検討した．50 μg/mL，及び100 μg/mLのTDBPPシクロヘキサン溶液2 mLをカラムに負荷し，20 mLのヘキサンで洗浄したところ，TDBPPはカラムに保持された．次に，30%エタノール含有ヘキサン30 mLで溶出したところ，90%以上のTDBPPが回収された．そこで，ヘキサン20 mLで脂溶性物質を溶出（除去）した後，30%エタノール含有ヘキサン30 mLでTDBPPを溶出することにした．

なお，フロリジルカラムに負荷するTDBPP溶液を，シクロヘキサンから酢酸エチル-ヘキサン混液（2：3）に変更し，同様の操作を行ったところ，試料溶液負荷から20 mLのヘキサンで洗浄するまでの工程で，100, 200 μg負荷のいずれにおいても約30%のTDBPPの溶出が観察された．

5. 市販防炎加工繊維製品の分析及び添加回収実験　市販防炎加工繊維製品5試料を今回構築した方法により抽出・精製した後に，メガボアカラムによるGC/FPDで定量したが，いずれの製品からもTDBPPは検出されなかった．しかし，GC/FPD測定でいくつかのピークが確認されたことから，様々なリン化合物が含まれている可能性が考えられた．

最も夾雑ピークの多かったカーペットのほかに，カーテン及び枕カバーの3試料に対し20及び200 μgのTDBPPを各3回添加した添加回収実験を行った（n=3）．その結果をTable 1に示す．いずれも100%前後の回収率が得られ，変動係数（CV）も小さく再現性も良好であった．なお，2 μgの添加回収実験も行ったが，夾雑ピーク（共存物質）の影響が大きく，良好な回収率及び再現性は得られなかった．TDBPPの難燃剤としての使用濃度は数%で，加工法によって残存量は異なるが，より高温で処理する練り込み加工でも製品中に数十ppmは存在する[12]ことから，20 μgでも十分な検出濃度と考えられる．

6. GC/MS測定の問題点と原因究明　現公定法は，リン化合物としてGC/FPDで検出する方法である．そこで，より正確に同定できるGC/MS法を公定法として導入するための検討を行った．ところが，TDBPP標準品の分解現象，特にGCのスプリットレス注入口での分解が観察された．TDBPPの標準品2種類（和光純薬工業製及びSUPELCO製A）をヘキサンに溶解し，GC/MS測定したところ，クロマトグラム上にいくつかのピークが出現した．そこで，新たに2種のTDBPP標準品（SUPELCO製B及びFluka製）を購入して，標準液を調製し測定したところ，この2種の標準品でも同様のピークが認められた．Figure 3にTDBPP標準（SUPELCO製B）溶液のSCAN測定から得られたトータルイオンクロマトグラム（TIC）及び各ピーク（①-④）のマススペクトルを示す［EI測定条件(1)］．いずれのピークのスペクトルもマスライブラリー（NIST98及びWiley275）から検索したTDBPP（CAS No. 126-72-7）のマススペクトルとは一致しなかった．マスライブラリーは，直接導入法によるスペクトルを登録しているため一致しなかったものと考えられる．最大ピークは，Rt＝15.8 minのピーク④であったが，ピーク①，②，③など保持時間の短いいくつかのピークが観察された．マススペクトルでは，ともにm/z=137（C_3H_5BrO），m/z=201（$C_3H_5Br_2$），m/z=217（$C_3H_5Br_2O$），m/z=257（$C_3H_5Br_2O_2P$）など，TDBPP由来のプロピル基に

Table 1. Analytical Results and Recovery Rate of TDBPP in Textile Products

Sample No.	Usage	Materials	TDBPP (μg/g)	20 μg/g		200 μg/g	
				Recovery Rate (%)	CV (%)	Recovery Rate (%)	CV (%)
1	Carpet	Acryl 100%	N.D.	97.5	1.5	93.4	0.5
2	Curtain	Polyester 100%	N.D.	104	3.1	100	0.3
3	Curtain	Polyester 100%	N.D.	—	—	—	—
4	Pillow Cover	Cotton 100%	N.D.	118	6.3	105	3.0
5	Night Clothes	Acryl 60%＋Cotton 40%	N.D.	—	—	—	—

N.D.: not detected.

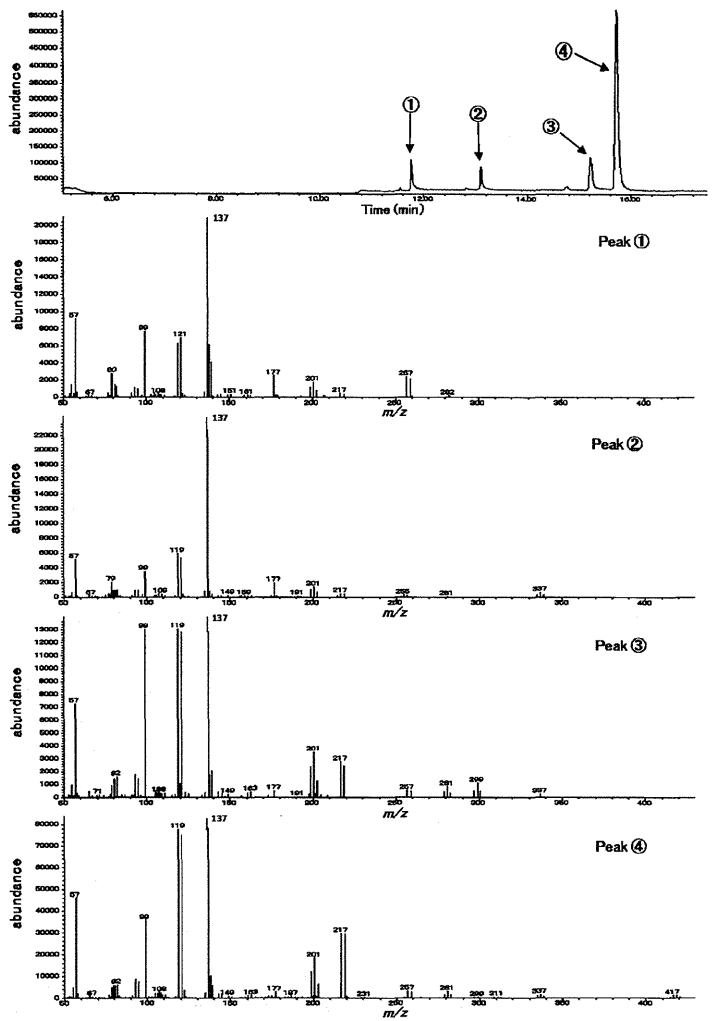

Fig. 3. Total Ion Chromatogram and Mass Spectra (peak①-④) of TDBPP Standard (Supelco B)
①-③: Pyrolysate of TDBPP, ④TDBPP. Operating conditions of GC/MS are given in text. Column temp.: 100°C (2 min)-20°C/min-290°C (6 min). Injection temp.: 290°C, Interface temp.: 290°C. Column: Inert Cap 5 MS/NP (0.25 mmφ×30 m×0.25 μm).

特徴的なフラグメントイオンが検出された．したがって，GC/MS（EI）だけでは，TDBPP のピークを判定し難かった．TDBPP は，260–300℃で主な熱分解が始まり，Br^- と PO_x を放出するとの報告[13]があることから，これらのピークは GC 分析中に TDBPP が分解したものと考えられた．

そこで，GC/MS と同じキャピラリーカラムで，GC/FPD による標準溶液の測定を行ったところ，同様に TDBPP 以外のリン化合物が検出された．装置・条件が若干異なるものの，主にピーク①–④に相当する保持時間のクロマトグラムが得られた．次に，同じキャピラリーカラム（ DB-5）で，GC/FID による測定も実施したところ，こちらも同様にピーク①–④に相当する保持時間にピークが検出され，炭素鎖を持つ化合物が生成していることが推測された．ところが，同じ標準品についてメガボアカラムを用いた直接導入法による GC/FPD での測定では，メインピーク以外のクロマトグラムは，ほとんど観察されなかった（Fig. 2）．そのため，注入方法の違いによって，注入口での熱分解挙動が変化することが考えられた．そこで，スプリット注入法やクールオンカラム法などの注入口での熱分解の影響が少ない注入法を，同じキャピラリーカラムを用いて検討した．その結果，やはりいくつかのピークが観察されたが，メインピーク④の面積比が相対的に大となった．このように，より適切な注入方法を選択することで，TDBPP の分解が少し改善された．しかし，そのような注入方法を用いても，いくつかの小さいピークが認められることから，注入口での分解だけではなくカラム内での分解の可能性も考えられた．城戸らは，キャピラリーカラムが長いと TDBPP のピークが消失し，短くするほどピークの感度が上昇することを報告している．[14] そこで，注入口やカラムでの熱分解を極力避けるために，注入口には石英ライナーを使用し，カラムの長さを短く（15 m）して測定したところ［EI 測定条件(2)］，ほぼ 1 つのピークとなった（Fig. 4）．このマススペクトルは，メーカー公開の標準品マススペクトルと近似していた．

GC 注入法のみが原因とは断定できないものの，標準品のピークはいくつにも分かれて検出されている．そこで，TDBPP 標準品のピークを確認するために，化学イオン化法（CI 法）による同定を行った［CI 測定条件(1)］．ここでも複数のピークが出現し，ピーク④に相当するピークのマススペクトルには，TDBPP から Br が 1 個脱離し，プロトン付加したと考えられる m/z=619 が，最も強いイオン強度で検出された．m/z=80（Br）と m/z=160（Br_2）が，それにつぐ強いイオン強度で検出された（Fig. 5）．プロトン付加した親イオン（m/z=698）は，

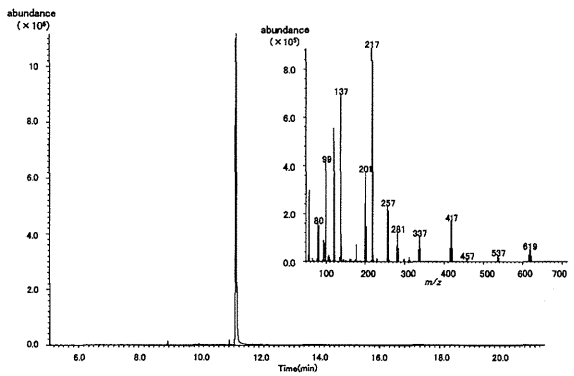

Fig. 4. Total Ion Chromatogram and Mass Spectrum of TDBPP Standard (Supelco B) Using GC/MS(EI) with 15 m Column
Operating conditions of GC/MS are given in text. Column temp.: 100℃ (2 min)–20℃/min–290℃(6 min). Flow rate: 2 mL/min. Injection temp.: 290℃, Ion source temp.: 160℃, Interface temp.: 250℃. Column: HP-5 (0.25 mmϕ×15 m×0.25 μm).

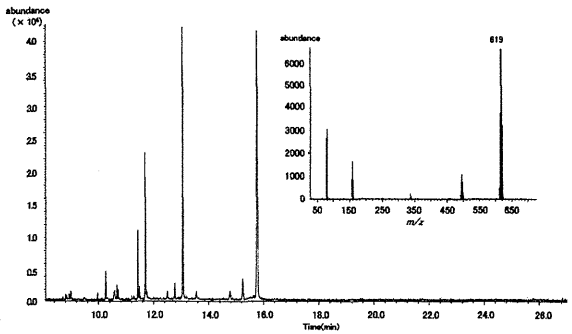

Fig. 5. Mass Spectrum of TDBPP Standard (Supelco B) Using Methane as the Reaction Gas
Operating conditions of GC/MS are given in text. Column temp.: 100℃ (2 min)–20℃/min–290℃(6 min). Injection temp.: 290℃, Ion source temp.: 160℃, Interface temp.: 250℃. Column: HP-5MSI (0.25 mmϕ×30 m×0.25 μm).

ほとんど検出されなかった．CI 法でも，イオン源内で TDBPP から Br が脱離することが示唆され，Br が 1 つ脱離したこのピークが TDBPP と推察された．CI 法で，ピーク③に相当するピークのスペクトルは，$C_3H_5Br_2$ が脱離した m/z=496 と，Br が 1 つ脱離した m/z=619 の 2 つが強いイオン強度を示した．ピーク②に相当するピークスペクトルは，$C_3H_5Br_2$ が脱離した m/z=496 と，Br イオンが 2 つ脱離した m/z=536 の 2 つが強いイオン強度を示した．ピーク①に相当するピークスペクトルは，Br が 3 つ脱離した m/z=457 が強いイオン強度を示した．ピーク③，②，①も m/z=80（Br）と，m/z=160（Br_2）のイオンが，比較的強い強度で検出された．

さらに，反応ガスにイソブタンを用いて，異なる GC/MS 装置（日本電子製）により CI 測定を行ったところ［CI 測定条件(2)］，感度は低いものの④に相当するピークから親イオンが検出され（Fig. 6），ピーク④は TDBPP 標準品のピークと考えられた．装置・条件によりイオン源内でも，標準品から Br が脱離する可能性が考えられた．

7. TDBPP 標準品の同定と純度検定　TDBPP 標準品の保証書には，GC/MS スペクトル，FT-IR 及び HPLC（UV-220 nm）のデータが記載されていた．純度検定は，HPLC のデータから 95.5％の含有量との記載があった．メーカー提供の MS スペクトルも親イオンはなかった．そこで，改めて標準品の同定と純度検定を行った．

前述したように，CI 法で標準品の同定を行ったところ，親イオンのみからなるスペクトルは検出できなかった．そこで，標準品を同定するため，高分解能 MS（ESI）の infusion 法で TDBPP 標準溶液を分析した．Positive モードでは $[M+Na]^+$（理論値：m/z=714.57004，実測値：m/z=714.57012，(−0.08 mmu)），Negative モードでは $[M-H]^-$（理論値：m/z=690.57354，実測値：m/z=690.57335，(−0.19 mmu)）と親イオンが検出され，理論値とほぼ同じ値であった．

次に，標準品の（SUPELCO 製 B）の純度検定を行った．前述したように，①–④のピークのマススペクトルからは，m/z=137（C_3H_5BrO），m/z=201（$C_3H_5Br_2$），m/z=217（$C_3H_5Br_2O$），m/z=257（$C_3H_5Br_2O_2P$）など，TDBPP 由来のプロピル基に特徴的なフラグメントイオンが検出されている．すなわち，TDBPP と同じ炭素数（C9）を持つ等モルの *n*-ノナンを GC/FID で同時測定し，各々のピーク面積比を比較して TDBPP 及び分解産物の含有量を推定することとした．100 μg/mL の TDBPP（SUPELCO 製 B）と等モル数（143 μmol/L）の *n*-ノナンを含有するヘキサン溶液を調製した．その溶液を GC/FID で測定し，各ピーク面積比からピーク①–④に相当する物質の炭素含量を求めた．GC/FID

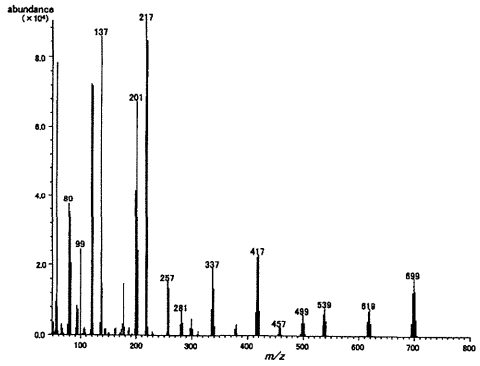

Fig. 6. Mass Spectrum of TDBPP Standard (Supelco B) Using 2-Methylpropane as the Reaction Gas
Operating conditions of GC/MS are given in text. Column temp.: 100℃ (2 min)–20℃/min–290℃ (6 min). Injection temp.: 290℃, Ion source temp.: 160℃, Interface temp.: 250℃. Column: DB-5MS (0.25 mmϕ×30 m×0.25 μm).

測定条件は，*n*-ノナン測定のため，昇温条件（40℃ (4 min)–20℃/min–290℃ (8 min)）のみを変更した．*n*-ノナンのピーク面積に対し，TDBPP である④の面積比は 86.5%，①は 8.0%，②は 2.5%，③が 1.8%であった．①–④の合計は 98.8%であり，ほぼ純品であることが分かった．

8. 公定分析法の改定　本研究は，「有害物質を含有する家庭用品の規制に関する法律」により使用禁止されている TDBPP の公定分析法改定を目的としている．ところが，検討過程で TDBPP は熱分解すること，それも GC/MS 装置の注入口，カラム，イオン源内で熱分解が起こることが分かった．つまり，GC/MS だけでは，TDBPP のピークを判定し難く，標準品については別途，7 項で記載したような標準品の同定及び純度検定を行う必要がある．

前述したように GC に適用可能な前処理法を確立した．しかし，測定法（定量・定性）には問題点が残った．今までの結果を踏まえた上で，公定分析法として，以下の方法が候補として考えられる．

(1) GC/FPD による定量及び GC/MS による定性法

現公定法のパックドカラムをメガボアカラムに変更し，直接注入法を用いた GC/FPD 測定で定量する．そして，ピークが検出された場合は，GC/MS (CI, EI) 法を用いてそのマススペクトルで確認する．例えば，EI 法によるピーク④のマススペクトルは（Fig. 3），メーカー提供の標準品マススペクトルと近似している．これをマスライブラリーに登録して，一致率から確認する．CI 法を用いる場合にも親イオンのみは検出できないことから，標準品のマススペクトルと試料ピークのマススペクトルを比較して，一致しているかを確認する．例えば Br が 1 つ脱離したスペクトル（Fig. 5）のピークを TDBPP として同定確認するとよい．

(2) GC/MS (EI) による定量・定性法

注入口に石英ライナー用い，カラムの長さを短く (15 m) することで，ほぼ 1 つのピークとなった [EI 測定法(2)]．このピークのマススペクトルとメーカー提供の標準品マススペクトルは，ほぼ一致した（Fig. 4）．そこで，定量イオンに m/z=217 を，確認イオンに m/z=137 を用いて，5–150 μg/mL の範囲で検量線を作成したところ，R^2=0.99 以上の良好な直線性を示した．検出限界は S/N=3 として 2 μg/mL であった．50 μg/mL 標準溶液を 5 回繰り返し注入したところ，再現性は CV=13.4%であった．再現性のばらつき及び低感度であることなど，少し問題点は残っているものの，定量分析法として適用可能と考えられる．

結　　論

「有害物質を含有する家庭用品の規制に関する法律」により，繊維製品への使用が禁止されている，トリス（2,3-ジブロムプロピル）ホスフェイト (TDBPP) の公定法を改定するために，分析法の検討を行った．

(1) キャピラリーカラムを用いた GC/MS 法では，TDBPP が熱分解すること，その現象は，注入口，カラムさらにイオン源でも生じることが分かった．(2) メガボアカラムを用いた GC/FPD 測定により，液–液分配及びフロリジルカラム精製で，100%前後の良好な回収率を得る前処理法を確立した．(3) 標準品の同定を ESI/MS で，純度検定を GC/FID で行い，ほぼ 100%であることを確認した．(4) 公定分析法としては，①GC/FPD 測定後に GC/MS での同定，②石英ライナー，ショートカラムを用いた GC/MS 測定を提案した．

謝辞　化学イオン化 GC/MS 測定の実施に御協力頂きました大阪府立公衆衛生研究所の小泉義彦主任研究員並びに北川陽子主任研究員，GC/MS での熱分解現象を解決するための御助言を頂きました大阪府立公衆衛生研究所の尾花裕孝博士並びに奈良県保健環境研究センターの陰地義樹博士，高分解能 MS 測定に御協力頂きました国立医薬品食品衛生研究所の齠島由二博士に感謝致します．

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Anal Bioanal Chem
DOI 10.1007/s00216-011-4707-y

ORIGINAL PAPER

Chiral analyses of dextromethorphan/levomethorphan and their metabolites in rat and human samples using LC-MS/MS

Ruri Kikura-Hanajiri · Maiko Kawamura · Atsuko Miyajima · Momoko Sunouchi · Yukihiro Goda

Received: 30 September 2010 / Revised: 7 January 2011 / Accepted: 22 January 2011

Abstract In order to develop an analytical method for the discrimination of dextromethorphan (an antitussive medicine) from its enantiomer, levomethorphan (a narcotic) in biological samples, chiral analyses of these drugs and their *O*-demethyl and/or *N*-demethyl metabolites in rat plasma, urine, and hair were carried out using LC-MS/MS. After the i.p. administration of dextromethorphan or levomethorphan to pigmented hairy male DA rats (5 mg/kg/day, 10 days), the parent compounds and their three metabolites in plasma, urine and hair were determined using LC-MS/MS. Complete chiral separation was achieved in 12 min on a Chiral CD-Ph column in 0.1% formic acid–acetonitrile by a linear gradient program. Most of the metabolites were detected as being the corresponding *O*-demethyl and *N*, *O*-didemethyl metabolites in the rat plasma and urine after the hydrolysis of *O*-glucuronides, although obvious differences in the amounts of these metabolites were found between the dextro and levo forms. No racemation was observed through *O*- and/or *N*-demethylation. In the rat hair samples collected 4 weeks after the first administration, those differences were more clearly detected and the concentrations of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites were 63.4, 2.7, 25.1, and 0.7 ng/mg for the dextro forms and 24.5, 24.6, 2.6, and 0.5 ng/mg for the levo forms, respectively. In order to fully investigate the differences of their metabolic properties between dextromethorphan and levomethorphan, DA rat and human liver microsomes were studied. The results suggested that there might be an enantioselective metabolism of levomethorphan, especially with regard to the *O*-demethylation, not only in DA rat but human liver microsomes as well. The proposed chiral analyses might be applied to human samples and could be useful for discriminating dextromethorphan use from levomethorphan use in the field of forensic toxicology, although further studies should be carried out using authentic human samples.

Keywords Levomethorphan · Dextromethorphan · Chiral analysis · Biological samples · LC-MS/MS · Enantioselective metabolism

Published in the special issue *Forensic Toxicology* with Guest Editors Frank T. Peters, Hans H. Maurer, and Frank Musshoff.

R. Kikura-Hanajiri (✉) · M. Kawamura · Y. Goda
Division of Pharmacognosy, Phytochemistry and Narcotics,
National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya,
Tokyo 158-8501, Japan
e-mail: kikura@nihs.go.jp

A. Miyajima
Division of Medical Devices,
National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya,
Tokyo 158-8501, Japan

M. Sunouchi
Division of Pharmacology, National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya,
Tokyo 158-8501, Japan

Published online: 11 February 2011

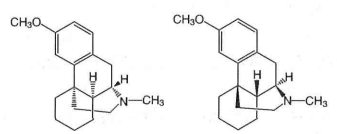
Introduction

Dextromethorphan is widely used all over the world as an over-the-counter antitussive medicine. It produces little or no central nervous system depression at therapeutic doses, but it has dissociative effects similar to ketamine and phencyclidine in large doses as an *N*-methyl-*D*-aspartate receptor antagonist [1–6]. To obtain its hallucinogenic effect, young people abuse this drug by large doses and many fatalities from overdoses have been reported [7, 8]. In contrast, its enantiomer, levomethorphan, is a potent narcotic analgesic [9] (Fig. 1), and an *O*-demethyl compound of levomethorphan, levorphanol, is known to have stronger opioid pharmacological effects [9, 10]. Levomethorphan is strictly controlled in the world as a narcotic and is never used for therapeutic purposes.

In humans, as shown in Fig. 2, it has been reported that dextromethorphan is primarily metabolized to dextrorphan via *O*-demethylation by cytochrome P450 2D6 (CYP2D6), which is polymorphically expressed in humans, who can be classified as poor, intermediate, and extensive metabolizers [11, 12]. Dextromethorphan is *N*-demethylated via an additional route to 3-methoxymorphinan (3-MEM), primarily mediated by CYP3A4 in human liver microsomes [11, 13]. Dextrorphan and 3-MEM are then demethylated to 3-hydroxymorphinan (3-HM) via CYP3A4 and CYP2D6, respectively. Moreover, dextrorphan and 3-HM are glucuronized to their *O*-glucuronides and these are mainly excreted into human urine [14, 15].

A variety of analytical methods for the determination of dextromethorphan and its metabolites in biological samples have been reported using capillary electrophoresis (CE) [16, 17], HPLC [18–22], GC-MS [23–26], and LC-MS (/MS) [15, 27–30]. However, there is little information regarding the metabolic properties of levomethorphan. Although a chiral separation method of dextromethorphan and levomethorphan using HPLC with fluorometric detection [22] or using CE [17] has been reported, there has been no report that describes a simultaneous determination of dextromethorphan, levomethorphan, and their metabolites in biological samples after administration of these drugs. Considering the possibility of the adulteration or substitution of dextromethorphan with levomethorphan due to its chemical similarities for illegal purposes, it is necessary to establish the enantiometric separation of dextromethorphan and levomethorphan in biological samples.

In order to develop an analytical method for the discrimination of dextromethorphan from levomethorphan in biological samples, we first investigated chiral analyses of these drugs and their *O*-demethyl and/or *N*-demethyl metabolites in plasma, urine, and hair samples of rats administered with each enantiomer, using LC-MS/MS. In addition, detailed metabolic properties of these drugs were investigated using rat and human liver microsomes.



Dextromethorphan Levomethorphan

Fig. 1 Chemical structures of dextromethorphan and its enantiomer, levomethorphan

Experimental

Materials

Levomethorphan was obtained from Cerilliant (Round Rock, TX, USA). Dextromethorphan hydrobromide, dextrorphan tartrate, (+)-3-HM hydrobromide, (+)-3-MEM hydrochloride, and levallorphan tartrate (used as internal standard, IS) were from Sigma-Aldrich (St. Louis, MO, USA). Levorphanol tartrate was given by Professor T. Nagano (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan). A β-glucuronidase solution (EC 3.2.1.31, 103,000 units/mL, Source: *Helix pomatia*) was purchased from Wako Chemicals (Osaka, Japan). A solid-phase extraction column (OASIS HLB, 3 cc, 60 mg) was obtained from Waters (Milford, MA, USA), and the membrane filter (Ultrafree-MC, 0.45 μm) was from Millipore Corporation (Bedford, MA, USA).

Liver microsomes from individual male dark agouti (DA) rats (n=4, 6 weeks old, around 125 g mean weight) were prepared by ultracentrifugation as described [31, 32]. The concentrations of microsomal protein were estimated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Human liver microsomes (50-donor pool, 20 mg/mL) were purchased from BD Biosciences (Woburn, MA, USA). Nicotinamide adenine dinucleotide phosphate (NADP) and glucose 6-phosphate (G-6-P) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) and G-6-P dehydrogenase (G-6-PDH) was from Roche Diagnostics (Indianapolis, IN, USA). All other chemicals and solvents were of an analytical reagent grade or HPLC grade (Wako Chemicals, Osaka, Japan).

Instrumentation

The UPLC analysis was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA). The separations were achieved using a Chiral CD-Ph column (150×2.1 mm i.d., 5 μm) from Shiseido (Tokyo, Japan). The column temperature was maintained at 30 °C, and the following gradient system was used with a mobile phase A (0.1% formic acid) and mobile phase B (0.1% formic acid/acetonitrile) delivered at 0.25 mL/min: 80% A/20% B (2 min hold)—70% A/30% B (15 min). The mobile phase was used as a wash solvent to avoid any carry-over from previous injections. The autosampler was maintained at 4 °C, and the injection volume was 2 μL. Quantitation was achieved by MS/MS detection in a positive ion mode using a Quattro Premier XE mass

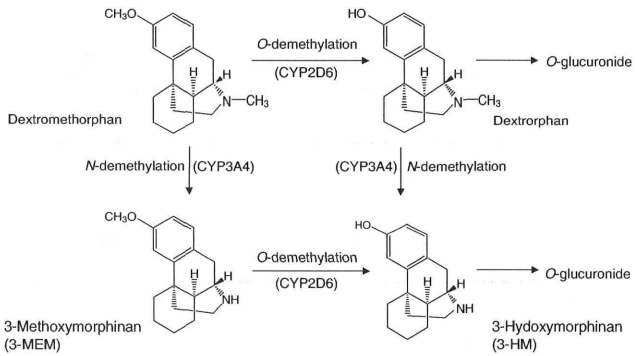


Fig. 2 Main metabolic pathway of dextromethorphan in humans

spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. Quantification was performed using multiple reaction monitoring of the transitions of precursor ions to product ions with each cone voltage and collision energy as shown in Table 1. The optimal MS parameters obtained were as follows: capillary 3.0 kV, source temperature 120 °C, and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas, with a flow rate of 800 and 50 L/h, respectively. Argon was used as the collision gas, with a flow rate of 0.25 mL/min. All data collected in the centroid mode were processed using MassLynx™ NT4.1 software with a QuanLynx™ program (Waters, Milford, MA, USA).

Since the standard compounds of (−)-3-MEM and (−)-3-HM were not available, these peaks were confirmed by comparison of their retention times and mass fragmentations with those of the standard compounds of the dextro forms ((+)-3-MEM and (+)-3-HM) using an ODS column. The analyses were performed using an Acquity HSS T3 column (100×2.1 mm i.d., 1.8 μm) from Waters (Milford, MA, USA). The column temperature was maintained at 40 °C, and the following gradient system was used with a mobile phase A (1% formic acid) and mobile phase B (1% formic acid/acetonitrile) delivered at 0.3 mL/min: 90% A/10% B (0 min)–70% A/30% B (8 min). The MS parameters were the same as for the analyses using the chiral column described above.

Animal experiments

The animal experimental model was designed as shown in our previous reports [33, 34]. All experiments were carried out with the approval of the Committee for Animal Care and Use of the National Institute of Health Sciences, Japan. Dextromethorphan hydrobromide (dissolved in an isotonic sodium chloride solution, 2.5 mg/mL, rat 1–3) or levomethorphan (dissolved in a mixed solution of 5% Emulphor™ EL-620/5% ethanol/90% isotonic sodium chloride solution, 2.5 mg/mL, rat 4–6) was administered to male DA pigmented rats, which were 5 weeks old and around 90 g mean weight (Japan SLC, Shizuoka, Japan). The drugs were given once daily at 5 mg/kg by intraperitoneal injection for ten successive days. Blood samples were collected 5, 15, 30, 60, 120, and 360 min after the first administration from the orbital vein plexus. Plasma samples were prepared by centrifugation at 10,000×*g* for 3 min and stored at −20 °C until analysis. The area under the plasma concentration time curve (AUC) was calculated by the conventional method. Urine samples were collected 0–24, 24–48, and 48–72 h after the last administration and stored at −20 °C. Each animal had been shaved on the back just before the first drug administration. The new growing hair samples were collected 28 days after the first administration.

Extraction of parent compounds and their metabolites from rat plasma and urine samples

For the quantitative analysis of *O*-demethyl and *N*,*O*-didemethyl metabolites in the rat plasma and urine samples, the analytes were measured as free compounds after the hydrolysis of *O*-glucuronides. The optimal condition of the hydrolysis was evaluated, with the peak of putative *O*-glucuronide at nearly 2 min (*m/z* 434→258) on the MRM chromatogram disappearing from rat plasma and urine samples after the hydrolysis.

A 25-μL plasma sample with 50 μL of added 10 mM ammonium formate buffer (pH 5.0) was reacted with 20 μL of the β-glucuronidase solution at 37 °C for 20 h. To precipitate the proteins in the plasma, 40 μL of the IS methanol solution and 100 μL of methanol were poured into each tube, and the mixtures were then vigorously mixed. The mixed solution was centrifuged at 1,200×*g* for 3 min and filtered prior to the injection for the LC-MS/MS analysis.

Table 1 Analytical conditions of LC-MS/MS using the Chiral CD-Ph column

Compounds	Retention time min	Precursor ions *m/z*	Cone voltage V	Product ions *m/z*	Collision energy eV
Dextromethorphan	10.6	272	40	171	45
Dextrorphan	6.1	258	45	157	40
(+)-3-MEM	8.1	258	40	170	35
(+)-3-HM	3.9	244	30	156	35
Levomethorphan	11.3	272	40	171	45
Levorphanol	5.5	258	45	157	40
(−)-3-MEM	9.8	258	40	170	35
(−)-3-HM	4.5	244	30	156	35
Levallorphan (IS)	7.5	284	40	157	40

To a 50-μL urine sample (20 μL for 0–24 h samples) was added 100 μL of the β-glucuronidase solution, 1 mL of 10 mM ammonium formate buffer (pH 5.0) and 50 μL of the IS aqueous solution, respectively. The mixed solution was incubated at 37 °C with gentle shaking. After an OASIS HLB column was pre-activated with 2 mL of methanol and distilled water, the reaction mixture was applied to the column. Following the wash of the column with 2 mL of distilled water, 1 mL of methanol was passed through the column to elute the target drugs. A 2-μL of the solution was automatically injected into the UPLC-MS/MS.

Extraction of parent compounds and their metabolites from rat hair samples

Hair samples were washed three times with 0.1% sodium dodecyl sulfate under ultrasonication, followed by washing three times with water under the same condition. After the sample was dried under a nitrogen stream at room temperature, approximately 10 mg of finely cut hair was precisely weighed and extracted with 1 mL of methanol/5 M hydrochloric acid mixed solution (20:1) containing 50 μL of the IS methanol solution for 1 h under ultrasonication. Following overnight storage at room temperature, the hair was filtered off, the filtrate was evaporated with a nitrogen stream, and the residue was dissolved in 1 mL of distilled water. The solution was treated with an OASIS HLB column and analyzed as described above.

Linearity, precision, and recovery of the analytical method for the rat samples

An individual standard solution of 1.0 mg/mL of each drug, dextromethorphan, levomethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan, and levorphanol, was prepared in methanol and stored at 4 °C. The IS solutions of 1 μg/mL of levallorphan in methanol for the analysis of hair samples and those of 1 μg/mL of levallorphan in distilled water for plasma and urine samples were also prepared.

The drug concentrations in the samples were calculated using the peak–area ratios of the ions monitored for the target compounds versus IS. The calibration curves for the determination were constructed by analyzing extracted drug-free control samples spiked with the standard solution, as described above. The calibration samples containing 0, 1, 2, 4, 20, 40, 200, and 400 ng/mL of the target drugs for the rat plasma, 0, 5, 10, 50, 100, 500, 1,000 2,500, 5,000, and 10,000 ng/mL for the urine samples and 0, 0.1, 0.5, 1.0, 5.0, 10, 25, and 50 ng/mg for the hair samples were prepared just before analysis. The limit of quantitation (LOQ) of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance, while the limit of detection (LOD) was defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise ratios (*S/N*) of 3.

The precision of the method was evaluated by five consecutive analyses of the plasma and urine samples that were spiked with the standard solutions containing 2, 20, and 200 ng/mL for the rat plasma samples and 5, 500, and 5,000 ng/mL for the urine samples, respectively. For the hair analyses, the control samples spiked with the standard solutions each containing 0.1, 5, and 50 ng/mg of the targeted drugs were evaluated. The recoveries of the four analytes from the rat samples were determined using each sample spiked with the analytes at a concentration of 80 ng/mL for the plasma, 500 ng/mL for the urine, and 10 ng/mg for the hair, respectively. To determine the recoveries, the responses of the analytes in the standard solutions and in the extracts from the rat control samples were compared. For the quantitative analysis of (−)-MEM and (−)-HM, the calibration curves of (+)-MEM and (+)-HM were used.