Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 809 (2004) 131–136
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of tetrabromobisphenol A in human serum by liquid chromatography–electrospray ionization tandem mass spectrometry

Tadashi Hayama [a,b], Hideyuki Yoshida [a], Suzuko Onimaru [b], Sayuri Yonekura [c], Hiroaki Kuroki [c], Kenichiro Todoroki [a], Hitoshi Nohta [a], Masatoshi Yamaguchi [a,*]

[a] *Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Johnan, Fukuoka 814-0180, Japan*
[b] *Environmental Science Center, Foundation for Kyushu Environmental and Occupational Health, 6-4-23 Higashi-Aikawa, Kurume, Fukuoka 839-0809, Japan*
[c] *Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami, Fukuoka 815-8511, Japan*

Received 24 February 2004; received in revised form 24 May 2004; accepted 10 June 2004

Available online 6 July 2004

Abstract

A method for the determination of tetrabromobisphenol A (TBBPA) in human serum utilizing solid-phase extractions (SPEs) and liquid chromatography (LC) with electrospray ionization tandem MS (MS/MS) has been developed. After purification and concentration of TBBPA using consecutive SPEs on reversed-phase and normal-phase cartridges, the serum sample was subjected to LC. TBBPA was separated on a C18 reversed-phase column by gradient elution with a mixture of water, methanol, and acetonitrile as the mobile phase, and then detected with electrospray ionization MS/MS in negative ion mode. $^{13}C_{12}$-TBBPA was suitable as an internal standard for the reproducible determination of TBBPA in human serum samples (5 g). The method has been validated in TBBPA concentration range of 5–100 pg per g serum, and the recoveries in the concentration range were higher than 83.3%. The repeatabilities of the proposed method of non-spiked control serum (6.3 pg per g serum) and spiked serum (added 5–100 pg per g serum) were within 10.0% as relative standard deviations. The limit of quantification (LOQ) for TBBPA was 4.1 pg per g serum, which was corresponded to 0.63 fmol on column.

Keywords: Tetrabisphenol A

1. Introduction

A large number of compounds have been used as flame-retardants to protect different products from catching fire. Tetrabromobisphenol A (TBBPA, Fig. 1), which is one of the most widely used flame-retardants, is utilized as a reactive additive in polymerization and is incorporated into epoxy resins used in printed circuit boards [1]. Leakage of unreacted TBBPA in the resins into the environment might occur in many electronic equipments, as discharges from industry or from wastes at dismantling plants. Indeed, TBBPA has been found in sediments [2,3], sewage sludge [3,4], and indoor air [5,6]. Trace amounts of TBBPA in the environmental samples have been determined by gas chromatography (GC) with electron-capture detection (ECD) [2,5,7], GC–mass spectrometry (MS) with electron ionization [2] or GC–ECMS [3,4,8]. Recently, the determination methods of TBBPA in human plasma or serum have been developed [9–11] and applied to the survey of the relationships between the TBBPA concentration in blood and occupation [11,12] or aging [13]. However, the tedious pretreatments and derivatization as well as purification are necessary for the determination of TBBPA with GC.

In recent years, the instrumentations of liquid chromatography (LC) with MS or tandem MS (MS/MS) detection and the related techniques have been evolved rapidly, and so the LC–MS methods have been utilized to so many fields. The greatest advantage of LC–MS against GC–MS is that the derivatization step is not necessary for the detection in many cases. That will lead to simple procedure, rapid analysis, and high reproducibility [14–19].

The objective of this study is to develop a simple method for the determination of TBBPA in human serum.

* Corresponding author. Tel.: +81 92 871 6631; fax: +81 92 863 0389.
E-mail address: masayama@fukuoka-u.ac.jp (M. Yamaguchi).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.06.013

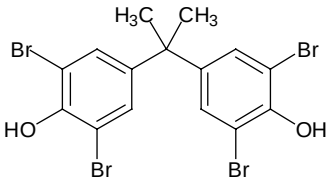

Fig. 1. Structural formula of tetrabromobisphenol A.

Solid-phase extraction (SPE) offers efficient methods with lower solvent consumption, less risk of contamination, and higher selectivity. In this work, a method for sample preparation using SPE is presented. As the concentrations of TBBPA in human serum are expected to be low, LC–MS/MS with electrospray ionization (ESI) interface was chosen for the determination, due to its high selectivity and sensitivity towards phenolic compounds. $^{13}C_{12}$-TBBPA was used as an internal standard to compensate for random errors throughout the whole procedure and for differences between the samples. Tetrabromo-*o*-cresol (TBCr) was used to check the system suitability of the LC apparatus.

2. Experimental

2.1. Reagents and solutions

TBBPA standard (environmental analytical grade) and $^{13}C_{12}$-TBBPA (99%, 50 μg/mL in methanol) were obtained from Kanto Chemical (Tokyo, Japan) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively. All organic solvents and water were of LC-grade or pesticide-grade from Wako Pure Chemicals (Osaka, Japan), and used as received. All other chemicals were of analytical grade. Nitrogen gas (99.999%) for drying and argon gas (99.999%) for MS/MS were obtained from Watanabe Corporation (Kurume, Japan).

Stock solutions of 100 μg/mL TBBPA and 100 μg/mL TBCr were prepared by dissolving an accurate amount in 10.0 mL of methanol. The solutions of TBBPA, $^{13}C_{12}$-TBBPA and TBCr were diluted further with methanol to the required concentrations before use.

2.2. Materials

Abselut Nexus column (polystyrene divinylbenzene, 200 mg, 6 mL) and Strata SI-1 column (silicagel, 100 mg, 1 mL) were purchased from Varian (Palo Alto, CA, USA) and Phenomenex (Torrance, CA, USA), respectively. An Abselut Nexus column was conditioned with dichloromethane (10 mL), methanol (5 mL), and water (5 mL), and a Strata SI-1 column was with dichloromethane (3 mL) and hexane (2 mL).

All glassware was washed in diluted Clean 99-L (Clean Chemical, Ibaraki, Japan), rinsed with water and then heated at 200 °C for at least 12 h.

2.3. Serum samples

A serum sample (male) was purchased from Sigma (St. Louis, MI, USA) and used as a control serum. Other serum samples were obtained from whole blood (8.5 mL) of healthy male volunteers in our laboratory with vacuum blood collecting tube SST-II (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). They understood the purpose and the importance of this experiments, and presented their blood by their own free will. Each sample was allowed to stand for at least 30 min at room temperature (24 ± 4 °C) and then centrifuged at 3000 × *g* for 5 min. Aliquots of the serum samples were transferred to respective screw-capped tubes and kept frozen at −20 °C until use.

2.4. Pretreatment of serum sample

The extraction of TBBPA from serum was according to the previous method [9,10] with minor modifications as follows. Five milliliters of formic acid–methanol (4:1, v/v) and 1.0 μL of $^{13}C_{12}$-TBBPA (0.5 μg/mL) were added to the serum sample (5.0 g), and the resulting sample was sonicated in an ultrasonic bath (Yamato B-3200, Tokyo, Japan) for 5 min. After the resulting sample was diluted with 5 mL of water–methanol (1:1, v/v) and sonicated for 5 min, whole the diluted sample was applied to the preconditioned Abselut Nexus cartridge. The cartridge was washed with 5 mL of water and dried by passing through nitrogen stream more than 20 min, and TBBPA in the cartridge was eluted with 6 mL of dichloromethane. After the eluate was dried under nitrogen stream, the residue was dissolved to 1 mL of hexane and the solution was applied to the preconditioned Strata SI-1 cartridge. The cartridge was washed with 1 mL of hexane, eluted with 2 mL of hexane–dichloromethane (2:1, v/v), and the eluate was dried under nitrogen stream. To the residue, 1.0 μL of TBCr (2.5 μg/mL) was added, and the volume was adjusted to 0.50 mL with methanol. The solution was set to the auto-sampler of LC–MS/MS system.

It was so difficult to obtain a TBBPA-free serum as a serum blank that the procedure blank was used instead of the serum blank. To prepare the procedure blank, a 5.0 mL of water in place of serum sample was subjected to the same procedure. The blank was used for the subtraction of contaminated TBBPA from solvents, reagents, and the environment including air.

2.5. Instrumentation

A Thermo Electron (San Jose, CA, USA) Surveyor LC–MS/MS system consisted of a chromatograph pump, an auto-sampler, a gradient unit, an on-line degasser, a column oven and a TSQ Quantum mass spectrometer was used. A reversed-phase Mightysil RP-18 GP (150 mm × 2.0 mm i.d., particle size 3 μm; Kanto Chemical) was used. Injection of samples at 10 μL each was carried out automat-

ically. Water (mobile phase A) and a mixture of methanol and acetonitrile (4:1, v/v) (mobile phase B) were used as mobile phases for the gradient elution (gradient curve: 0–0.5 min, 40% B; 0.5–2.5 min, linear change from 40 to 95% B; 2.5–10 min, 95% B; 10–10.1 min, linear change from 95 to 40% B; a run-time, 25 min). The flow-rate of the mobile phase and the column oven temperature were set at 0.2 mL/min and 40 °C, respectively. The effluent from LC column was flowed directly, without splitting, into the ion source of mass spectrometer.

The LC–MS/MS, TSQ Quantum mass spectrometer, was operated in the negative ESI mode. The TSQ Quantum was tuned using the built-in auto-tuning system. Operating parameters of the ESI interface of MS/MS were optimized in full scan mode (*m/z* 100–1000) using an infusion system of TBBPA in a carrier solution (methanol–acetonitrile–water; 8:2:1, v/v) at 20 μL/min by means of a syringe pump. The spray needle voltage was −3.0 kV, heated capillary temperature 350 °C, sheath gas pressure 45, and auxiliary gas setting 15. Both the sheath gas and auxiliary gas used were nitrogen obtained from N_2 supplier 12ES (Sic, Tokyo, Japan). The collision gas was argon at a pressure of 1.5 mTorr for all studies. For the MS/MS analysis, the optimized relative collision energies for collision-induced dissociation (CID) were 38 V (0–8.5 min: TBBPA, *m/z* 542.7 → 445.8; $^{13}C_{12}$-TBBPA, *m/z* 554.7 → 457.9) and 27 V (8.5–12.0 min: TBCr, *m/z* 422.7 → 342.7), respectively.

2.6. Quantification and validation

Peak areas were used for quantification of TBBPA. All peaks were integrated automatically. The TBBPA amounts were calculated using the calibration curve by the ratio of the peak area of TBBPA to that of $^{13}C_{12}$-TBBPA. The concentration data reported have been corrected for the procedure blank.

The calibration graph and repeatability of the present method were determined throughout the full analytical procedures (sample pretreatment and LC–MS/MS analysis). The linearity of the method for TBBPA was checked by preparing a calibration graph ($n = 6$ each) at five different concentrations: the control serum added with 0, 5, 10, 50, and 100 pg TBBPA per g serum. The equation of the calibration line was calculated by least-squares linear. The method was validated using the serum samples (five levels) used for calibration graph.

3. Results and discussion

3.1. Detection conditions

The optimization studies for chromatographic conditions were carried out using TBBPA standard solution (10 ng/mL). It is known that the addition of organic bases promotes the ionization of phenolic compounds and causes the increase of sensitivity to LC–MS analysis [14–19]. However, the sensitivity of TBBPA did not increase by the post-column addition of several concentrations of acids (formic acid, acetic acid, trifluoroacetic acid), bases (ammonia, triethylamine), and their salts, because organic solvents in the mobile phase interfere with the additives from accelerating the ionization of TBBPA. As an organic modifier in the mobile phase, a mixture of methanol and acetonitrile was used. With increasing the concentration of methanol, the sensitivity for TBBPA increased, but the column pressure also increased. A mixture of methanol and acetonitrile (4:1, v/v) was selected as a compromise.

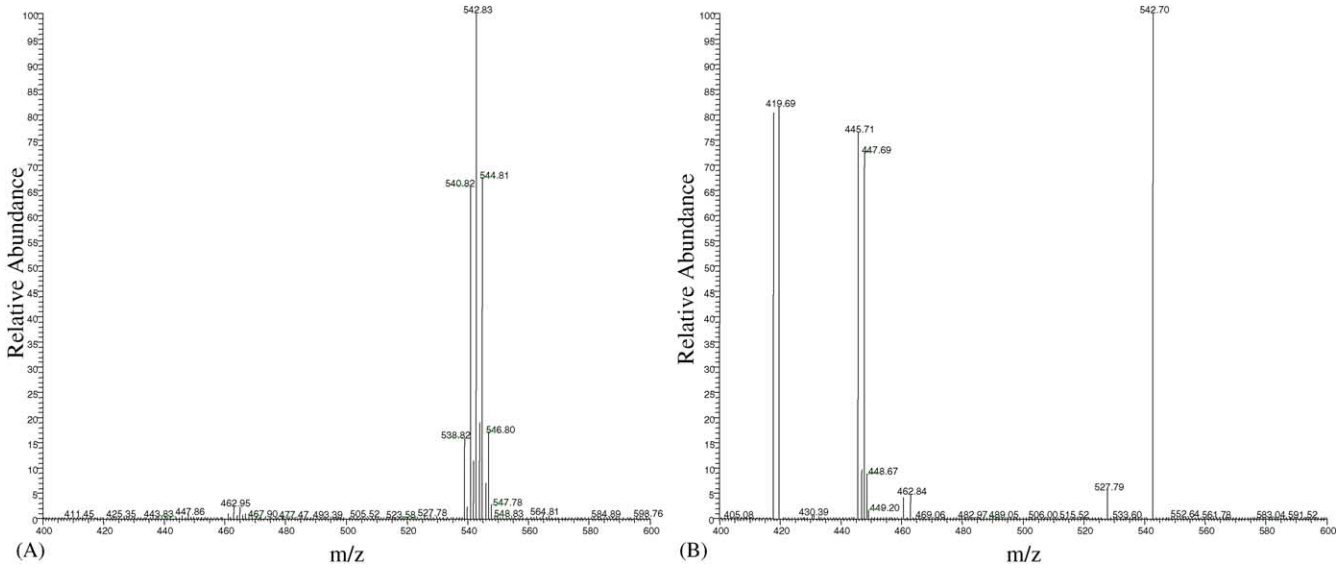

Fig. 2. Negative (A) ESI-MS and (B) MS/MS spectra of TBBPA obtained by infusion system of 10 ng/mL solution (in methanol–acetonitrile–water; 8:2:1, v/v). Precursor ion of MS/MS is *m/z* 542.7.

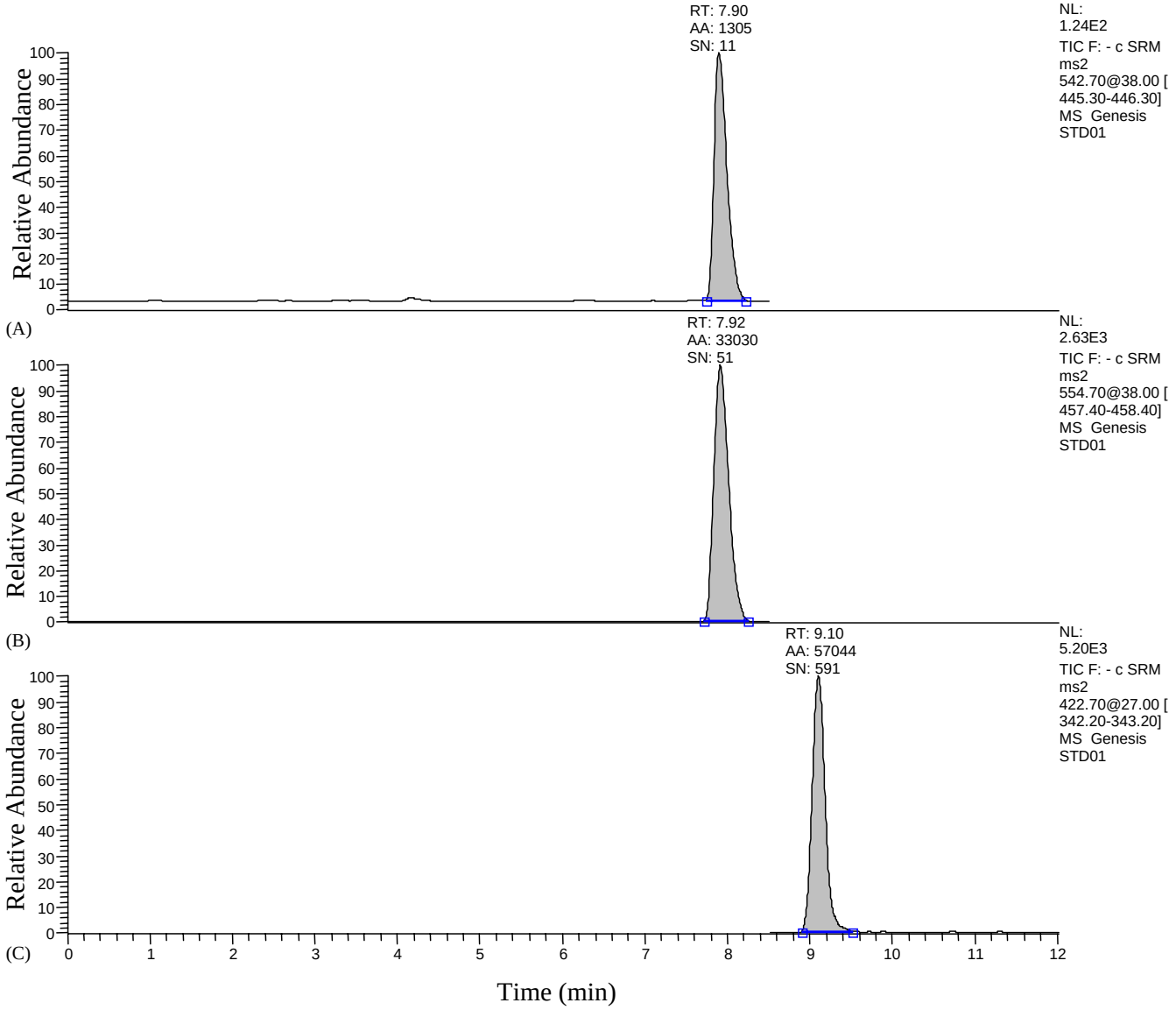

Fig. 3. Ion chromatograms for the LC–MS/MS analysis of TBBPA, $^{13}C_{12}$-TBBPA, and TBCr. (A) Selected reaction monitoring MS/MS chromatogram of TBBPA (0.05 ng/mL; *m/z* 542.7 → 445.8) (B) selected reaction monitoring MS/MS chromatogram of $^{13}C_{12}$-TBBPA (1 ng/mL; *m/z* 554.7 → 457.9) (C) selected reaction monitoring MS/MS chromatogram of TBCr (5 ng/mL; *m/z* 422.7 → 342.7).

The MS/MS detection conditions (ion source voltage, temperature of the heated capillary, the flow-rate of nitrogen gas, and the energy of CID) were also optimized to afford the highest relative intensity. The conditions described in Section 2.5 were selected. Under the selected conditions, the MS and MS/MS spectra of TBBPA and typical ion chromatograms were depicted in Figs. 2 and 3, respectively.

3.2. Pretreatment of serum samples

The previous methods developed for the determination of brominated flame-retardants (TBBPA and polybrominated diphenyl ethers) in human serum [9,10] was modified for extraction of TBBPA from serum. Though concentrated sulfuric acid had been used to decompose lipids in serum samples [9,10], we used SPE on the silica cartridge to remove the lipids for reproducible results and experimental safety. Under the selected SPE conditions, spiked TBBPA in serum were recovered satisfactory. In the present method, firstly hydrophilic concomitants in serum were removed by SPE on the reversed-phase cartridge, and then hydrophobic ones (mainly lipids) were removed by the normal phase SPE.

The recoveries were determined using the calibration solutions for the LC–MS/MS method after correction of instrumental errors by the peak area of TBCr. The recoveries (percentage, mean ± standard deviation, $n = 6$ each) of 5, 10, 50, and 100 pg TBBPA per g serum added to the control serum sample were 83.3 ± 8.3, 84.0 ± 8.4, 103.8 ± 6.7, and 101.9 ± 7.6, respectively, throughout the whole procedure.

3.3. Calibration graph, precision and limit of quantification

The detection limit (signal-to-noise ratio (S/N) = 3) of TBBPA in standard solution was 7.2 pg/mL, which was corresponded to 0.13 fmol on column. The relationship between the ratio of the peak area of TBBPA to that of $^{13}C_{12}$-TBBPA (internal standard) and the amounts of TBBPA standard was linear over the concentration range of 20–2000 pg/mL, and the linear correlation coefficient was 0.9999 ($n = 6$). The inter-day precision values were established by repeated determinations ($n = 5$) using standard solutions (50, 100, 500, and 1000 pg/mL); the relative standard deviations were within 6.8%.

The detection limit ($S/N = 3$) of TBBPA in serum sample by extrapolation using non-spiked and spiked serum samples was 0.8 pg per g serum. The limit of quantification for serum sample set to the value (average of the procedure blank ($n = 10$) + 10 × standard deviation of the procedure blank ($n = 10$)) was 4.1 pg per g serum, which was corresponded to 0.63 fmol on column. A good linear relationship was observed between the ratio of the peak area of TBBPA to that of $^{13}C_{12}$-TBBPA and the amount of TBBPA added to control serum in five concentration levels (non-spiked and spiked 5, 10, 50, and 100 pg per g serum); the linear correlation coefficient ($n = 6$) was 0.9996. The inter-day precision values were established by repeated determinations ($n = 5$) using non-spiked control serum (6.3 pg per g serum) and the serum spiked with TBBPA (added 5, 10, 50, and 100 pg per g serum); the relative standard deviations were 7.0, 9.9, 10.0, 6.5, and 7.4%, respectively.

These results show that the present internal standard method permits the sensitive and precise determination of TBBPA in serum samples.

3.4. Determination of TBBPA in human serum

Fig. 4 shows typical ion chromatograms obtained with the control serum samples. The concentrations of TBBPA in

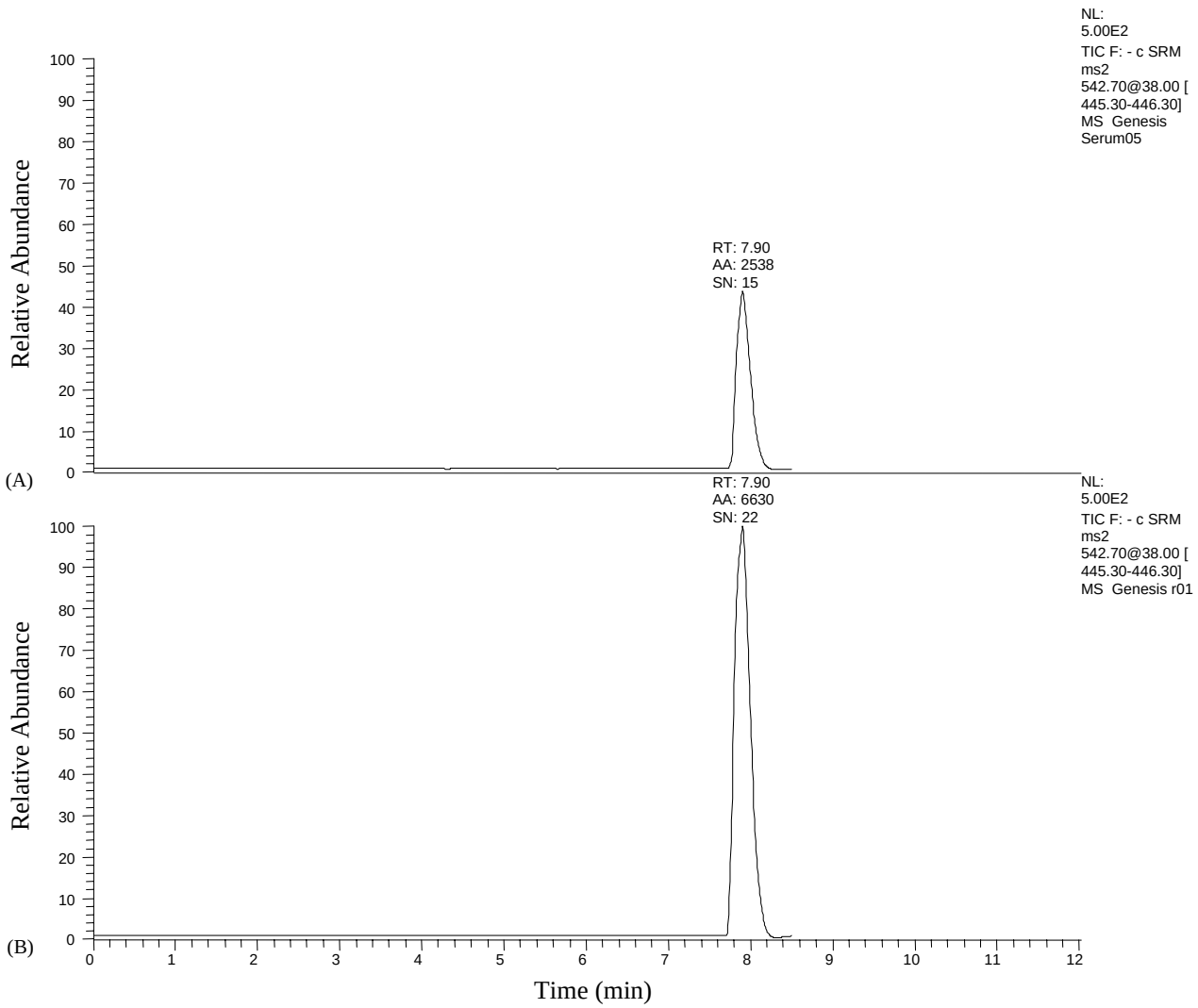

Fig. 4. Selected reaction monitoring MS/MS chromatograms obtained with control serum samples. (A) Control serum (6.3 pg per g serum) (B) the serum spiked with TBBPA (added 10 pg per g serum).

Table 1
Concentration of TBBPA in normal human serum

Sex[a]	TBBPA (pg per g serum)
M	6.7
M	6.2
M	8.3
M	7.1
M	8.7
Mean	7.40
S.D.	1.06
M (control)	6.3

[a] M, male.

serum samples from healthy volunteers and control serum sample are given in Table 1. Our quantification values of the concentration of TBBPA in serum samples from Japanese healthy volunteers are almost the same as European levels [11–13], though Asia is the largest consumer of brominated flame-retardants containing TBBPA in the world (more than 70%) [20].

4. Conclusion

The proposed LC–MS/MS method for the determination of TBBPA in human serum is simpler than the previous GC methods, because the derivatization step is not necessary. The validation data of this method shows the satisfied results in wide dynamic range. Therefore, proposed LC–MS/MS method should be useful for the clinical and environmental studies. Further examinations concerning the relationship between the blood concentrations of TBBPA and environment such as age, occupation and so on are now in progress.

Acknowledgements

This work was supported in part by Grant-in-Aids for Encouragement of Young Scientist (B) (Nos. 13771424 and 16790095) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] WHO, Environmental Health Criteria 172, Tetrabromobisphenol A and derivatives, World Health Organization, Geneva, Switzerland, 1995.
[2] I. Watanabe, T. Kashimoto, R. Tatsukawa, Bull. Environ. Contam. Toxicol. 31 (1983) 48.
[3] U. Sellström, B. Jansson, Chemosphere 31 (1995) 3085.
[4] U. Sellström, A. Kierkegaard, T. Alsberg, C. Wahlberg, C. de Wit, Organohalogen Compd. 40 (1999) 383.
[5] Å. Bergmman, C. Östman, R. Nybom, A. Sjödin, H. Carlsson, U. Nilsson, C.A. Wachtmeister, Organohalogen Compd. 33 (1997) 414.
[6] A. Sjödin, H. Carlsson, K. Thuresson, S. Sjölin, Å. Bergman, C. Östman, Environ. Sci. Technol. 35 (2001) 448.
[7] L. Hovander, M. Athanasiadou, L. Asplund, E. Klasson-Wehler, Organohalogen Compd. 35 (1998) 115.
[8] E. Klasson-Wehler, L. Hovander, Å. Bergmman, Organohalogen Compd. 33 (1997) 420.
[9] C. Thomsen, K. Janák, E. Lundanes, G. Becher, J. Chromatogr. B 750 (2001) 1.
[10] C. Thomsen, E. Lundanes, G. Becher, J. Sep. Sci. 24 (2001) 282.
[11] K. Jakobsson, K. Thuresson, L. Rylander, A. Sjödin, L. Hagmar, Å. Bergman, Chemosphere 46 (2002) 709.
[12] C. Thomsen, E. Lundanes, G. Becher, J. Environ. Monit. 3 (2001) 366.
[13] C. Thomsen, E. Lundanes, G. Becher, Environ. Sci. Technol. 36 (2002) 1414.
[14] M. Careri, A. Mangia, M. Musci, J. Chromatogr. A 794 (1998) 263.
[15] W. Mück, Pharmazie 54 (1999) 639.
[16] D. Zhong, X. Chen, J. Gu, X. Li, J. Guo, Clin. Chim. Acta 313 (2001) 147.
[17] J. Sherma, J. AOAC Int. 86 (2003) 873.
[18] M.J. Lopez de Alda, S.D. Cruz, M. Petrovic, D. Barceló, J. Chromatogr. A 1000 (2003) 503.
[19] R. Kostiainen, T. Kotiaho, T. Kuuranne, S. Auriola, J. Mass Spectromet. 38 (2003) 357.
[20] T. Hyötyläinen, K. Hartonen, Trends Anal. Chem. 21 (2002) 13.