HBCD stereoisomers: Thermal interconversion and enantiospecific trace analysis in biota

Robert Köppen[a], Roland Becker[a], Marcus Weber[b], Vedat Durmaz[b], Irene Nehls[a]

[a] Department of Analytical Chemistry, Reference Materials, BAM Federal Institute for Materials Research and Testing, 12489 Berlin, Germany
[b] Department of Numerical Analysis, Computational Drug Design, Zuse Institute Berlin (ZIB), 14195 Berlin, Germany

Introduction

1,2,5,6,9,10-Hexabromocylcododecane (HBCD), Fig. 1, is a major flame retardant and increasingly found in environmental compartments[1] and biota[2, 3]. The complex stereoisomerism[4] of HBCD is a challenge for its trace quantification and a comprehensive risk assessment requires knowledge on the behaviour of diastereomers and enantiomers during analysis in relevant matrices and in the food chain. Here, recent experimental and theoretical contributions to the understanding of the behaviour of HBCD stereoisomers under thermal stress are presented. Furthermore, the enantiospecific quantification of HBCD enantiomers in fish by LC-MS/MS using a gradient-free eluent is described.

Materials and Methods

The purification and characterisation of the investigated HBCD enantiomers is described in detail somewhere else[5]. For the interconversion experiments neat (+)-γ-HBCD was exposed to a time series at 160 °C and afterwards analysed for the resulting composition of stereoisomers[6]. For this purpose HPLC-DAD was applicable. Simulations of the interconversion of α-, β-, and γ-HBCD were done with the Merck Molecular Force Field designed for molecules of the size of HBCD[7, 8]. The hybrid Monte-Carlo method (HMC)[9, 10] a combination of a Markov Chain Monte-Carlo approach with short time molecular dynamics (MD) simulations (78 fs, using a velocity verlet integrator[11] with a time-step of 1.3 fs) was applied. The HMC-samplings for the three HBCD-stereoisomers required 30 million force field evaluations (CPU-time on a standard PC: 6 hours).
Fish fillets were cryo-ground on a centrifugal mill (liquid N_2, 500 µm, ZM 1000; Retsch GmbH, Haan, Germany), then lyophilised on a Lyovac GT2 (FINN-AQUA GmbH, Hürth, Germany), homogenised, and stored at -20 °C until extraction. Between 0.2 and 1.5 g of the fish powder were extracted (ASE 200, Dionex, Sunnyvale, USA) with ethyl acetate after addition of 50 µL of a 450 ng g^{-1} solution of $^{13}C_{12}$-labelled α-, β- and γ-HBCD (11 mL cells; void volume filled with hydromatrix; 3 cycles: 100 °C; 5 min; 140 bar). The extracts were concentrated to 10 mL under a stream of nitrogen and co-extracted lipids were removed using an automated GPC system (GPC VARIO, LCTech, Dorfen, Germany) equipped with an automatic injector, a fraction collector, and a S-X3 Bio-Beads gel permeation column (500 mm x 40 mm, L x OD, 50 g of 200 – 400 mesh). Injection volume was 6 mL of extract and cyclohexane/ethyl acetate (1:1, v:v) was used as mobile phase (4 mL min^{-1}). The fraction from 20.0 to 30.0 min was evaporated to dryness, re-dissolved in n-hexane and cleaned further on 1 g pre-treated florisil (24 h heated at 160 ºC) with n-hexane (5 mL) and n-hexane/dichloromethane (1:1, v:v, 13 mL). Extracts were evaporated to dryness using a gentle stream of nitrogen and re-dissolved in 300 µL methanol for LC-MS/MS analysis. The lipid contents of the biota samples were determined gravimetrically from the respective GPC fraction. Quantification of HBCD was performed on an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) HPLC binary pump system equipped with a vacuum degasser, a thermostatted autosampler and a thermostatted column compartment, which was coupled to a API 4000™ triple-quadrupole mass spectrometer from Applied Biosystems / MDS SCIEX (Foster City, Ca. USA / Concord, Ontario, Canada) run in the electrospray negative ionisation (ESI^-) mode. Stereoisomers were separated using a Zorbax XDB-C18 (double end-capped, pore size: 80 Å, Agilent Technologies, Waldbronn, Germany) followed by a NUCLEODEX β-PM (pore size: 100 Å, Macherey-Nagel GmbH & Co, Düren, Germany) analytical column maintained at 15 °C (both columns: 5 µm particle size, 200 x 4.6 mm). Isocratic LC runs using 10 mM ammonium acetate buffer and acetonitrile:methanol (90:10, v:v) lasted 35 min per sample (flow rate:

350 µL min^{-1}). The MS/MS parameters were optimized by flow injection analysis. The monitored transitions for HBCD were 640.6 → 79 (native) and 652.6 → 79 ($^{13}C_{12}$ labelled). Data were processed by the Analyst 1.4.1 software package (Applied Biosystems / MDS SCIEX). Details regarding MS parameters, the control of blank values, the determination of detection limits and enantiomeric fractions are given elsewhere[12].

Results and Discussions

Interconversion of HBCD stereoisomers

The alteration of the composition of technical HBCD at elevated temperatures was reported by Peled[13] and confirmed recently[14]. γ-HBCD predominates in the technical product and α-HBCD is the main component after thermal equilibration.

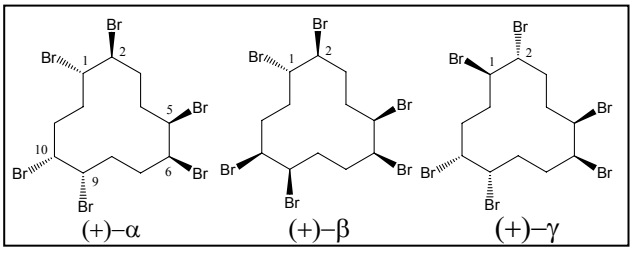

Fig. 1: HBCD diastereomers

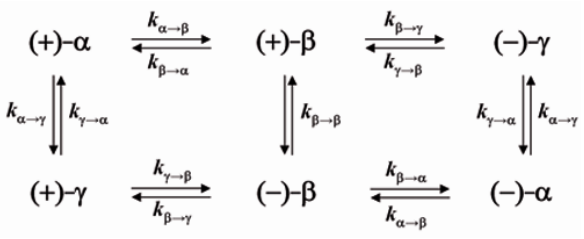

Fig. 2: Pathways of thermal interconversion

Pure enantiomers of α-, β-, and γ-HBCD (Fig. 1) were recently characterised[5] and their availability enabled the elucidation of the interconversion pathways and kinetics[6]. Fig. 2 shows the isomerisation reactions, which drive any given composition of α-, β-, and γ-HBCD towards the oscillating mixture of all six stereoisomers with the α-enantiomers as main component. This leads to the idea that classical simulations can be used in order to characterise the interconversion processes qualitatively. Since an interconversion requires the less favoured *anti* position of the bromine atoms of the involved (BrHC-CHBr)-moiety it was investigated if the energy differences between *anti* and *gauche* positions as derived from MD simulation correlate with the experimentally observed interconversion rates and can be used to describe the HBCD stereoisomer composition at equilibrium. Thus, the respective parts of the configurational space corresponding to the *gauche*- and the *anti*-positions were compared via computer simulation for each (BrHC-CHBr)-moiety of α-, β-, and γ-HBCD and expressed in terms of *free energy* differences (Table 1). The simulations were carried out for the vacuum as an approximation and are based on the *Boltzmann* distribution (canonical ensemble) of states, which is the most likely distribution of states at constant temperature, constant number of particles and constant volume.

Table 1: Free energy differences $\Delta_{ga}A$ (kJ mol^{-1}) and mean potential energies $\langle V_{pot} \rangle$ (kJ mol^{-1})

	(+)-*α*-HBCD		(+)-*β*-HBCD		(+)-*γ*-HBCD	
	$\Delta_{ga}A$	interconverts to:	$\Delta_{ga}A$	interconverts to:	$\Delta_{ga}A$	interconverts to:
C_1C_2	-13	(+)-*γ*	-9	(+)-*β*	-1	(+)-*α*
C_5C_6	-33	(+)-*β*	-30	(+)-*γ*	-28	(+)-*β*
C_9C_{10}	-32	(+)-*β*	-25	(+)-*α*	-28	(+)-*β*

The simulated free energy differences $\Delta_{ga}A$ between *gauche* and *anti* conformational spaces are lowest in case of the C_1C_2-moiety of α-HBCD followed by the C_1C_2-moiety of β-HBCD[15]. This corresponds to the experimental observation[6] that the interconversion α→γ is the fastest followed by the racemisation reaction (+)-β ↔ (-)-β. The dependency of the rates *k* on the respective free energy differences $\Delta_{ga}A$ according to the *Arrhenius* equation (1) allowed to derive the ratio $k_{\gamma\to\alpha} : k_{\beta\to\beta} : k_{others}$ as 1 : 0.6 : 10^{-1}-10^{-2} for 160 °C, which is in good agreement with experiment[6] and explains well the stereoisomer composition at thermal equilibrium.

$$k \propto \exp\left(-\frac{1}{T}\Delta_{ag}A\right) \qquad (1)$$

Quantification of HBCD stereoisomers

So far, trace determinations of HBCD were mostly done diastereomer-specific using LC-MS/MS. Few reports on the enantiomer-specific determination of HBCD in fish samples[14,16] are currently available. A comprehensive risk assessment would benefit from detailed knowledge on enantiomeric ratios in relevant biota.

Usually, HPLC analyses are done with a gradient of eluent in order to improve separation of analytes. The mass spectrometer employed for this work did not enable constant ionisation conditions as long as a solvent gradient was used. Therefore, isocratic LC conditions were applied and appropriate separation of the stereoisomers was achieved by a combination of an achiral column and a chiral cyclodextrin-based column. The described conditions enabled base line separations of all six HBCD stereoisomers (Fig. 3) and direct quantification from area counts. Advantageously, this procedure circumvented the consideration of baseline corrections.

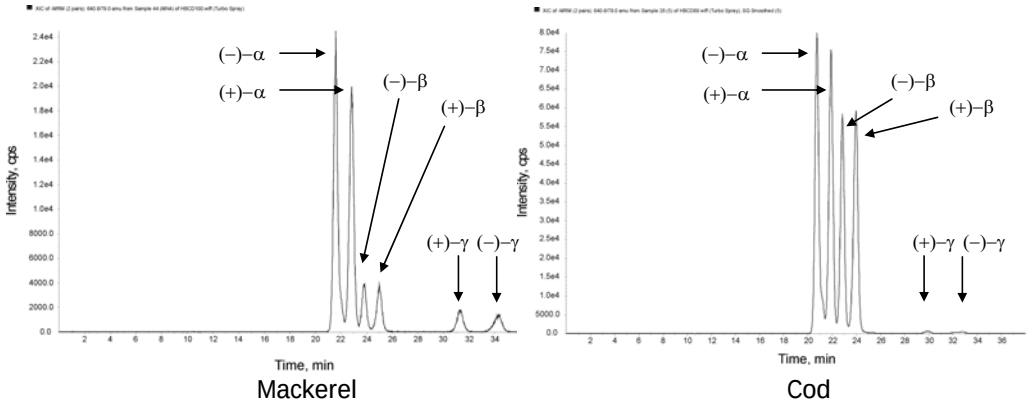

Fig. 3: LC separation of the HBCD enantiomers form fish extracts using a C18 and a chiral column

The relative response factors of α, β, and γ behaved like 1.00:3.35:1.38, which correlates with a literature report[17]. Limits of detection of a given enantiomer ranged from 6 to 20 pg g^{-1} and limits of quantification between 20 and 70 pg g^{-1}, which was seen to be sufficiently low to quantify the six main enantiomers in all investigated biota samples. The chromatographic behaviour of HBCD under the applied conditions includes the separation of the diastereomers by the achiral C18 phase in the well established order of elution (α, β, γ) and then the chiral separation in the known[5, 14] order (-)-α, (+)-α, followed by (-)-β, (+)-β, and then (+)-γ, (-)-γ. Other than in case of the exclusive use of a chiral column both α enantiomers elute prior to the β enantiomers. Each pair of enantiomers interacts with the cyclodextrin phase such that the (1R, 2R) configurations elute first. Table 2 comprises the results obtained from a number of fishes sampled at one specific site. The enantiomeric fractions could be determined with an uncertainty of 5–11% depending on diastereomer and concentration and were in most cases significantly shifted towards (-)-α and (-)-β. HBCD levels were always above the limit of quantification and as generally observed in biota[2, 3] α-HBCD was throughout the dominating diastereomer. A look at the trophic level as estimated from the $\delta^{15}N$ and $\delta^{13}C$ values and from general knowledge on the relative position of investigated species in the food chain the observed HBCD concentrations and stereoisomeric patterns may be comprised as follows: There is a clear tendency for HBCD to accumulate in fish species of higher trophic level. The predominance of α over β and γ correlates with the elution from the achiral C18 phase as does the accumulation of the (-)-enantiomers in case of α and β with the same behaviour on the chiral phase. The ratio of γ-HBCD to α and β is greatest in skate and flounder which correlates with their living on the ground whose HCBD contamination is known[14] to reflect the isomer pattern of the technical product while the relatively high concentration of β-HBCD in cod is surprising. The alteration of the isomeric pattern with passage from abiotic to biotic matrices tempts to assume a bioisomerisation[18,19] of HBCD similar to the thermally induced interconversion discussed above. Detailed investigations may provide evidence for this concept and answer the question if the solubility of the diastereomers in water, which decreases along the line α, β, γ[20, 21] may be another factor for their biomagnification.

Table 2: Concentrations of HBCD isomers (ng g^{-1}, lipid weight) in fish samples from the Etnefjord (Norway)

Species	(-)-α	(+)-α	(-)-β	(+)-β	(+)-γ	(-)-γ
Mackerel *Scomber scombrus*	98.35 ± 9.01	85.79 ± 7.77	5.83 ± 0.55	5.41 ± 0.50	11.53 ± 1.05	12.02 ± 1.08
Codfish *Gadus morhua*	11140 ± 970	10110 ± 890	4000 ± 350	4850 ± 420	117.8 ± 10.22	98.99 ± 8.81
Thorny skate *Amblyraja radiata*	853.7 ± 74.46	622.8 ± 54.83	51.27 ± 4.49	35.27 ± 3.08	85.22 ± 7.62	68.23 ± 5.98
Pollack *P. pollachius*	577.7 ± 51.15	512.9 ± 44.30	80.01 ± 7.01	70.47 ± 6.25	26.87 ± 2.37	27.95 ± 2.46
Flounder *Platichthys flesus*	245.1 ± 21.24	165.5 ± 14.41	30.15 ± 2.64	22.54 ± 1.96	101.71 ± 8.78	116.4 ± 10.04

$\delta^{13}C$ and $\delta^{15}N$ values (‰): Mackerel: (-21.80; 11.71), Cod: (-17.93; 13.49), Skate: (-19.15; 11.53), Pollack: (-23.49; 13.84), Flounder: (-18.69, 11.78), References: (VPDB; Air).

Acknowledgements

The simulation of the interconversion of HBCD was supported in the framework of the BAM-ZIB cooperation on common research and development in the field of scientific computing. Date of agreement: July 10th, 2007.

References

1. De Wit CA. *Chemosphere* 2002; 40: 583.
2. De Wit CA, Alaee M, Muir DCG. *Chemosphere*, 2006; 64: 209.
3. Covaci A, Gerecke ASC, Law RJ, Voorspoels S, Kohler M, Heeb NV, Leslie H, Allchin CR, De Boer J. *Environ Sci Technol* 2006; 40: 3680.
4. Becher G. *Chemosphere* 2005; 58: 989.
5. Koeppen R, Becker R, Emmerling F, Jung C, Nehls I. *Chirality* 2007; 19: 214.
6. Köppen R, Becker R, Jung C, Nehls I. *Chemosphere* 2008; 71: 656.
7. Halgren TA. *J Am Chem Soc* 1992; 114: 7827.
8. Halgren TA. *J Comp Chem* 1996; 17: 490.
9. Duane S, Kennedy AD, Pendleton BJ, Roweth D. *Phys Lett B* 1987; 195: 216.
10. Schütte C, Fischer A, Huisinga W, Deuflhard P. *J Comput Phys* 1999; 151: 146.
11. Frenkel D, Smit B, Understanding Molecular Simulation – From Algorithms to Applications, in Computational Science Series, Vol. 1, Academic Press, 2002.
12. Köppen R. Thesis (2008), Humboldt University, Berlin.
13. Peled M, Scharia R, Sondack D, in Advances in Organobromine Chemistry. Eds.: J.R. Desmurs, B. Gerard, Elsevier, Amsterdam, 92 (1995).
14. Janak K, Covaci A, Voorspoels S, Becher G. *Environ Sci Technol* 2005; 39: 1987.
15. Weber M, Becker R, Durmaz V, Köppen R. *Mol Simulat*, accepted.
16. Marvin CH, MacInnis G, Alaee M, Arsenault G, Tomy GT. *Rapid Commun Mass Spectrom* 2007; 21: 1925.
17. Suzuki S, Hasegawa A. *Anal Sci* 2006; 22: 469.
18. Tomy G, Budakowski W, Halldorson T, Whittle DM, Keir MJ, Marvin C, MacInnis G, Alaee M. *Environ Sci Technol* 2004; 38: 2298.
19. Zegers BN, Mets A, van Bommel R, Minkenberg C, Hamers T, Kamstra JH, Pierce GJ, Boon N JP. *Environ Sci Technol* 2005; 39: 2095.
20. Hunziker RW, Gonsior S, MacGregor JA, Desjardins D, Ariano J, Friederich U. *Organohalogen Compd* 2004; 66: 2300.
21. Kuramochi H, Suzuki S, Kawamoto K, Sakai S. 4th Conference on Brominated Flame Retardants. 24-27 April 2007, Amsterdam.