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POLYAROMATIC HYDROCARBONS AS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CALIBRATION STANDARDS FOR THE LOW LEVEL DETERMINATION OF CHLORINATED DIBENZO-*p*-DIOXINS AND CHLORINATED DIBENZOFURANS IN BIOLOGICAL SAMPLES

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SUMMARY

A series of polyaromatic hydrocarbons (PAHs) has been used to calibrate the retention times of chlorinated dibenzo-*p*-dioxins (dioxins) and chlorinated dibenzofurans (furans) on high-performance liquid chromatography (HPLC) in the ultra-trace analysis of biological samples. Fraction collection is then based on the HPLC retention times of the PAHs without the need for the dioxins or furans. Because of the variety of PAHs, a particular one can be chosen that will co-chromatograph near any given dioxin or furan. It has been shown that the effect of solvent changes and sample co-extractives on the HPLC eluting properties of the PAHs is minimal. The use of PAHs as secondary standards for HPLC calibration in dioxin trace analysis diminishes both the potential for contamination of the sample by the standard and the handling of the chlorinated dioxins and furans. The PAH standards could also be useful for those laboratories who are limited in their safety facilities or in the number and amount of dioxin–furan standards available to them.

INTRODUCTION

The determination of chlorinated dibenzo-*p*-dioxins (dioxins) and chlorinated dibenzofurans (furans) in biological samples at part per trillion (ppt)* levels has attracted a great deal of effort in the last five years[1]. This activity has resulted from the high toxicity of some of the dioxins and furans combined with their occurrence in diverse environmental and food samples. The analytical schemes that have been devised to carry out this determination must be highly specific and extremely sensitive. As a result these techniques are often elaborate with many steps. One of the cleanup and separation steps that is currently used[2–6] by many laboratories carrying out this type of analysis is reversed-phase high-performance liquid chromatography (HPLC). A typical scenario for a biological matrix at ppt levels would be: extraction, purification by column chromatography, solvent concentration, and injection of the

* Throughout this article the American trillion (10^{12}) is meant.

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entire sample on reversed-phase HPLC followed by fraction collection for gas chromatography–mass spectrometry (GC–MS) determination. The most sensitive HPLC detection mode for the dioxins and furans is ultraviolet (UV) absorbance (5–50 ng) which contrasts markedly with the sensitivity of GC–MS (0.1–10 pg). Moreover, the presence of UV absorbing coextracted material in sample extracts often completely obscures the HPLC pattern at low attenuation. As a result the retention time of the HPLC column must first be calibrated with relatively high levels (10–100 ng) of dioxins or furans before the samples containing small amounts (10–1000 pg) of the same compounds can be injected and fractions collected. The possibility of contamination of the low-level sample by the high-level HPLC calibration standard is a distinct possibility. In addition, each calibration (usually carried out at least once a day) necessitates the handling of relatively large amounts of the highly toxic chlorinated standards. This report describes the use of polyaromatic hydrocarbons (PAHs) as secondary standards for the calibration of the HPLC column in the determination of low levels of several dioxins and furans in biological samples. Fraction collection from the HPLC is then based on the retention time of PAHs without the need for the dioxins or furans.

EXPERIMENTAL

Reagents

All solvents for HPLC were HPLC grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). These were filtered through a Millipore filter (type FH; 0.5 μm) and degassed before use. Other solvents for sample extraction and cleanup were from Caledon Labs. (Georgetown, Canada).

Standards

Some dioxin standards originated as reported[4]. Other dioxins and furans were generous gifts of either D. Firestone (Food and Drug Administration, Washington, DC, U.S.A.) or D. Hallett (Canadian Wildlife Service, Hull, Canada). PAHs were purchased from ICN Pharmaceuticals (Montreal, Canada). Other chlorinated aromatics were obtained from either Analabs (North Haven, CT, U.S.A.) or RFR (Hope, RI, U.S.A.). Standards were used as received. Typically, a stock solution of 100–500 μg/ml was prepared by weighing an aliquot on an analytical balance and by dissolution in methanol or toluene. A more dilute solution (1–10 μg/ml) in methanol was then made from the stock solution.

HPLC

The system used for the sample cleanup consisted of a Waters M-45 pump (Waters Assoc., Milford, MA, U.S.A.) connected to a 250 × 4.6 mm I.D., 10 μm, LiChrosorb C_{18} (Merck) column (Brownlee Labs., Santa Clara, CA, U.S.A.), at room temperature with 100% methanol at 1.0 ml/min as mobile phase. Injection was carried out on a Rheodyne Model 7125 valve (Catali, CA) with a 100-μl loop. Detection was achieved by a Schoeffel SF 770 variable-wavelength detector (Kratos, Westwood, NJ, U.S.A.) containing an 8-μl flow-cell with output to a 1-mV recorder. Usually the wavelength chosen was 235 nm, near the absorbance maximum of 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD). Absorbance setting on the detector was 0.01 units when a 10–50 ng standard was injected.

Sample preparation and analysis

Fish and liver samples were routinely extracted and purified as in reference[4] except for the following: [^{13}C]2,3,7,8-TCDD and [^{37}Cl]octachlorodibenzodioxin (OCDD) were added as internal standards at the beginning for recovery purposes and the wash of the Florisil column was carried out with 2% dichloromethane in hexane instead of 100% hexane. The gas chromatograph was fitted with a 15-m fused-silica wall-coated chromatographic column (0.25 mm I.D.) containing DB-5 (J & W Scientific) (equivalent to SE-54) as stationary phase, coupled directly to the mass spectrometer. The amount of native TCDD in the extract was obtained by comparison of the signal between the extract and a standard at m/z 320 while recovery values were obtained similarly at m/z 332. The gas chromatograph was cold-trap injected at 80°C for 1 min, heated quickly to 180°C, held for 1 min, then temperature programmed between 180–250°C at 5°C/min with helium (linear velocity 40 cm/sec) as the carrier gas giving a retention time between 9 and 11 min for 2,3,7,8-TCDD. Similarly, GC conditions were chosen for the other dioxins to obtain a retention time between 9–14 min.

RESULTS AND DISCUSSION

In our low-level analysis of biological matrices for dioxins using HPLC as a cleanup technique, usually three to four HPLC fractions were taken whose number and content are outlined in Table I. Not all fractions from each sample were assayed by GC–MS as collection depended on the nature and history of the sample. The large collection volume did allow a wide spectrum of both dioxins and furans to be determined. Because of contamination and safety concerns we looked for a set of HPLC

TABLE I

HPLC FRACTIONS COLLECTED FROM REVERSED-PHASE HPLC FOR DIOXIN AND FURAN DETERMINATION AND CONTENT OF EACH FRACTION

HPLC conditions as in experimental. T = tetra; P = penta; H = hexa; Hp = hepta; O = octa; CD = chlorinated dibenzo; D = dioxin; F = furan. Numbers from 1 to 8 not interrupted with commas refer to positions on either the dioxin or furan ring system substituted with chlorine.

Name of fraction	*Elution time, min (vol., ml)*	*Content*
Tetra	7–12 (5)	TCDD —2378, 1234, 1368, 1379, 1237/1238, 1278, 1378 TCDF —2378, 2368 PCDD —12478 (part) PCDF —23478, 23467
Hexa	12–17 (5)	PCDD —12378, 12478 (part) PCDF —12367, 12378 HCDD —123678, 123789, 123478, 123679, 12478 HCDF —234678
Hepta	17–23 (6)	HCDF —123678 HpCDD —1234678, 1234679
Octa	23–30 (7)	OCDD
Hepta-F	30–37 (7)	HpCDF
Octa-F	37–45 (8)	OCDF

standards which: (a) eluted on reversed-phase HPLC in methanol within 0.5 to 1.0 min of the dioxins/furans; (b) were less toxic than the dioxins/furans; (c) had a lower molecular weight; (d) did not contain chlorine; (e) had reproducible HPLC retention times for slight changes of solvent polarity and (f) were not unduly effected by coextracted substances from the sample.

In the beginning our efforts focussed on halogenated aromatic compounds such as polychlorinated biphenyls (PCBs), polybrominated biphenyls and polychlorinated diphenylethers. While some of these were promising, most members of this group eluted earlier on reversed-phase HPLC than the dioxins and furans so that none were suitable for the later eluting higher chlorinated congeners. In addition, since they contained chlorine, their mass spectra exhibited a halide cluster and often showed molecular or fragment ions with the same nominal mass as the dioxins and furans. Consequently, they could not be used.

A series of polyaromatic hydrocarbons was available from a previous study and these were evaluated. Table II lists the HPLC retention times of a number of these with conditions as in the experimental section. The spectrum of the retention time is quite large running from almost unretained to over 30 min. In general, the larger the molecular weight of these compounds the longer the retention time on reversed-phase HPLC with some exceptions. Wise *et al.*[7] carried out a study of retention times of PAHs reversed-phase HPLC. Their finding of an empirical relationship between length/breadth and retention time appears to hold for the series of PAHs in Table II.

Since we had been in the habit of using 2,3,7,8-TCDD, 1,2,3,6,7,8-hexachlorodibenzodioxin (1,2,3,6,7,8-HCDD) and OCDD to calibrate the retention time of our HPLC instrument, three of the PAHs were chosen with retention times similar to the above three dioxins. For this purpose, 1,2,5,6-dibenzanthracene (DBA), 1,2,7,8-di-

TABLE II

RETENTION TIMES OF A SERIES OF PAHs ON REVERSED-PHASE HPLC WITH METHANOL ELUTION

PAH	*No. rings*	*Mol. wt.*	*Retention time (min)*
9,10-Diphenylanthracene	5	254	6.0
2,3-Dibenzofluorene	5	266	6.3
Benzo[*e*]pyrene	5	252	8.6
Perylene	5	252	8.8
1,2,3,4-Dibenzanthracene	5	278	9.0
Rubrene	8	532	9.5
Benzo[*a*]pyrene	5	252	10.0
1,2,5,6-Dibenzanthracene	5	278	10.8
2,3,6,7-Dibenzanthracene	5	278	13.2
1,2,7,8-Dibenzphenanthrene	5	278	14.2
3-Methylcholanthrene	5	268	14.5
1,2,4,5-Dibenzpyrene	6	302	17.5
Coronene	7	300	23.2
3,4,9,10-Dibenzpyrene	6	302	26.0
3,4,8,9-Dibenzpyrene	6	302	30.3

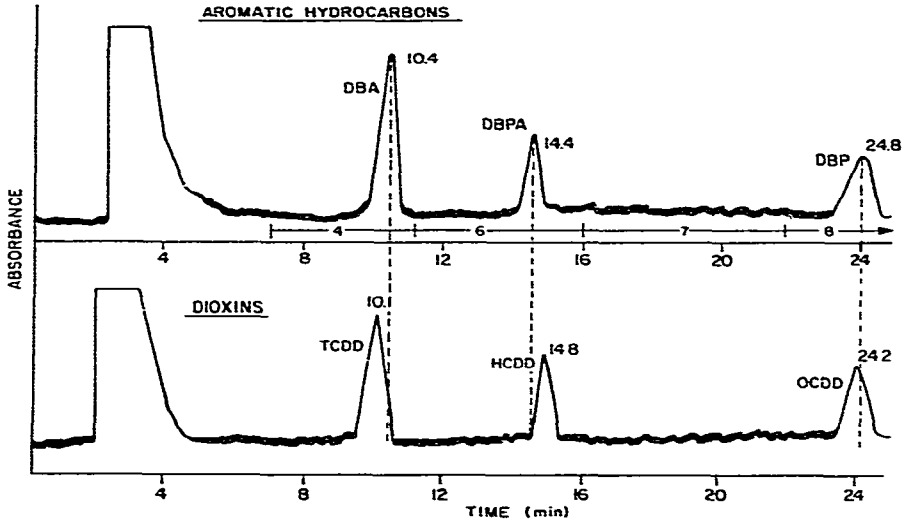

Fig. 1. Diagram of chromatograms obtained from reversed-phase HPLC of a set of PAH standards (upper) and dioxin standards (lower). Absorbance units are 0.01 full scale and 20–50 ng of each standard have been injected with UV detection at 235 nm with a mobile phase of 100% methanol at 1.0 ml/min on a 250 × 4.6 mm I.D. LiChrosorb C_{18} column. DBA = 1,2,5,6-Dibenzanthracene; DBPA = 1,2,7,8-dibenzphenanthrene; DBP = 3,4,9,10-dibenzpyrene; TCDD = 2,3,7,8-tetrachlorodibenzodioxin; HCDD = 1,2,3,6,7,8-hexachlorodibenzodioxin.

benzphenanthrene (DBPA) and 3,4,9,10-dibenzpyrene (DBP) were taken and studied further. Fig. 1 shows a tracing of an HPLC chromatogram of these three PAHs compared to the three dioxin congeners. The three chosen PAHs elute within one minute of their respective dioxins. UV spectra of these PAHs in methanol show a variety of absorbance maxima between200 and 350–400 nm such that almost any UV wavelength could be used for detection including 210, 254 and 280 nm. Typically, about 20 ng of each of the three PAHs in methanol were injected into the HPLC system and retention times noted. The valve was then washed with methanol and the entire low-level sample in 50–70 μl acetonitrile plus a 25-μl methanol wash was injected onto the column. Fractions were then collected as follows: tetra fraction —5 min before the end of the 1,2,5,6-DBA peak; hexa fraction–end of tetra fraction to 1–1.5 min after end of 1,2,7,8-DBPA peak; hepta fraction— between hexa and octa fractions; octa fraction —2.0 min after end of 3,4,9,10-peak and 1 min before start of this peak. Fig. 1 (upper) just above the time scale gives an example of the fractionation chosen. Each fraction contains those dioxins and furans as listed in Table I plus some others for which standards are not available.

The partially purified sample extracts before HPLC still contained a large amount of extraneous material as evidenced by the HPLC–UV detector and GC–electron capture response. There was a possibility that the retention time of either the PAH standards or the dioxins/furans would change vis-a-vis each other or absolutely when the sample polarity changed. To investigate this more fully, the HPLC conditions for the mobile phase were changed slightly by the addition of either water (2%; longer retention time on reversed-phase HPLC) or dichloromethane (2%; shorter retention time) and the effect on the retention times noted. Table III lists these changed

TABLE III

EFFECT OF SOLVENT CHANGES ON THE REVERSED-PHASE HPLC RETENTION TIMES IN MIN OF SELECTED DIOXIN AND PAH STANDARDS

DBA = Dibenzanthracene; DBPA = dibenzphenanthracene; DBP = dibenzpyrene.

Compound	*Mobile Phase*		
	Methanol 100%	*2% Dichloromethane in methanol*	*2% Water in methanol*
1,2,5,6-DBA	10.4	9.9	14.0
2,3,7,8-TCDD	10.1	9.4	13.8
1,2,7,8-DBPA	14.4	12.7	18.3
1,2,3,6,7,8-HCDD	14.8	13.3	21.6
3,4,9,10-DBP	24.8	22.7	34.0
OCDD	24.2	22.1	40.5

retention times. For dichloromethane addition, both dioxins and PAHs decrease in retention time in almost the same amount and the effect is small. For water addition, the retention times increase for both classes of compounds with the dioxins, particularly OCDD, being more affected than the PAHs. Hence a large amount of water in a sample extract could effect the fraction collection and recovery while a non-polar solvent or co-extracted material in the sample extract would not appear to alter the retention time and recovery as much. Additionally, an oily fish (smelt) extract was cleaned up as described in the experimental part and, just before HPLC, spiked with a large amount (400 ng) of the three PAH standards (the high levels of PAHs were necessary because of other UV-absorbing extraneous material in the fish). This extract was run on the HPLC and the retention times of the PAHs in the sample were the same within experimental error as the PAH calibration standards. Values found for the three PAH were: 11.0, 14.8, 26.8 min, respectively, for the three calibration standards, and 10.8, 14.4, 26.0 min, respectively, for the three high-level PAH standards in the fish extract. An additional precaution exists in analysis of dioxin containing samples with the routine use of isotopically labelled internal standards in the MS system to monitor recoveries. Any change in the PAH retention time for whatever reason which could lead to false negatives would be picked up by MS in the recovery data and corrective steps could be taken where necessary. These experiments demonstrate the versatility of the PAHs in the analysis of sample extracts for dioxins and furan. Moreover, recoveries of the isotopically labelled [^{13}C]2,3,7,8-TCDD or [^{37}Cl]-OCDD have not been affected by the use of the PAH standards in fish, liver, fat and other matrices where low detection limits are paramount.

This technique of HPLC retention time calibration has not been applied to normal-phase HPLC, particularly silica gel, which has been used for the further separation of dioxin isomers in a complex mixture[3]. Nor would it prevent cross contamination between samples (false positives) particularly if analysis were being carried out with high levels as are found, for example, in fly ash. However, these PAH do have the advantage that they have little or no electron capture response, do not contain chlorine as do dioxins and furans, and have maximum m/z values in MS lower than tetrachlorinated dioxins and furans. While they are not harmless substances (some have toxic effects similar to or less than benzo[*a*]pyrene), they are

relatively less toxic than the dioxins and furans. The main virtues in trace analysis are their wide applicability and versatility to the dioxin–furan field, the minimization of contamination between standards and samples, and the need not to handle toxic chlorinated standards on a routine basis. Their use should be particularly suited in ultra-trace analysis to those laboratories which do not have elaborate safety facilities or are limited in the number and amount of standards available to them.

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