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SIMULTANEOUS DETERMINATION OF MONOPHENYLTIN, DIPHENYLTIN AND TRIPHENYLTIN BY HPLC WITH A UV DETECTOR AND ROLE OF THIOL COMPOUNDS

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ABSTRACT:

Simultaneous determination of monophenyltin (MPT), diphenyltin (DPT) and triphenyltin (TPT) by a UV detector was developed using high performance liquid chromatography (HPLC) with methanol–acetic acid–water mixtures containing thiol compounds as mobile phase. Peaks of TPT, DPT and MPT were completely separated and they were detected using UV detector at 257 nm. The effects of thiol compounds, *i.e.* dithiothreitol (DTT), dithioerythritol (DTE) and 2-mercapoethanol (ME) on the peak heights and retention times of phenyltin compounds were investigated, and the optimum performance was achieved with methanol–acetic acid–water (60:10:30 [v/v/v]) containing 5 mM DTE. The different affinity of thiol group with different phenyltins was found helpful to the separation of phenyltins. The developed method was successfully applied to the analysis of phenyltins in organotin biodegradation by *Pseudomonas aeruginosa*.

INTRODUCTION:

The production and use of synthetic chemicals in industry have led to the entry of many xenobiotics into the environment. One such group of xenobiotics is phenyltins, which has caused environmental concerns due to their acute toxicity such as impotence of neogastropods and gastropods (Bryan et al., 1988), inhibition of photosynthesis and respiration in marine micro algae (Mooney and Patching 1995), disruption both of physiological and morphological components of ion regulatory functions of an estuarine fish (Hartl et. al., 2001) and resistance to degradation. Triphenyltins are organo- metallic compounds used mainly as anti-fouling agents, pesticides, biostabilizers and fungicides. Environmentally much of the current interest in organotins focuses on fate and effect of triphenyltins on organisms (Fent et. al., 1998, Lo et. al., 2003, Hoch 2000), Studies have shown that even trace amount of phenyltins can have a significant detrimental effect on aquatic organisms (Hoch 2000).

Measurement of phenyltins in environmental samples has so far been proposed (Gonzalez- Toledo et. al., 2002, Gonzalez-Toledo et. al., 2001), especially by HPLC with fluorimetric detection (Gonzalez- Toledo et. al., 2002) as applied to determine diphenyltin (DPT) and tri- phenyltin (TPT). Lu et. al., (2004) have also reported the determination of TPT and DPT in culture medium by using HPLC with UV detection. Monophenyltin (MPT) is one of the major degradation products of TPT, however, the simultaneous determination of MPT together with DPT and TPT proved a complex matrix (Gonzalez-Toledo et. al., 2001, Lu et. al., 2004). Inoue et. al., (2003) used HPLC with fluorescent detector to detect MPT, DPT and TPT, requiring post-column derivation and its related accessory.

The purpose of this study was to develop

a simple and reprocible method for simultaneous analysis of TPT, DPT, and MPT phenyltin species in biodegraded samples. The effect of thiol compounds in the mobile phase of HPLC on phenyltins separation has also been investigated.

MATERIALS AND METHODS:

Chemicals and reagents: Monophenyltin trichloride (MPT, 98%), diphenyltin di-chloride (DPT, 96%) and triphenyltin chloride (TPT, 95%) from Aldrich Chemical Co. (Milwaukee,WI,USA) were used with out further purification. Dithiothreitol (DTT) obtained from Sino-American Biotech- nology Co. (Shanghai, China), 2- mercapto- ethanol (ME) from Amresco (Solon, OH, USA), dithioerythritol (DTE) from Feinbio chemica GmbH&Co. (Overath, Germany) and 5, 5'-dithiobis(2-nitroben zoate) (DTNB) from Sigma (St. Louis, MO, USA) were used. Methanol of HPLC grade (Shanghai Lab Reagent Co. Ltd., Shanghai, China) and all other reagents were of analytical reagent grade.

Standard stock solutions (1 mM) of TPT, DPT and MPT were prepared by dissolving the corresponding organotin chlorides in methanol and stored at 4ºC in dark glass bottles. The appropriate working solutions were prepared daily from the stock solutions and subsequent dilutions were freshly prepared in mobile phase not containing thiol compounds. DTNB (2 mM) was also prepared in methanol and stored at 4ºC before use.

HPLC analysis: Chromatographic separa--tion was performed on a Shimadzu (Kyoto, Japan) HPLC equipment (LC-10ATvp) with UV-Vis detector and a 20µl injection loop. A 250×4.6 mm ID reversed-phase column packed with 5 µm particles (Kromasil C_{18}) (Dalian Elite Analytical Instruments Co. Ltd., Dalian, China) was used. HPLC mobile phase consisted of methanol-acetic acid- water (60:10:30 v/v/v) containing DTT,DTE or ME and the mixture was degassed for 10 min by ultrasonic at 257 nm. All chromato- graphic separations were carried out at room temperature (25ºC) with a flow rate of 0.75 ml/min. Samples were filtered and 200µl of sample was injected each time. For fluorescent detection, it was carried out as reported by Inoue et. al., (2003).

Free thiol determination: Free thiol was reported to reduce one molecule of DTNB to two molecules of TNB, with a subsequent increase in absorbance at 412 nm (Ellman 1959). The absorbance (at 412 nm) of 100 µM DTNB in water was monitored after addition of 50 µM DTT by using a spectrophotometer (Unico UV2100, Shanghai, China). To check whether the organotin compounds react with the thiol group, they were preincubated with DTT for 3 min before the DTNB addition, and absorbance was measured at 412 nm.

Cultivation of TPT degrading bacterium and sample preparation for HPLC analysis: TPT degrading bacterium was screened in our laboratory and *Pseudomonas aeruginosa* CGMCC 1.860 was found to be the best strain [WQ Zhou, M.S. thesis, ECUST, Shanghai, 2004]. The strain was stored at –18ºC in LB culture with glycerol (1:1 v/v) and was routinely cultured at 30ºC in liquid LB medium. Culture medium was based on M9 medium, which consisted of Na_2HPO_4, 17.6 g/L; KH_2PO_4, 3g/L; NaCl, 0.5 g/L; and NH_4Cl, 5 g/L. Initial pH value was adjusted to 7.0, and 0.1 ml of 1 M $MgSO_4$ was added to 50 ml medium after sterilization. Sodium succinate (4 g/L) was added to M9 medium as a carbon source. TPT was added into the culture medium (at 200µM) for degradation. The micro- organism was cultured in 250ml shake flasks (at 120 rpm and 30ºC) in the dark for 24 h.

Each sample of 50ml culture medium was added with 5ml of 1M HCl and 150mg NaCl, then extracted three times with 10 ml ethyl acetate for each time. The moisture in the pooled organic phase was removed with anhydrous sodium sulfate and organic solution

was evaporated to dryness on a rotary evaporator at 40ºC. The residue was dissolved in 10ml methanol. All samples were stored at –20°C before analysis.

RESULTS AND DISCUSSION:

Detection and separation of phenyltins by HPLC with DTT: The initial mobile phase consisted of methanol-acetic acid-water. An increase in the acetic acid concentration significantly reduced the peak tailing of DPT and MPT peaks (Gonzalez-Toledo et. al., 2000). An acetic acid concentration of 10% (v/v) was optimal, and retention times of phenyltins were adjusted by changing the ratio of methanol/water. However, attempt to use this mobile phase without addition of thiol compound proved unsuccessful for simultaneous separation of all three phenyltins. As shown in Fig. 1A, when the mobile phase consisting of methanol-acetic acid-water (60:10:30 v/v/v) was used and standard solution of a mixture of TPT, DPT and MPT (200 µl) with the final concentration of each phenyltin being 0.1 mM was injected, in the chromatogram MPT was eluted in the void volume, which coincided with other reports (Hoch 2000, Gonzalez-Toledo et, al., 2000).

A different chromatogram (Fig. 1B) was obtained when 1 mM DTT was added into the above mobile phase. The addition of DTT in the mobile phase increased the retention time of all phenyltins and MPT was successfully separated from the void volume. It is clear that all the phenyltin species were separated here and could be detected by HPLC with UV detector.

Another set of experiments was done by mixing DTT with phenyltin standard solution at first and then the mixture was analyzed by HPLC without DTT in its mobile phase. Almost the same chroma- togram as Fig. 1A was obtained (data not shown), implying that treatment of samples with DTT did not help in complete separation of the phenyltins.

Effects of different thiol compounds on phenyltins separation: Thiol compounds such as DTT and DTE were reported to be able to protect cells from the attack of organotins and prevent inhibitory effects of organotins on enzymes (Lo et. al., 2003). Organotin was also utilized to separate and identify thiols in biological materials (Wronski 1996). In this work, it was observed that addition of DTT in HPLC mobile phase led to the separation of phenyltins, which is in agreement with a previous report (Inoue et. al., 2003). Based on these facts, it is assumed that the interaction between thiol compounds and phenyltins in the column might be critical to the phenyltins separation.

In order to investigate the influence of concentration of different thiol compounds on phenyltins separation, a different level (1 to 10mM) of DTT, DTE and ME was applied in the mobile phase. As shown in Table 1, all thiol compounds significantly affected both retention time and peak height of phenyltins. The retention time of all three organotins was prolonged when DTT concentration was increased from 0 to 1 mM. When DTT level was higher than 1 mM, the retention time of DPT and MPT did not change so much, but the retention time of TPT was apparently increased with increasing concentration of DTT up to 10 mM. For the peak heights of TPT, DPT and MPT, they were quite different at different levels of DTT. The peak height of both DPT and MPT was significantly increased with an increase of DTT concentration from 0 to 10 mM. In contrast, the peak height of TPT showed a significant decrease (to about 60%) when the DTT level was changed from 0 to 1 mM, and it almost did not change at 1-10 mM of DTT (Table 1).

The effect of DTE was almost the same as DTT. This might be due to the similar molecular structure of DTE and DTT as they are isomeric compounds. But, an increase of DTE concentration from 5 to 10 mM did not improve the retention time of TPT. ME at a concentration of 1 mM was not able to

separate MPT from void volume. At ME concentration of 5mM, MPT was successfully separated from void volume.

The above results show that thiol compounds had a strong influence on the elution time and peak height of phenyltins. Because one molecular ME has only one thiol, while DTT or DTE has two thiols, it is apparent that at the same concentration of ME, DTT and DTE, the thiol content of ME is lower than that of DTT and DTE. It is considered that relatively low concentration of thiol in the mobile phase might lead to relatively weak performance of ME; the separation of phenyltin species is associated with thiol content, and the concentration of free thiol is responsible for the separation of phenyltins.

Optimum performance of HPLC was achieved with mobile phase containing 5 mM DTE. This condition was used in experiments for checking reproducibility and limits of detection (LOD). Reproduci- bility was studied by evaluating the relative standard deviations (RSD) for six replicate injections within 1-10 µM. The relative standard deviations were in the range of 2-5% for all three phenyltin compounds at 10 µM. The LOD values were calculated from a signal that was three times the noise. Detection limits of TPT, DPT and MPT were 0.01, 0.02 and 0.01 nM, respectively.

Interaction of phenyltins with thiol compounds: In our experiments, thiol compounds were found helpful to the separation and detection of phenyltins by HPLC. It is reported that organotin has special affinity with thiol group and could form metal mercaptide (Wronski 1996). This might contribute to phenyltins separation in our case. To confirm whether the concentration of free thiol would decrease in the presence of phenyltin compounds due to the affinity binding with each other, free thiol content was measured with DTNB in the mixture of DTT and phenyltins. Theoretically, one mole of DTNB would be reduced to two moles of TNB by two moles of free thiol, increasing absorbance at 412 nm (Ellman 1959). Apparently the concentration of free thiol will decrease if the absorbance (A_{412}) decreases.

As seen in Fig. 2, curve A is control, in which case DTT directly reacted with DTNB. In cases of curve B, C and D, phenyltins were preincubated with DTT for 3 min before an addition of DTNB. Compared with the control (Fig. 2A), the decrease of A_{412} (curve D) indicates that free thiol content was reduced. It was confirmed that TPT, DPT and MPT didn't alter the absorbance (A_{412}) by mixing DTNB or TNB with phenyltins (data not shown). The results suggest that TPT may decrease the concentration of free thiol by acting on it. Similarly absorbance for TPT, DPT (Fig. 2B) and MPT (Fig. 2C) also decreased by reducing the concentrations of free thiol, but the degree of reduction of free thiol was less than that by TPT (Fig. 2D). It may be assumed that the different affinity of TPT, DPT and MPT with thiol affected the retention times of phenyltins, and eased simultaneous separation of all the phenyltin species.

Application of the developed method to a microbial degradation system: The investigation of TPT-degradation by *P. aeruginosa* CGMCC 1.860 has been performed in our laboratory, and here we used the developed analytical method to observe the degradation of TPT and its products DPT and MPT. Samples were prepared as described in Materials and Methods. As recommended above, the mobile phase was a 60:10:30 (v/v/v) mixture of methanol, acetic acid and water containing 1 mM DTT. The analytical results obtained for TPT, DPT and MPT are given in Table 2. Same samples were analyzed using another method of HPLC with fluorescence detector (Inoue et. al., 2003). The accuracy of the method was evaluated by comparing the results obtained from both methods. All concentrations of organotin compounds were

in good agreement with each other as relative deviation was within 5% suggesting that the proposed method of this work is reliable for simultaneous analysis of phenyltin compounds in bio- degraded systems.

A convenient HPLC based method for the simultaneous determination of phenyltin compounds (TPT, DPT and MPT) with UV detector at 257nm has been developed. The method employs the mobile phase of methanol–acetic acid–water (60:10:30 v/v/v) containing thiol compound, which enables the successful separation and detection of TPT, DPT and MPT mixtures. This method could be successfully used for analyzing phenyltins in organotin bio- degradation.

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Table 1: Effects of thiol compounds on retention times and peak heights of TPT, DPT and MPT. Column, C_{18} Kromasil; flow-rate, 0.75 ml min^{-1}; mobile phase system, methanol-acetic acid-water (60:10:30,v/v/v) containing DTT, DTE and ME at different concentrations.

	Compound concentration (mM)	Thiol concentration (mM)	Retention time (RT) and peak height					
			TPT		DPT		MPT	
			RT (min)	Height (mAU)	RT (min)	Height (mAU)	RT (min)	Height (mAU)
DTT	0	0	10.2	8.2	5.8	6.8	ND	ND
	1	2	14.9	4.8	11.5	15.2	5.2	1.7
	5	10	27.4	4.4	12.7	22.4	5.6	2.4
	10	20	45.1	4.7	12.7	23.1	5.6	3.5
DTE	0	0	10.2	8.2	5.8	6.8	ND	ND
	1	2	13.0	5.7	9.5	16.4	5.1	1.6
	5	10	22.0	4.5	10.2	22.3	5.1	6.5
	10	20	22.1	4.9	9.9	23.2	5.1	3.9
ME	0	0	10.2	8.2	5.8	6.8	ND	ND
	1	1	12.1	6.3	7.2	13.1	ND	ND
	5	5	18.3	5.4	7.8	22.4	5.2	0.9
	10	10	25.1	5.2	8.4	27.1	5.4	2.3

ND: not detected

Table 2: Degradation of TPT by *P. aeruginosa* CGMCC 1.860. TPT (200 □M) was added into the medium for 24h. Phenyltins were analyzed with UV detector at 257 nm and the mobile phase was methanol:acetic acid:water (60:10:30 [v/v/v]) in the presence of 1 mM DTT. The same samples were also analyzed using the method with fluorescence detector

.HPLC detector	Time (h)	Organotin concentration (□M)		
		TPT	DPT	MPT
UV	0	190.31±6.72	3.16±0.53	0.13±0.19
	24	156.70±12.05	20.75±1.01	10.06±1.12
Fluorescence	0	195.212±8.258	3.514±0.627	0.163±0.136
	24	149.363±13.471	18.732±2.611	11.465±1.877

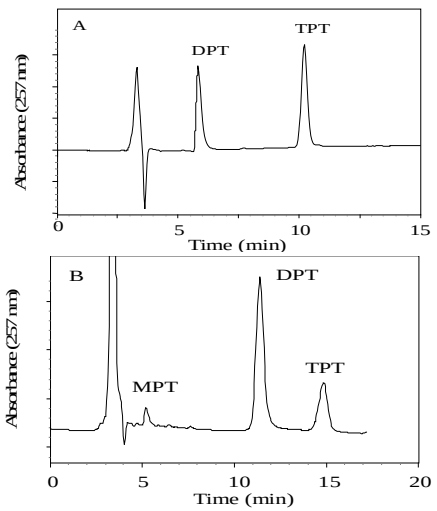

Fig. 1: Chromatograms of a standard mixture of organotins containing 100 □M of TPT, DPT and MPT for each. The mobile phase consisted of methanol-acetic acid-water (60:10:30, [v/v/v]) in the absence (A) or presence (B) of 1 mM DTT. The organotins were detected at 257nm with UV detector.

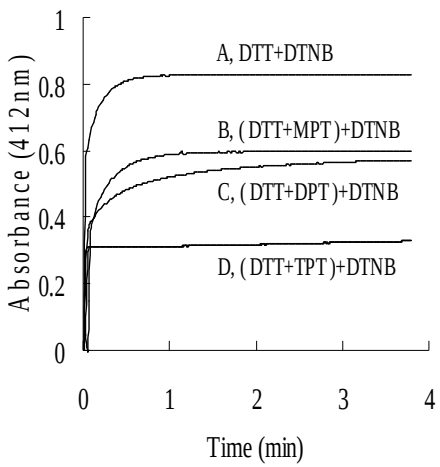

Fig. 2: Interaction of phenyltins (TPT, DPT and MPT) with free thiol (DTT). Curve A is control, which DTT reacted with DTNB directly. For curve B, C and D, 200 □M of MPT, DPT or TPT was incubated with a solution of 50 □M DTT for 3 min respectively, and then100 □M DTNB was added. The absorbance was monitored at 412 nm with UV spectrophotometer

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