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IDENTIFICATION OF TRIMETHYLLEAD IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COLUMN SWITCHING AND CHEMICAL REACTION DETECTION AND BY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Incorporated tetraalkyllead compounds are metabolized in the liver and the highly toxic trialkyllead species are excreted via the urine. The procedure for the determination of these metabolites in urine consists of solid-phase enrichment, reversed-phase pre-column high-performance liquid chromatography (HPLC) and chemical reaction detection. As urine is a very complex matrix, it must be questioned whether the retention time alone is a sufficient criterion for the identification of the analytes. For the trimethyllead ion the validity of the results was examined by selectivity checks of the chemical reaction detector, by the application of different stationary and mobile phases in single and dual pre-column HPLC systems and by the use of thermospray LC–mass spectrometry as an independent method. The results demonstrated that the recommended method is accurate for the determination of trimethyllead in urine samples.

INTRODUCTION

For more than five decades tetraalkyllead (TAL) compounds have been produced as antiknock additives for gasoline. Employees who work in industrial TAL production, as tank cleaners, at gasoline stations, as mechanics or in garages, come into contact with these substances and can be regarded as exposed. Regulations in the F.R.G. limit the maximum concentrations of tetramethyllead (TML) and tetraethyllead (TEL) at workplaces to 75 $\mu g/m^3$. TALs are volatile, lipophilic, toxic substances which are resorbed through the skin or via the respiration tract. Dealkylation of TAL to inorganic lead via the tri- and dialkyllead derivatives takes place in the liver and the metabolites are excreted in the urine. The ultimate hazardous toxic species are trialkyllead compounds, the toxic potentials of which were found to be 10–100 times higher than those of inorganic lead.

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In cases of TAL exposure, analytical techniques for the quantitative determination of trialkyllead species in biological materials are a prerequisite for effective biological monitoring. Urine samples are preferred because they contain all organolead metabolites and the sampling is non-invasive. Accordingly, an analytical procedure for the determination of trialkyllead species in urine was developed.

The system consists of an off-line solid-phase enrichment and an on-line high-performance liquid chromatographic (HPLC) pre-column enrichment[1] followed by HPLC separation and detection by a chemical reaction detector[2]. The chromatographic columns (RP C_{18} pre-column and RP C_{18} analytical column) are run with methanolic acetate buffers as mobile phases. In the post-chromatographic chemical reaction detector, tetraalkyllead compounds and trialkyllead ions are decomposed into dialkyllead species by iodine and measured spectrophotometrically via their 4-(2-pyridylazo)resorcinol (PAR) complexes at 515 nm.

As urine, like other biological samples, is a very complex matrix, it must be questioned whether the retention times and high recovery rates are sufficient criteria for the identification of the analytes. Having in view the practical situation of an exposure to tetramethyllead, the overall specificity, *i.e.*, the selectivity of the separation and detection part of the procedure, was checked for the trimethyllead ion.

The aim of the investigation was the validation of the trimethyllead analysis in order to achieve exact quantification. The following three approaches were chosen from different possibilities: (1) selectivity check of the chemical reaction detector; (2) enhancement of the chromatographic selectivity by combining different stationary and mobile phases; (3) identification of the analyte by thermospray liquid chromatography–mass spectrometry (LC–MS).

EXPERIMENTAL

Most of the equipment, materials, chemicals and solutions used have been described elsewhere[1]. The following investigations were carried out with additional chromatographic materials and mobile phases. The reversed-phase (RP) analytical column (Partisil ODS III, 5 μm; 250 mm × 4.6 mm I.D.) was run with a mobile phase consisting of 200 ml methanol + 8.20 g sodium acetate + 5.9 ml of 96% acetic acid per 1000 ml as optimal eluent for $(CH_3)_3Pb^+$. The analysis of $(CH_3)_3Pb^+$ by ion chromatography was performed with 700 ml methanol + 7.71 g ammonium acetate + 1 ml of 96% acetic acid per 1000 ml eluent on a Partisil SCX (10 μm) column (250 mm × 4.6 mm I.D.). The flow-rate of the eluent for both analytical columns was set at 1 ml/min.

Two stationary phases were used for the pre-columns. The RP pre-column was packed with Nucleosil 10 C_{18} and the ion-exchange pre-column (SA pre-column) with Nucleosil 10 SA material using the slurry packing technique. Both pre-columns (20 mm × 4.6 mm I.D.) were connected to ports 1 and 4 of a Rheodyne 7010 valve using ManuFIT MFII guard column holders (Bischoff, Leonberg, F.R.G.). In the single pre-column arrangements the loading with the analyte was carried out at a flow-rate of 2 ml/min with borate buffer III (pH 8) for the RP pre-column and with doubly distilled water for the SA pre-column.

In the dual pre-column system the flushing solution for the first pre-column (RP) was borate buffer of pH 8 (see above) at a flow-rate of 2 ml/min. The same

flow-rate was applied for the elution of this column with methanol–water (20:80) and in addition with 1 ml of push eluent [100 ml methanol + 8.20 g sodium acetate + 5.9 mg of 96% acetic acid per 1000 ml]. This elution simultaneously implied the loading of the second pre-column (SA). The mobile phase of the analytical column was used to elute the second pre-column and to separate the analytes (1 ml/min). The abbreviations SA and SCX both represent ion exchange. The monitoring of the analytes was carried out by the post-column chemical reaction detector, except in the LC–MS coupling. The detector sensitivity is indicated by the absorption ranges of the spectrophotometer given in the legends of the figures.

The sample loop was a knitted PTFE capillary (2 m × 0.8 mm I.D.) with a volume of 1 ml. Sample loops made of stainless-steel capillaries were used for the injection of 20-μl amounts. The injection valve and the pre-column valves were of the type Rheodyne 7010. The connections in the arrangement of the column switching were all made with stainless-steel capillaries (0.4 mm I.D.). The HPLC pump 1 was a Waters Model 6000 A, which makes fast eluent changeovers possible by simply setting the solvent select valve to the solvent reservoir desired. In the single and dual pre-column technique the course of events was controlled manually with the aid of a stop-watch. The flushing interval of the sample loop was set to 40 s in order to guarantee total transport of the injection volume from the loop through the connections on to the pre-column.

The nomenclature for the borate buffers used is the same as in the previous paper[1]: borate buffer II (pH 10) consists of 23.17 g of boric acid + 27.97 g of potassium chloride + 12.16 g of sodium hydroxide per 1000 ml and borate buffer III (pH 8) of 3.09 g of boric acid + 3.73 g of potassium chloride + 0.156 g of sodium hydroxide per 1000 ml.

The thermospray MS detector was coupled to a LiChrosorb RP-18 (7 μm) column (200 mm × 4 mm I.D.) run with a methanolic ammonium acetate buffer [200 ml methanol + 7.71 g ammonium acetate per 1000 ml adjusted to pH 4.5 with 1% acetic acid]. The mass spectrometer (MAT CH5), the LC–MS coupling and the mass spectrometric working conditions will be described later.

RESULTS AND DISCUSSION

Selectivity of the chemical reaction detector

The chemical reaction detector consists of a chemical reaction system as a post-column derivatization unit and a spectrophotometer as a physical detector. Four reactors (knitted PTFE capillaries) form the chemical reaction system in which the following reactions take place[2]:

$$R_nPb^{(4-n)+} \xrightarrow[(2)\ Na_2S_2O_3]{(1)\ I_2} R_2Pb^{2+}/Pb^{2+}\ (n = 2\text{–}4) \qquad (1)$$

$$R_2Pb^{2+}/Pb^{2+} \xrightarrow[pH\ 9\text{–}10]{PAR} R_2Pb\text{–}PAR^{+}/Pb\text{–}PAR^{+} \qquad (2)$$

The selectivity of this system for trialkyllead compounds is determined by two reactions: conversion of R_3Pb^+ into R_2Pb^{2+} through reaction with iodine (eqn. 1) and

complexation of the formed dialkyllead by PAR (eqn. 2). The complex is stable within the pH range 9–10 and has its maximal absorption at 515 nm. Being a common metal indicator, PAR also reacts with many other metals and some organometallics. The formation of these complexes and with it the absorption maximum are dependent on pH. Interferences, which reduce the selectivity of the detector, can be caused either by red compounds from the sample or red substances generated by iodination and by the change in the pH. Further interferences can be introduced by PAR complexes of other metals having an absorption maximum at or close to 515 nm.

The selectivity of the detector was checked by cross-experiments. In these tests, the chemical reagents iodine and PAR were omitted from the reaction system one by one and in combination. In order not to displace the equilibrium within the reactor, only the solvents of the reagents mentioned were pumped in. All experiments were performed in combination with the preliminary enrichment step and with different chromatographic systems. $(CH_3)_3Pb^+$ standards, an extract from a 1.5-l pool urine and an extract from a 50-ml urine sample (No. 73) from a person probably exposed to tetramethyllead were prepared for the tests. The high sample volume of 1.5 l of urine was chosen in order to obtain a more concentrated eluate. In contrast to the published procedure[1], the enrichment was carried out starting with sample volumes of 100 ml instead of 50 ml. The procedure was performed simultaneously with fifteen aliquots of the sample. The combined extracts were diluted to 250 ml and adjusted to pH 10. A second solid-phase enrichment step followed with this volume and finally yielded a 2-ml extract. This volume was split into five aliquots. After dilution and pH adjustment with 800 μl of borate buffer II, samples of 1.2 ml were available for injection.

The results of the investigations are given in Table I and can be summarized as follows: (1) by omitting iodine from the reaction system no signal for $(CH_3)_3Pb^+$ could be recorded for either the standard or the samples; (2) accordingly, by omitting the PAR reagent no $(CH_3)_3Pb^+$ signals could be recorded; (3) the same results were obtained when both reagents were omitted from the reaction system. The cross-ex-

TABLE I

SELECTIVITY CHECKS OF THE CHEMICAL REACTION DETECTOR FOR $(CH_3)_3Pb^+$ ANALYSIS

+, With reagent; −, without reagent.

Sample	I_2	$Na_2S_2O_3$	*Buffer*	*PAR*	*Signal*	*Columns*
$(CH_3)_3Pb^+$ standard	+	+	+	+	+	Nucleosil 10 C_{18}
	−	+	+	+	−	Partisil ODS III
	+	+	+	−	−	Partisil SCX
	−	+	+	−	−	
Extract of 1.5 l of urine	+	+	+	+	+	Nucleosil 10 C_{18}
	−	+	+	+	−	Partisil SCX
	+	+	+	−	−	
	−	+	+	−	−	
Extract of 50 ml of urine No. 73	+	+	+	+	+	Nucleosil 10 C_{18}
	−	+	+	+	−	Partisil SCX

periments show that the chemical reaction detector coupled to a chromatographic system has a high selectivity for the detection of $(CH_3)_3Pb^+$ species.

Enhancement of the chromatographic selectivity by combining different stationary and mobile phases

For the validation of chromatographically separated analytes from very complex matrices, the dual column method is often applied in addition to mass spectrometry. The principle of this method is to separate the analytes on stationary phases of very different polarities, *i.e.*, by different separation mechanisms. This strategy guarantees a much higher degree of certainty for the identification than can be achieved by the single column method.

As all alkyllead compounds carry non-polar organic groups, they can be separated very well by LC on reversed-phases. A further possibility of separation results from the ionic properties of the lower alkylated lead species. According to their positive charge, di- and trialkyllead compounds can also be separated by ion chromatography. The use of RP and ion chromatography, alone or in combination, should lead to an additional certainty in the validation of the analytical procedure. The working conditions for the RP separation of trialkyllead compounds have already been published[1,3]; however, for the ion chromatographic separation they had to be worked out.

The ionic alkyllead species $(C_2H_5)_2Pb^{2+}$, $(C_2H_5)_3Pb^+$, $(CH_3)_3Pb^+$ and $(CH_3)_2Pb^{2+}$ were separated on a Partisil SCX column in the order given with a methanolic ammonium acetate buffer within 20 min and with a chromagraphic resolution greater than unity (Fig. 1).

As only the trialkylated lead species and especially the trimethyllead ion were of interest, the mobile phase for the ion chromatographic separation of $(CH_3)_3Pb^+$ was optimized in order to reduce the time of the chromatographic run. Finally, 700 ml methanol + 7.71 g ammonium acetate + 1 ml of 96% acetic acid per 1000 ml showed good properties for this purpose ($(C_2H_5)_3Pb^+$, 8.7 min; $(C_2H_5)_2Pb^{2+}$, 8.9 min; $(CH_3)_3Pb^+$, 11.0 min; $(CH_3)_2Pb^{2+}$, 14.1 min). The retention time differed slight-

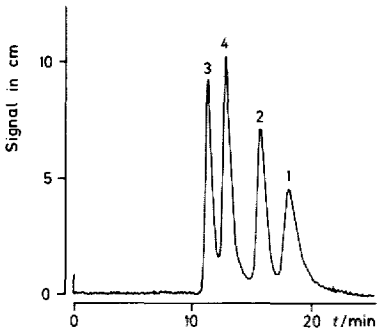

Fig. 1. Ion chromatogram of organolead salts. Stationary phase, Partisil SCX; flow-rate, 1 ml/min (275 ml of methanol + 0.45 ml of 96% acetic acid + 7.71 g of ammonium acetate per 1000 ml). Peaks: 1 = $(CH_3)_2PbCl_2$; 2 = $(CH_3)_3Pb(CH_3COO)$; 3 = $(C_2H_5)_2PbCl_2$; 4 = $(C_2H_5)_3PbCl$. Concentrations: 1 μg per 20 μl of mobile phase; 0.1 a.u.f.s.

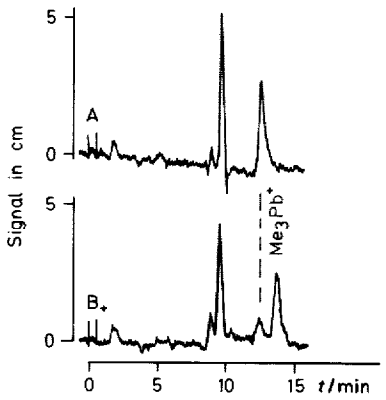

Fig. 2. RP chromatograms with RP pre-column. PC, Nucleosil 10 C_{18}; AC, Partisil ODS III. A, 42 ng of $(CH_3)_3Pb(CH_3COO)$ standard; B_+, 333 μl of a 2-ml extract from 50 ml of urine spiked with 40 ng of $(CH_3)_3Pb(CH_3COO)$. 0.015 a.u.f.s.

ly depending on the chromatographic system applied. With the given mobile phase $(CH_3)_3Pb^+$ was sufficiently separated from the other lead species.

As the concentration of $(CH_3)_3Pb^+$ in urine is very low, an enrichment step has to precede the determination. This procedure, a solid-phase enrichment on silica gel, leads to a 2-ml methanolic extract with a pH of 4.2. For the purpose of low detection limits, a large amount of this extract up to the total volume should be injected into the HPLC system. As the extract had a disadvantageous composition for the analytes to be retained on the pre-columns, it had to be diluted and simultaneously adjusted to pH 8 by the addition of borate buffer II in the proportions 1:2. This means that with an injection volume of 1 ml, 333 μl of the original extract were injected on to the pre-column. The standard samples were prepared in the same manner. After the injection of the extract, the analytes were enriched on the pre-column and eluted on to the analytical column in the backflush mode. Solid-phase enrichment, pH adjustment of the extract and the single pre-column technique with reversed phases have been described in detail elsewhere[1].

Pre-columns and analytical columns with different selectivities were coupled for correct peak identification of the $(CH_3)_3Pb^+$ signal. The RP materials applied were different C_{18} phases and the ion-exchange materials were strong acid cation exchangers with SO_3H groups. The analyses were carried out with the following combinations of pre-column and analytical column: RP–RP, RP–SCX and SCX–SCX. The SCX–RP column combination could not be tested because, under optimized conditions, the elution of $(CH_3)_3Pb^+$ from the SCX pre-column needs an eluent containing 70% of methanol. This mobile phase, however, cannot effect a retention on the RP analytical column.

Figs. 2 and 3 show the chromatograms obtained by the three combinations of pre-column (PC) and analytical column (AC). A standard and a spiked real urine sample were measured with RP–RP coupling. In addition, a non-spiked real urine sample was chromatographed using the other two combinations of columns. The evaluation of all chromatograms showed that the determination of $(CH_3)_3Pb^+$ was achieved without interferences. Further, in the chromatograms in Fig. 3, it can be

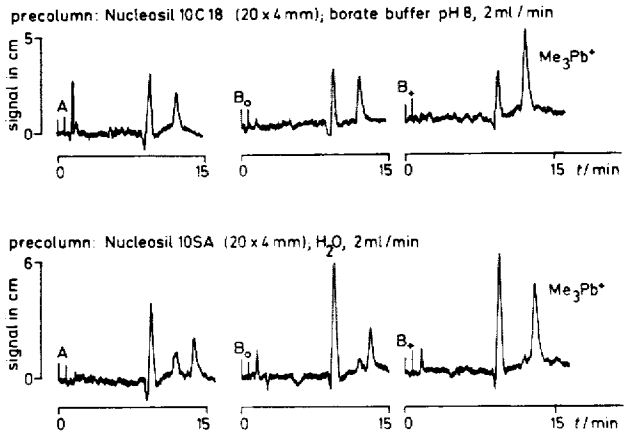

Fig. 3. Ion chromatograms with RP pre-column (upper part) and SA pre-column (lower part). AC, Partisil SCX. A, 42 ng of $(CH_3)_3Pb(CH_3COO)$ standard; B_0, 333 μl of a 2-ml extract from 1500 ml of pool urine; B_+, 333 μl of a 2-ml extract from 1500 ml of pool urine spiked with 42 ng of $(CH_3)_3Pb(CH_3COO)$. 0.02 a.u.f.s.

clearly seen that the non-spiked 1.5-l urine sample contained 180 pg/ml of $(CH_3)_3Pb^+$ although originating from an unexposed person.

For further validation of the $(CH_3)_3Pb^+$ signal, the chromatographic system was extended by a second pre-column. The set-up is given schematically in Fig. 4. In this arrangement the complete system consisted of two HPLC pumps, three six-port switching valves, two pre-columns and one analytical column. The only possible series of the pre-columns and the analytical column (RP–SCX–SCX) is laid down by the mobile phases. For each step of the procedure the current pathways of the different mobile phases are also shown in Fig. 4. The first pre-column (RP) was eluted in the forward direction and the second pre-column (SA) in the backflush mode.

In the first enrichment step, the eluate was transported from the sample loop to the first pre-column by borate buffer (pH 8). The elution of this pre-column and the simultaneous enrichment on to the second pre-column was carried out with methanol–water (20:80) as the mobile phase. For more efficient elution of the first pre-column the sample loop was filled with 1 ml of a push eluent [200 ml of methanol + 8.20 g sodium acetate + 5.9 ml of 96% acetic acid per 1000 ml]. During the elution of the first pre-column this segment was inserted into the flow of the second mobile phase of pump 1. The higher ionic strength of the solution accelerates the elution of alkyllead species on reversed phases. The elution of the second pre-column was carried out in the backflush mode with the mobile phase of the analytical column.

After the elution of the second pre-column the analytes were chromatographed on the analytical column and detected by the chemical reaction detector. The time scale of the procedure for switching the valves, the flow of the mobile phases and the hold time of the single steps are illustrated in Fig. 5. The chromatograms obtained with this complex system are given in Fig. 6. As in the single pre-column technique, standards and non-spiked and spiked urine samples were measured. Even with this complex chromatographic system no interfering effects were observed. The results of these investigations with various stationary and mobile phases, leading to totally

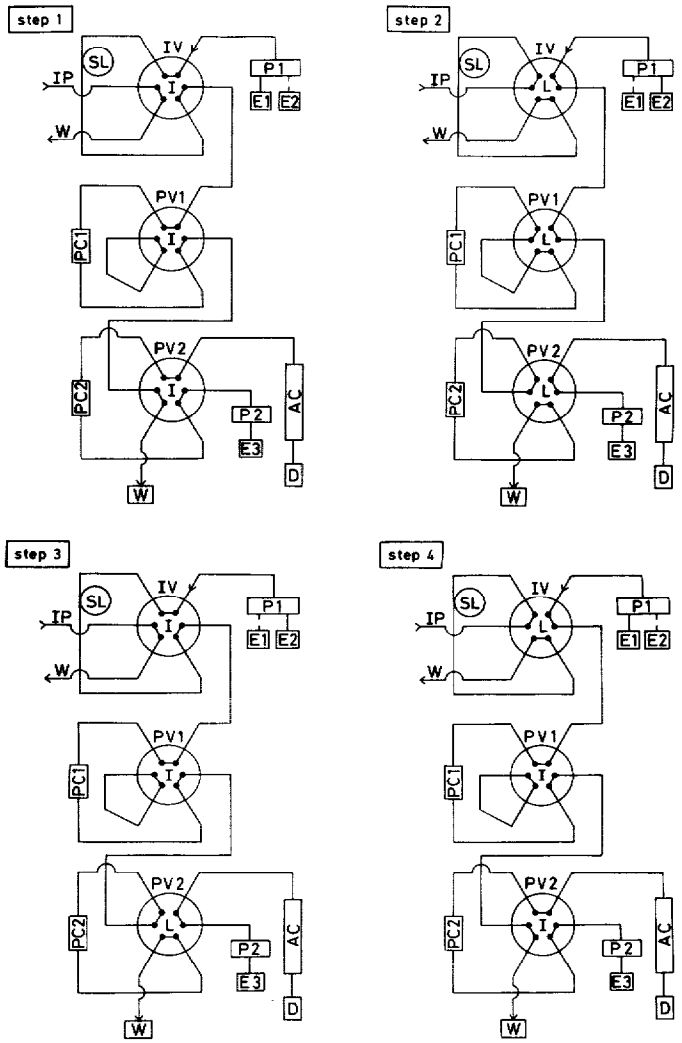

Fig. 4. Schematic diagram of the four steps of the column switching in the dual pre-column technique. IP = injection port; W = waste; SL = sample loop; PC1 = pre-column 1; PC2 = pre-column 2; IV = injection valve; PV1 = pre-column valve 1; PV2 = pre-column valve 2; I = inject; L = load; P1 = pump 1; P2 = pump 2; E1 = eluent 1; E2 = eluent 2; E3 = eluent 3; AC = analytical column; D = detector.

different selectivities, strongly increase the probability of an accurate analysis of $(CH_3)_3Pb^+$ with the designed procedure.

Identification of the analyte by thermospray LC–MS

In a search for an independent strategy to validate our results, we found thermospray LC–MS[4] to be the most promising method owing to its inherent ability to produce mass spectra of pre-formed cations. The instrument (MAT CH5) was modified with respect to the previously delineated basic design[5] only by changing from

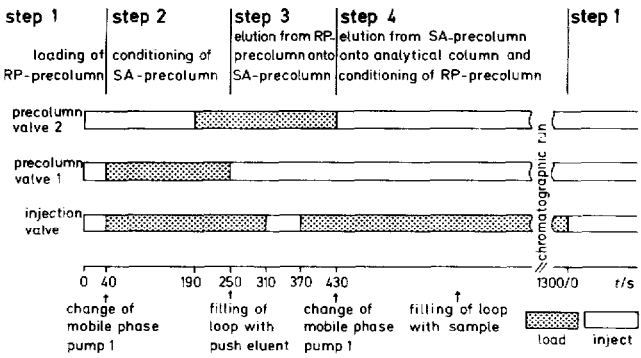

Fig. 5. Time scale of the chromatographic steps in the dual pre-column technique.

indirect to direct heating of the interface by means of a temperature-regulated power supply [maximum current of 10 A through the stainless-steel capillary (60 cm × 200 μm I.D.), heated region 40 cm, *ca.* 1 ohm resistance] and by replacing the orifice from the source into the mass spectrometer by a 0.2 × 3.0 mm slit in order to enhance the sensitivity of the sector field instrument. The HPLC system now consists of an LC 2600 syringe pump (ISCO) and an RP C_{18} column which is connected to the heated interface by a fused-silica capillary of length 50 cm. For the experiments depicted here, the mobile phase was a methanolic ammonium acetate buffer. Instead of the sodium acetate buffer used for the preceding RP chromatograms, an ammonium acetate buffer was chosen because of its better vaporizability in the LC–MS coupling technique. The flow-rate was set to 1.1 ml/min to achieve optimal conditions. The extracts of the solid-phase enrichment were evaporated to dryness and redissolved in water (enrichment factor = 10). For calibration, standard solutions of trimethyllead acetate in buffer were prepared in the range 5–500 ng, for which a linear calibration function was obtained. With such an external calibration, series of standards, blanks and samples of urine were analysed and one example is given in Fig. 7. For reasons of time and because of different urine volumes, the samples for the investigations by HPLC–chemical reaction detector and LC–MS were not identical. A urine sample with a lower starting volume (0.2 l) for the enrichment step was

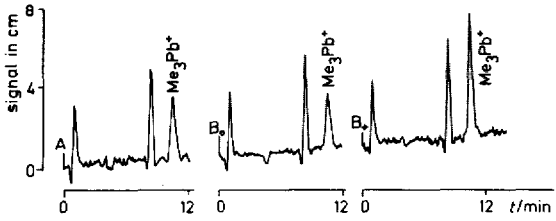

Fig. 6. Ion chromatograms with the dual pre-column technique. PC1, Nucleosil 10 C_{18}; PC2, Nucleosil 10 SA; AC, Partisil SCX. A, 42 ng of $(CH_3)_3Pb(CH_3COO)$ standard; B_0, 333 μl of a 2-ml extract from 1500 ml of pool urine; B_+, 333 μl of a 2-ml extract from 1500 ml of pool urine spiked with 42 ng of $(CH_3)_3Pb(CH_3COO)$. 0.015 a.u.f.s. Me = methyl.

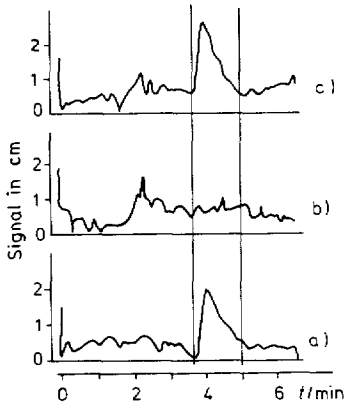

Fig. 7. RP chromatograms of $(CH_3)_3Pb^+$ with thermospray MS detection. (a) 5 ng of $(CH_3)_3Pb^+$ standard per 20 μl; (b) 20-μl extract of a blank urine sample; (c) 20-μl extract of a spiked urine sample. Thermospray interface, MS (MAT CH5); single ion detection, m/z 253.

measured by LC–MS. In the chromatogram of the blank sample (trace b) no $(CH_3)_3Pb^+$ could be recorded, whereas in the spiked sample the amount of added $(CH_3)_3Pb^+$ was determined with an overall recovery of 80%.

Although the reproducibility of 10% so far obtained is not to our satisfaction, it becomes evident that (a) the results of the post-column reaction detection are accurate and (b) the LC–MS method itself is a sensitive and accurate means for the detection of charged metal organic species such as alkyllead cations in biological matrices.

CONCLUSIONS

The results of the investigations carried out in order to validate the $(CH_3)_3Pb^+$ signal can be summarized as follows.

(1) The chemical reaction system with the reagents iodine and PAR, which are the main chemical derivatization reagents, and spectrophotometric measurement show a high selectivity for the detection of $(CH_3)_3Pb^+$ ions enriched from urine samples.

(2) The application of RP and ion-exchange phases, alone or in combination, with single or dual pre-column HPLC techniques coupled to the chemical reaction detector allows the determination of $(CH_3)_3Pb^+$ in urine without interferences.

(3) The use of thermospray LC–MS as an independent method confirms the results obtained by the chromatographic systems coupled to the chemical reaction detector.

The developed method, consisting of solid-phase enrichment, RP pre-column HPLC and chemical reaction detection, is an accurate procedure for the determination of trialkyllead species in the complex matrix urine.

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