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Journal of Chromatography B, 716 (1998) 139–145

JOURNAL OF CHROMATOGRAPHY B

Speciation of ionic alkyllead compounds in human urine by gas chromatography–mass spectrometry after butylation through a Grignard reaction

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Received 24 March 1998; received in revised form 23 June 1998; accepted 23 June 1998

Abstract

One analytical procedure for the determination of ionic alkyllead in human urine has been studied. The system consists of the extraction of Me_3Pb^+, Et_3Pb^+ and Pb^{2+} at pH 9.0 with diethyldithiocarbamate to an organic phase. Then, the ionic compounds are butylated with BuMgCl and the final organic solution is analyzed by GC–MS–SIM. The elimination of both foam and gels in the extraction step and the general procedure for the urine are discussed. The recovery of compounds ranges from 105.1% for Me_3Pb^+ to 97.2% for Et_3Pb^+ using hexane as extracting agent and detection limits are 18.4 pg/ml of Me_3Pb^+ and 19.2 pg/ml of Et_3Pb^+ in urine. The speciation of ionic alkylleads in the urine of a petrol station worker showed a value of 27.9 pg/ml of Me_3Pb^+ in urine and Et_3Pb^+ was below the detection limit.

Keywords: Alkyllead compounds; Lead

1. Introduction

Organolead compounds have been added to gasoline for years. As volatile compounds, they are emitted to the environment in which they can be transformed into ionic alkylcompounds, such as R_3Pb^+ (trialkyllead), R_2Pb^{2+} (dialkyllead), RPb^{3+} (monoalkyllead) and even Pb^{2+} (inorganic lead) [1–3].

Tetraalkylleads are liposoluble compounds and consequently, they are easily absorbed by the skin, lungs and intestinal tract of living organisms. This absorption depends on the type of compound, exposure time and the type of organism. It has been confirmed [4] that with long exposure, alkylleads are much more toxic in human beings than inorganic lead. Once in the body, organoleads are degraded by the liver to ionic alkylleads or Pb^{2+} and these metabolites are excreted in the urine [4–7].

As lead is well-known to be toxic to the human body, it is an important parameter to analyse in biological fluids and for this reason its analytical methodology has been considerably improved [8,9].

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0378-4347/98/$19.00
PII: S0378-4347(98)00319-3

However, most of the studies concerning lead in the human body refer to inorganic or total lead and only a few are focused on alkyllead derivatives, although the toxic potentials of the trialkyllead can be from 10 to 100 times higher than those of inorganic lead [4,7,10–12]. The first study of lead speciation in urine [6], carried out by flameless atomic absorption spectrometry, showed a high concentration of dialkyllead in the urine of individuals strongly contaminated by lead, but organoleads in urine from low contaminated workers could not be detected. As the expected concentration of organoleads in the urine of normal individuals is lower, a more sensitive procedure is necessary.

Further studies have allowed the determination of alkylleads in urine using high-performance liquid chromatography (HPLC) and posterior chemical reaction detection [5,7,12–14]. All of them used spiked samples of urine, but these procedures were not able to determine the low concentration of alkylleads in the urine of individuals without intoxication symptoms of lead. That is, with a medium exposure time to lead.

Very low detection limits as well as a good change of signal by changing the concentration (sensitivity) can be achieved by hyphenated techniques which involve gas chromatography, but a derivatization step is necessary to transform the ionic compounds into volatile compounds. Among the derivatization systems, the Grignard reaction has been shown to be a very efficient system [15–19].

Based on Grignard derivatization, the analysis of alkylleads in environmental samples such as rain water or road dust has been carried out [21–24]. In these studies, the hyphenated technique: gas chromatography–mass spectrometry in selective ion monitoring mode (GC–MS–SIM mode) can be pointed out as one of the most sensitive ones [20].

Extraction of components from urine to an organic phase is difficult and often gels or foams are formed, which add difficulties to the analytical procedure.

This paper shows the optimization of an analytical procedure for determination of Me_3Pb^+, Et_3Pb^+ and Pb^{2+} in urine samples by extraction, Grignard derivatization and final determination by GC–MS–SIM mode. The extraction of ionic lead compounds from urine is studied in depth to avoid losses of compounds. The results obtained are discussed.

2. Experimental

2.1. Reagents

Deionized water, further purified in a Millipore Milli-Q system, was used throughout.

The standard solutions were prepared with commercially pure solid Me_3PbCl and Et_3PbCl supplied by Alfa Products.

A standard solution of Et_4Pb (200 μg/g) was prepared in hexane (Merck of residue analysis quality) with commercially pure liquid supplied by Alfa Products.

n-Hexane, ethylacetate and other organic solvents (ethanol, octanol) of residue analysis quality were supplied by MercK. *n*-Pentane was from Carlo-Erba, and *n*-amyl and isoamyl alcohols were from Panreac.

A 2 *M* solution of butylmagnesium chloride (BuMgCl) in tetrahydrofuran was obtained from Fluka.

Diethyldithiocarbamate, citric acid and NaCl were of analytical-reagent grade, H_2SO_4 and NH_3 were superpure-grade, all supplied by Merck.

Anhydrous Na_2SO_4 from Panreac, and Whatman (1-PS) Phase-separator paper were used to dry the sample.

A 0.45-μm nylon filter syringe was obtained from Tecknokroma.

2.2. Apparatus

A Heidolph rotary evaporator, a vibrator and a Centromix S-549 centrifuge were from Selecta (Barcelona, Spain).

A GC–MS Hewlett-Packard 5890 Series II with a 5971 A mass selective detector were used. A SGL-1 capillary column of 25-m length×0.25-mm I.D.× 0.25-μm film thickness was used. The chromatographic conditions are shown in Table 1.

2.3. Urine samples

Urine samples were obtained from voluntary workers. The urine for 24 h was placed in a glass bottle and it was homogenized and stored at 4°C before the analysis (never more than a week).

Masses of 5, 15, 25, 35 and 50 ng of Me_3Pb^+ and Et_3Pb^+ in aqueous solution were added to 50 ml of

Table 1
Chromatographic conditions

Column	Capillary SGL-1 25 m×0.25 mm I.D.×0.25 μm						
Precolumn	2 m (empty)×0.25 mm I.D. fused-silica						
Injection volume	2 μl						
Injection mode	Splitless						
Injection *T*	250°C						
Detector *T*	280°C						
Column temperature program	Initial *T* (°C)	Time (min)	$Rate_1$	T_1 (°C)	$Rate_2$	Final *T* (°C)	Time (s)
	50	1.5	15	90	10	260	5
Solvent delay	3.5 min						
Carrier gas	Helium: 40 kPa of head pressure						
	Group	Retention time (min)	Start time[a] (min)	*m/z*			
SIM mode	Me_3BuPb	5.16	3.50	223–253			
	Et_4Pb	6.70	5.00	237–295			
	Et_3BuPb	8.86	7.80	237–265			
	Bu_4Pb	14.24	13.50	265–379			

[a] Time at which the detector begins to measure.

urine to prepare the spiked samples. These spiked samples were shaken for 8–10 h (at room temperature) to facilitate the compounds linking to the urine matrix.

2.4. Procedures

To 50 ml of urine sample, 0.4 g of citric acid and NH_3 were added to fix the pH at 9.0. Then 0.5 ml of a 0.25 *M* aqueous solution of diethyldithiocarbamate (DDTC) and 5 ml of organic solvent (pentane, pentane–alcohol mixtures, pentane–ethyl acetate mixtures, pentane+salts, hexane or hexane+salts) were added. The mixture was shaken and the aqueous phase was separated. One gram of NaCl was added to the remaining organic phase and then it was centrifuged at 4500 rpm (2263 *g*) for 10 min. This extraction step was repeated three times and all the extracts were collected together in a round bottom flask. The organic supernatant solution was filtered through a Whatman PS paper and concentrated to 0.5 ml under nitrogen current. Then BuMgCl was added under N_2 atmosphere. A volume of 10 ml of 0.5 *M* H_2SO_4 solution was added to hydrolyse the excess of Grignard reagent. The organic phase was washed three times with highly pure water, the appropriate amount of Et_4Pb (0.1 ml) was added as internal standard and the final solution (about 0.8 ml, gravimetrically controlled) was analyzed by GC–MS–SIM under the experimental conditions listed in Table 1.

3. Results and discussion

3.1. Extraction solvent

One of the critical steps of the analytical procedure for lead speciation is the extraction of ionic compounds from the aqueous matrix.

The presence of several salts in the matrix did not influence the extraction, as was previously demonstrated [21]. However, urine has urea, proteins, organic metabolites and quite a lot of different compounds which can strongly affect the extraction. The first attempt was to use pentane as extracting

agent, the same as was used before for aqueous samples [2,3,15–19]. However, when the urine with DDTC and pentane at pH 9.0 were shaken, a thick foam was formed as well as a colloidal gel, and the two phases were not separated. The addition of salts, such as sodium chloride, to break the colloidal dispersion was not effective enough, and it was necessary to centrifuge the mixture in order to get a separated phase.

It was thought that perhaps the urine sample was too concentrated, but once diluted, the gel appeared again.

The gel formation in the extraction step from urine is quite common in other analytical procedures, and other authors suggested the addition of alcohols to the main extracting agent in order to avoid the gels. Ethanol, amyl alcohol, octanol, isopropyl alcohol, isoamyl alcohol and also ethylacetate were added to the urine–DDTC–pentane mixture. Table 2 shows the results obtained using pentane or hexane as extracting agents and several additional substances. Although the addition of isoamyl alcohol, ethylacetate or a mixture of salts (sodium chloride and ammonium sulphate) is efficient for avoiding the gel formation, the recoveries obtained of Me_3Pb^+ and Et_3Pb^+ are very low. These low values can be also attributed to the further evaporation step, since the presence of the solvents with high boiling point increases the evaporation temperature and consequently, the losses of alkylleads.

Hexane with the addition of 1 g of NaCl and further centrifugation to separate the organic phase is the most efficient system, with recoveries ranging from 82.9 to 118.9%. An additional advantage of hexane is that the evaporation to dryness is not necessary and derivatization with BuMgCl can be carried out in this solvent. Three sequential extractions were applied in all cases to be sure that the extraction was quantitative, and all the extracts were filtered through a Whatman 1 PS paper to dry the organic solution before the derivatization.

3.2. Concentration step and derivatization

In order to reach a high signal, the total volume of the extract has to be reduced. This reduction is

Table 2
Different solvents and treatments used in the extraction of the organolead complexes

Main extraction solvent	Added substance	Is the formation of gels reduced?	Range of recovery (%) obtained	Observations
Pentane	Water (dilution of the sample)	No	<30	More fluid gel
Pentane	Ethanol	No	<30	Diffuse interphase
Pentane	Amyl alcohol	No	<30	High evaporation temperatures (losses)
Pentane	Octanol	No	<30	High evaporation temperatures (losses)
Pentane	Isopropyl alcohol	No	35–45	High evaporation temperatures (losses)
Pentane	Isoamyl alcohol	Yes	35–60	High evaporation temperatures (losses)
Pentane	Ethyl acetate	Yes	35–70	High evaporation temperatures (losses)
Pentane	Salts mix[a]	Yes	35–65	High evaporation temperatures (losses)
Hexane	–	No	70–80	It's not necessary to evaporate to dryness
Hexane	Salts mix[a]	Yes	92–110	Its not necessary to evaporate to dryness

Concentration range used: 20–40 ng of Me_3Pb^+ and Et_3Pb^+ added to 50 ml of urine sample.
[a] In all cases the samples were centrifuged in order to obtain better separation of the phases. These salts were added in the centrifugation step.

usually carried out by evaporation under N_2 current or in a rotatory evaporator at controlled temperatures. Previous studies [15–19] showed that working with pentane, no losses were observed in this step, since the evaporation temperature was quite low (room temperature). However, in this case, it is necessary to warm the hexane solution to evaporate it under vacuum. A maximum temperature of 30°C and vacuum (rotary evaporator) gave better results and no losses were observed under these conditions.

A 2 *M* solution of BuMgCl in THF was used for the Grignard reaction. N_2 was used to transfer this solution to the round bottom flask were the organic extract was placed and the total system was held in an inert atmosphere to avoid the decomposition of the Grignard reagent. Once the reagent was added, the mixture was shaken to facilitate the reaction. The excess of reagent was eliminated under the experimental conditions described above.

The final extract, containing the internal standard and the analytes, was passed through a little glass column of 3×0.5-cm I.D. containing Na_2SO_4, and it was encapsulated in a 2-ml vial for chromatographic analysis. All the extracts were gravimetrically controlled (about 0.55 g). No decomposition compounds from Grignard reaction were obtained working in SIM mode of a GC–MS.

3.3. Determination of Me_3Pb^+ and Et_3Pb^+ by GC–MS–SIM

As no standards of $BuMe_3Pb$ or $BuEt_3Pb$ were available, calibration plots were obtained considering the initial concentration of Me_3Pb^+ or Et_3Pb^+ with respect to the internal standard (Et_4Pb) versus the relative value of area counts found in the chromatographic analysis.

Two values of m/z ratio as characteristic masses of each compound were taken in SIM mode. The value of $m/z=208$, used before for these compounds [21,22] was eliminated, since the background noise was too high.

Fig. 1 shows a typical chromatogram of an urine-spiked sample. Table 3 shows the analytical features for lead speciation in human urine. Detection limits were calculated from the background

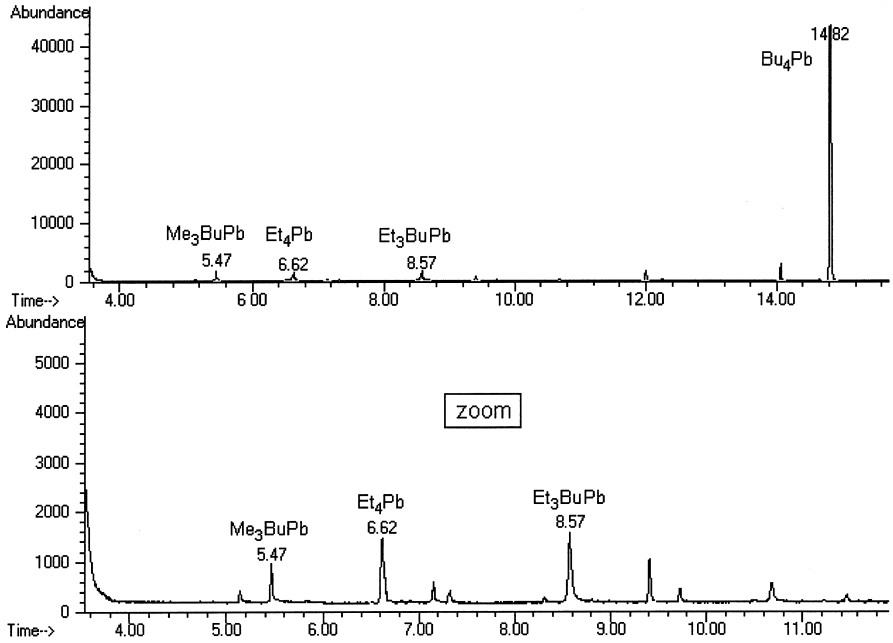

Fig. 1. Chromatogram of a urine sample spiked with 31.5 ng of Me_3Pb^+, 34.3 ng of Et_3Pb^+, 13.5 ng/g of Et_4Pb (internal standard) and Pb^{2+} in excess.

Table 3
Analytical parameters for the determination of Me_3Pb^+ and Et_3Pb^+ in urine samples

Compound	Linear limit pg/2 μl (a)	Detection limit		Quantitation limit	
		pg/2 μl[a]	pg/ml[b]	pg/2 μl[a]	pg/ml[b]
Me_3Pb^+	3.00–500.00	2.00	18.43	3.00	27.62
Et_3Pb^+	3.70–625.00	2.78	19.21	3.70	25.57

[a] Expressed as pg of compound injected into the column (volume 2 μl).
[b] Expressed as pg/ml of compound in urine sample (50 ml).

noise of the real urine sample, as the concentration equivalent to three times the background noise (experimentally observed), whereas the quantitation limit was calculated as the concentration equivalent to ten times the background noise.

3.4. Recovery studies

To obtain the recovery values in the global process, the concentration of the compounds added versus the concentration found were plotted. The slope of the straight line obtained is the recovery value. The quantification was made in two calibration curves of different ranges for the added samples. As shown in Table 4, quantitative recoveries were obtained in all cases.

The relative standard deviation is higher in the case of Et_3Pb^+, although it is an acceptable value according to the concentration level of the spiked urine samples (ng/g) and attributed to its more instability [3,21,23]. The five samples correspond to different days and also different calibration plots, which is under the Quality Control scheme [21,23,24].

3.5. Speciation of alkylleads in raw samples of urine

A urine sample from a petrol station worker with low exposure (temporary worker) to alkylleads was analyzed following the procedure described above. A value of 27.9±4.2 pg/ml of Me_3Pb^+ was found, but Et_3Pb^+ was not detected. This result can be attributed to the use of gasolines with Me_4Pb as additive, typical in European gasolines. These results are expressed per volume of urine sample whereas the common analysis in human urine often expresses the results of contaminants per gram of creatinine excreted. Obviously, more concentrated urine samples from exposed workers could have higher levels of alkylleads. However, the aim of this work was not the systematic study of workers exposed to alkylleads but the development of an analytical method for application to a wide range of exposed workers, including those with low exposure.

Table 4
Recoveries obtained in the whole method applied to the urine samples

Test number	Recovery[a] of Me_3Pb^+ (%)	Recovery[a] of Et_3Pb^+ (%)
1	99.2	95.1
2	104.2	82.9
3	97.9	92.1
4	118.9	101.8
5	105.5	114.2
$\bar{X}$	105.1	97.2
R.S.D. (%)	8	12

[a] Each value corresponds to the slope of the straight line obtained when the concentration found of each compound is represented versus the concentration added. The concentration range used to obtain each plot was between 5 and 50 ng of Me_3Pb^+ and Et_3Pb^+ added to 50 ml of urine sample.

4. Conclusions

Several conclusions can be emphasized as follows:

1. The extraction of the complex between lead derivatives and DDTC from urine samples produces a thick foam and gels which make the phase separation difficult. These gels affect the recovery of Me_3Pb^+ and Et_3Pb^+ and their losses range from 30 to 70% when using pentane as extracting agent.
2. The addition of several alcohols or NaCl to the gel and further centrifugation does not allow the appropriate phase separation and losses are still too high.
3. Using hexane as extracting agent, and adding NaCl to the gel with further centrifugation, foam and gel are broken and recoveries reach from 85 to 100% when three sequential extractions are applied.
4. No additional matrix effects from urine were observed and working under the conditions described above, recoveries of 105.1% of Me_3Pb^+ and 97.2% of Et_3Pb^+ were obtained.
5. Both the detection and quantification limits obtained in the whole process for urine by GC–MS–SIM can be compared to those obtained working with aqueous samples [21] and GC–MS–SIM. The good sensitivity of this procedure allows the speciation of ionic alkylleads in raw samples of human urine.

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