Journal of Chromatography B, 879 (2011) 231–235

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Direct LC–ES-MS/MS determination of phthalates in physiological saline solutions

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ARTICLE INFO

Article history:
Received 30 September 2010
Accepted 1 December 2010
Available online 10 December 2010

Keywords:
Phthalates
LC–ES-MS/MS
Physiological saline solutions

ABSTRACT

A method for determining a group of phthalic esters (PAEs) in physiological saline solutions has been developed. The PAEs studied were dimethyl phthalate, diethyl phthalate, butyl benzyl phthalate and dibutyl phthalate. These groups of phthalates were determined by liquid chromatography–electrospray ionization-tandem mass spectrometry, working in positive ion mode. The compounds were separated by liquid chromatography working in gradient mode with acetonitrile–ultrapure water as a mobile phase. The separation was performed starting with 5% of acetonitrile and increasing to 75% in 5 min, followed by isocratic elution for 8 min. The method was precise (with relative standard deviation (RSD) from 1.0 to 6.8%) and sensitive, with LODs of 0.05, 0.38, 0.05 and 0.82 $\mu g\,L^{-1}$ for DMP, DEP, BBP and DBP, respectively. The proposed analytical method has been applied to determine these compounds in different physiological saline solutions commercialized in plastic bottles.

1. Introduction

Phthalates (PAEs) are a group of chemical compounds widely used in industry and commerce. Due to the ability to improve the softness and flexibility of plastics, they are widely used as polymer additives in plastics. These compounds are present in a wide variety of consumer products including children toys, cosmetics, personal care products, packaging, etc. [1–3]. Phthalates are not chemically bound to plastic; thus, they can be easily released from the plastic packaging to the contents and the environment [4].

The interest in the study of these types of chemical substances has increased in recent years because some of these compounds, such as dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and diethyl hexyl phthalate (DEHP), are suspected to be endocrine disruptors and carcinogenic to humans [5,6]. Therefore, it is essential to develop reliable and sensitive methods for determining this group of compounds at trace levels.

Several methods have been developed for PAEs determination in different matrices such as, biological samples, pharmaceutical drugs and environmental samples. The analysis of PAEs is mostly performed by gas chromatography (GC). Generally, GC methods present better sensitivity than HPLC methods, although these depend on the pre-treatment step, the instrumental conditions and the sample matrix [7]. Phthalates can be detected using electron capture detection (ECD) [8,9], flame ionization detection (FID) [10–12] and mass spectrometry (MS) [13–15]. HPLC can be used as an alternative technique and is especially useful for analysis of isomeric mixtures and phthalates metabolites without derivatization [16]. HPLC can be used in combination with different detectors such as UV [17–19], mass spectrometry [20–24] and using tandem mass spectrometry [16,25–28].

In some cases, due to the low levels of these compounds in the samples, a clean up/preconcentration step is necessary before the instrumental analysis. These sample pre-treatments include liquid–liquid extractions (LLE) [24,29,30], liquid-phase microextraction (LPME) [31], single drop microextraction (SDME) [32], solid phase extraction (SPE) [25,33], solid phase microextraction (SPME) [34,35], stir bar sorptive extraction (SBSE) [36,37] and solid–liquid extraction (SLE) [38]. The major problem in phthalate determination is the sample contamination during the sample pre-treatment. Due to the fact that these compounds are widely used, they are present in the environment and can be adsorbed onto the glass and other material. This problem can be diminished using different methods proposed in the literature to prevent phthalate contamination problems [20,21,27] and by reducing the number of steps necessary to prepare the sample.

The aim of this work was to develop a high sensitive method for phthalates determination in physiological saline solution samples by LC–ES-MS/MS without any sample pre-treatment.

2. Experimental

2.1. Reagents and standards

All reagents used were of analytical reagent-grade. Dimethyl phthalate (DMP) and butyl benzyl phthalate (BBP) were obtained from Supelco (Bellefonte, PA, USA). Diethyl phthalate (DEP)

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doi:10.1016/j.jchromb.2010.12.002

and dibutyl phthalate (DBP) were obtained from Riedel-de Haën (Seelze, Germany). The purity of these reagents was over 98%.

Stock standard solutions of each phthalate ester at a concentration of 1000 mg L^{-1} were prepared in methanol, kept in darkness and stored at 4 °C in a Teflon-capped glass vial. From these solutions, a working standard solution in methanol was prepared weekly containing all standards at concentrations of 100 mg L^{-1} each. Diluted working standard solutions were prepared daily by diluting the working solution.

Lichrosolv gradient grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Technical-grade acetone and glacial acetic acid (HPLC) for instrumental analysis were purchased from Panreac (Barcelona, Spain). Ultrapure (resi-analysed) water for environmental inorganic and organic trace analysis was supplied by J.T. Baker (Phillipsburg, NJ, USA).

Special care was taken to avoid the contact of reagents and solvents with plastic materials. Because of the ubiquity of plasticizers and the tendency of residues to persist, all glassware was cleaned prior to the analysis according to the recommendations specified in U.S. EPA Method 506 [39]. All material was washed with hot water and soap, rinsed with tap and ultrapure water and finally thoroughly rinsed with technical-grade acetone. Glassware was then sealed with aluminium foil and stored in a clean environment to avoid adsorption of phthalates from the air.

2.2. Instrumentation

Phthalates separation and quantification was performed using liquid chromatography/electrospray ionization-tandem mass spectrometry system.

A Series 1100 liquid chromatograph from Agilent Technologies (Waldbronn, Germany) was coupled to an API 4000™ Triple Quadrupole Mass spectrometer (Applied Biosystems, Concord, Canada) equipped with a Turbo Ionspray™ ionization source. Mass Spectrometry data were processed with Analyst 1.4.2 software.

A ZORBAX Eclipse XDB-C_8 column (2.1 mm × 50 mm, 3.5 μm particle size) supplied by Agilent Technologies was used for the separation of these compounds.

2.3. Chromatographic conditions

Ultrapure water and acetonitrile (both solvents containing 0.1%, v/v acetic acid) were used as a binary mobile phase. Phthalates were separated by LC working in gradient mode with acetonitrile–ultrapure water as a mobile phase. The separation was performed starting with 5% of acetonitrile and increasing to 75% in 5 min, followed by isocratic elution for 8 min and increasing to 75% in 5 min, remaining at this composition for 8 min.

The flow rate and the injection volume were 200 μL min^{-1} and 10 μL, respectively, and the chromatographic separation was performed at 40 °C. Under these conditions the separation time was less than 13 min. These optimal conditions are shown in Table 1.

Table 1
Operational conditions for LC–MS/MS.

HPLC (Agilent 1100)	
Column	Zorbax Eclipse XDB-C8 (3.5 μm 2.1 mm × 50 mm)
Mobile phase	Ultrapure water:acetonitrile (0.1%, v/v acetic acid)
Mode	Gradient
Flow rate	200 μl/min
Oven temperature	40 °C
Injection volume	10 μl
MS/MS (API 4000)	
Ion spray voltage	5500 V
Ionization mode	ESI-positive
Curtain gas	25 psi (nitrogen)
GS1 (nebulizer gas)	50 psi
GS2 (auxiliary gas)	60 psi
Ion source temperature	450 °C
CAD (collisionally activated dissociation)	4

2.4. Sample preparation

The samples were injected directly into the chromatograph, without any previous sample preparation process.

3. Results and discussion

3.1. ES-MS/MS conditions

The ES-MS/MS conditions for DMP, DEP, BBP and DBP determination by ES-MS/MS were studied. The ion source dependent (turbo ion spray) conditions were the same for all the analytes with an electrospray needle voltage of 5500 V in the positive ion mode. Nitrogen as a nebulizer and turbo heater gas (at 450 °C) was set as a pressure of 50 and 60 psi, respectively. The pressure of the curtain gas was also optimized selecting 25 psi as the optima pressure. Ion source collision-activated dissociation (CAD) was studied during the development of the method, selecting 4 V as the optimum condition.

To establish the MS/MS operating conditions used to determine these phthalates by ES-MS/MS, a standard solution (100 mg L^{-1}) of each phthalate were used. These solutions were infused directly into the MS/MS system using the syringe pump system of the API 4000. The phthalates studied were monitored at *m/z* 195, 223, 313 and 279, working in the scan mode, which were assigned to $[M+H]^+$. Moreover, in the product ion MS/MS measurement, the selective reaction monitoring ions (SRM) of DMP, DEP, BBP and DBP were set depending on their precursor ions. The combinations of precursor ion and product, as well the optimum potentials, are shown in Table 2.

3.2. Optimization of LC separation

After optimizing the detection conditions, the following experiments were conducted to optimize the chromatographic separation of the analytes.

Table 2
Optimal values of the compound parameters for the four phthalates studied, *m/z* transition selected and retention time (DP: declustering potential; EP: enhance potential; CE: collision energy; CXP: collision cell exit potential).

Compound	Acronym	*m/z* transition	Potentials optimization				t_R (min)
			DP	EP	CE	CXP	
Dimethyl phthalate	DMP	195 → 163	31	10	13	14	8.4
Diethyl phthalate	DEP	223 → 149	36	10	23	12	9.2
Butyl benzyl phthalate	BBP	313 → 91	41	10	23	6	11
Dibutyl phthalate	DBP	279 → 205	50	9	11	10	11.2

Table 3
Linear range, correlation coefficients, LODs and LOQs values obtained from the standard addition method in physiological saline solutions.

Phthalate	Linear range ($\mu g\,L^{-1}$)	Correlation coefficient (r)	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
DMP	0.5–50	0.9996	0.05	0.16
DEP	1–50	0.9978	0.38	1.27
BBP	1–50	0.9986	0.05	0.16
DBP	1–150	0.9956	0.82	2.74

Experiments were performed using acetonitrile:water, both solvents containing 0.1% (v/v) acetic acid as a mobile phase. This mobile phase was selected based on a previous work developed in our research group for phthalates determination in physiological saline solutions by LC–ES-MS [20]. Experiments were developed using a physiological saline solution spiked with 25 $\mu g\,L^{-1}$ of DMP, DEP and BBP, and 100 $\mu g\,L^{-1}$ of DBP. The best results were obtained starting the elution with 5% of acetonitrile, which was then increased linearly to 75% in 5 min. This composition was maintained for 8 min before returning to initial conditions. The column was equilibrated for 10 min.

Other parameters optimized were the temperature of the chromatographic column and the flow rate of the mobile phase. The optimum conditions selected were at temperature of 40 °C and a flow rate of 200 $\mu L\,min^{-1}$.

The chromatogram obtained for the physiological saline solution, spiked with 25 $\mu g\,L^{-1}$ of DMP, DEP and BBP, and 100 $\mu g\,L^{-1}$ of DBP, under the optimized conditions is shown in Fig. 1.

3.3. Analytical performances

After selection of the optimum conditions for LC–ES-MS/MS, the method was evaluated using DMP, DEP, BBP and DBP standard solutions.

The linearity of the response of this method was evaluated using a standard addition method. This addition was performed at seven different concentrations of the standard solution of these phthalates, using a commercial physiological saline solution supplied in a glass bottle. Linear regression was performed by plotting the peak area versus concentration, and was linear over the range of 0–50 $\mu g\,L^{-1}$ for DMP, DEP and BBP, and of 0–150 $\mu g\,L^{-1}$ for DBP. The equations obtained for each compound were as follows:

$$\text{DMP}: Q_A = 273725\,C + 265276 \quad r = 0.9996$$

$$\text{DEP}: Q_A = 325956\,C + 208430 \quad r = 0.9978$$

$$\text{BBP}: Q_A = 255127\,C + 185647 \quad r = 0.9986$$

$$\text{DBP}: Q_A = 129571\,C + 263706 \quad r = 0.9956$$

where Q_A is the peak area and C is the concentration in $\mu g\,L^{-1}$.

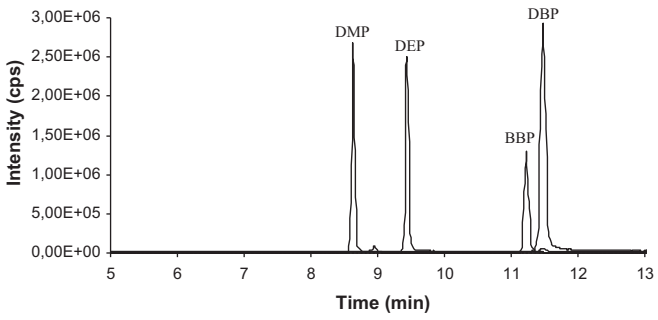

Fig. 1. LC–MS/MS ion chromatogram obtained from a physiological saline solution spiked with 25 $\mu g\,L^{-1}$ of DMP, DEP and BBP, and 100 $\mu g\,L^{-1}$ of DBP.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the IUPAC definition:

$$\text{LOQ} = \frac{10SD}{m} \qquad \text{LOD} = \frac{3SD}{m}$$

where SD is the standard deviation of ten blank solutions and m is the slope of the addition graph. A commercial physiological saline solution supplied in a glass bottle was used as a blank. The results obtained for LODs and LOQs are shown in Table 3. The LODs obtained are between 0.05 and 0.82 $\mu g\,L^{-1}$. The highest LOD obtained was for DBP. These LODs are lower than those obtained in a previous study to determine these compounds in the same type of samples by LC–MS [20]. Moreover, the method presents better or comparable sensitivity than other methods proposed in the literature for the determination of these phthalates using GC-MS in water samples. Serodio and Nogueira [2] developed a method for phthalates determination using stir bar sorptive extraction with liquid desorption followed by large volume injection and GC-MS obtaining LODs from 0.15 to 0.60 $\mu g\,L^{-1}$. Peñalver et al. [40] obtained LODs from 15 to 50 $\mu g\,L^{-1}$ for these phthalates using GC-MS, and obtained LODs from 0.007 to 0.17 $\mu g\,L^{-1}$ using SPME previous to the determination by GC-MS. Koch et al. [1] obtained LODs from 0.25 to 1.0 $\mu g\,L^{-1}$ for the determination of these phthalates in urine samples by LC–ES-MS/MS. The advantage of the proposed method is that present a good sensitivity when analyzing the sample directly, without any requiring preparation steps (e.g. preconcentration step).

Assays were developed to check intra- and interday precision. For the intraday study, aliquots of a physiological saline solution purchased in a glass bottle were spiked with two concentration levels of all phthalates studied and analysed six times in the same run. The interday assay was performed in the same way analyzing 12 aliquots of spiked samples in two different days. The results obtained for the intra- and interday assays are shown in Table 4. The RSD values were between 1.2 and 5.0% in the intraday assay and between 1.0 and 6.8% in the interday assay; thus, the method is precise for all the compounds studied.

The analytical recovery of the method was calculated using a blank sample (physiological saline solution commercialized in a glass bottle) spiked with three different concentrations of these compounds (5, 25 and 50 $\mu g\,L^{-1}$ for DMP, DEP and BBP and 40, 100 and 150 $\mu g\,L^{-1}$ for DBP). The spiked samples were prepared twice and analysed three times, and the recovery calculated using the standard addition graph. The recovery percentages obtained are shown in Table 5. The average analytical recoveries were 106.7, 92.6, 102.9 and 96.4% for DMP, DEP, BBP and DBP, respectively.

3.4. Application to physiological saline solution samples

The proposed analytical method has been applied to the analysis of different physiological saline solution samples, commercialised in plastic bottles, in order to check the presence of these phthalates and determine their concentration. Samples were directly injected into the chromatographic system; and no sample preparation process was necessary.

The results obtained for DMP, DEP, BBP and DBP are given in Table 6. The concentration levels obtained for BBP are lower than the LOD for all samples studied, and DBP was only detected in

Table 4
Results of intra- and interday assays to validate proposed LC–MS/MS method.

Phthalate	Intraday ($n = 6$)			Interday ($n = 12$)		
	Detected average (ng mL^{-1})	SD	RSD (%)	Detected average (ng mL^{-1})	SD	RSD (%)
DMP	29.86	0.36	1.20	29.88	0.29	0.97
	49.10	2.02	4.12	50.23	1.94	3.86
DEP	24.88	0.31	1.25	26.52	1.81	6.84
	46.24	1.04	2.25	46.95	1.33	2.84
BBP	24.28	0.46	1.89	24.44	0.61	2.51
	48.04	2.41	5.01	49.51	2.28	4.61
DBP	92.06	2.01	2.19	97.88	6.55	6.70
	148.91	6.91	4.64	146.74	5.40	3.68

Table 5
Recovery percentage for physiological saline solutions ± standard deviation to validate proposed LC–MS/MS method ($n = 3$).

Phthalate	% Recovery		
	5 μg L^{-1}	25 μg L^{-1}	50 μg L^{-1}
DMP	100.3 ± 2.5	118.3 ± 0.8	101.4 ± 3.2
DEP	81.4 ± 1.6	101.4 ± 0.5	94.9 ± 0.6
BBP	111.5 ± 2.8	98.7 ± 0.8	98.6 ± 2.7
Phthalate	% Recovery		
	40 μg L^{-1}	100 μg L^{-1}	150 μg L^{-1}
DBP	92.2 ± 2.2	93.6 ± 1.2	103.4 ± 1.6

Table 6
Concentration (μg L^{-1}) ± standard deviation (based on three replicates) found in different physiological saline solutions. <LOD: lower than the detection limit.

Physiological saline solution	DMP	DEP	BBP	DBP
Brand 01	17.4 ± 0.6	14.5 ± 0.4	<LOD	7.7 ± 0.6
Brand 02	0.4 ± 0.1	<LOD	<LOD	<LOD
Brand 03	19.2 ± 1.5	3.9 ± 0.2	<LOD	<LOD
Brand 04	346.8 ± 0.8	2.7 ± 0.1	<LOD	<LOD

brand 1. The concentration levels varied from 0.4 to 346 μg L^{-1} for DMP and from 0.4 to 14.5 μg L^{-1} for DEP. The brand 2 sample presented the lowest concentration of phthalates, being DMP the only phthalate detected. Phthalate esters are used in the manufacture of plastic containers; thus, the presence of phthalates in the samples can be attributed to the release of these compounds from the plastic containers. As an example, the chromatogram obtained when analyzing the brand 1 sample is shown in Fig. 2.

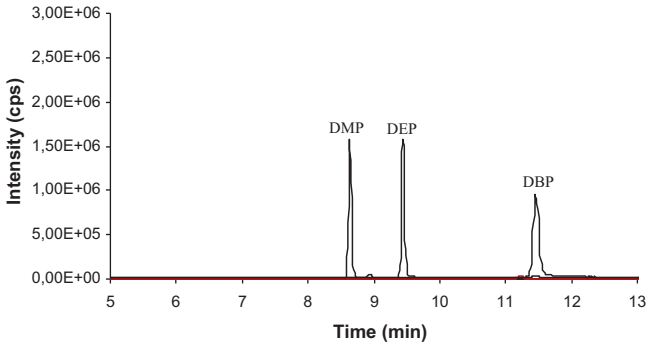

Fig. 2. LC–ES-MS/MS ion chromatogram obtained from brand 1 physiological saline solution.

4. Conclusion

A rapid (less than 13 min), sensitive and accurate method for the determination of DMP, DEP, BBP and DBP by LC–ES-MS/MS was developed. The main advantage of this method, compared with the methods proposed in the literature, is that the compounds can be detected at very low concentration without any sample pre-treatment. Moreover, the limits of detection obtained are comparable with the LODs found in the literature for determining of these phthalates by researches who performed a preconcentration step before the determination by GC-MS. Another advantage is that the reduction of the number of sample pre-treatment steps decreases the risk of the sample contamination during the analysis, which is a very common problem in the analysis of phthalates.

The method was applied for the determination of these compounds in four physiological saline solutions commercialized in plastic bottles. The presence of these compounds in the samples can be attributed to the different compositions of the plastic containers. Thus, control of material used in the manufacture of the plastic containers is essential to avoid human exposure to these toxic contaminants.

Acknowledgement

The authors wish to thank the Xunta de Galicia (Grupo de Referencia Competitivos 2007 (000047-0) for financial support.

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