A NEW METHOD FOR DETERMINATION OF PHTHALATES IN BREAST MILK BY HPLC-UV-MS WITH ON-LINE SAMPLE PROCESSING

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Introduction

Phthalates are ubiquitous pollutants due to their huge widespread use in various indoor as well as industrial applications, e.g in PVC products, as ingredients in printing inks, paints, wall-papers, wood finishes, in adhesives, in cosmetics, etc[1]. They seriously contaminate food, however significant exposure from air cannot be excluded[2]. In humans, dialkylphthalates are rapidly metabolised to monoalkylphthalates, which have been in many cases shown to have similar health effects as the parent compounds. Both are toxic to kidney, liver and testes[3]. While dialkylphthalates have a potential for bioaccumulation, monoalkylphthalates are easily glucuronidated and thus supposed to be excreted fast. This might be , however, argued for higher alkylated phthalates. Some data[4] shows a disproportion in ratio of a probable daily intake of different dialkylphthalates to the amount of corresponding monoalkylphthalate excreted in urine. For several reasons, infants belong to the highest exposed group of population (toys, teethers, PVC flooring, food). However, is the exposure to phthalates from breast milk significant?

So far, neither dialkylphthalates, nor monoalkylphthalates were determined in breast milk. Analysis of dialkylphthalates is difficult due to contamination problems. Recently a method for analysis of monoalkylphthalates in urine based on solid phase extraction and HPLC-MS-MS has been published[1]. To our opinion both dialkylphthalates and monoalkylphthalates has to be measured to reveal their accumulation in breast milk. Contamination problems must be overcome by limited contact of the sample with laboratory environment and by limited usage of possibly contaminated chemicals and materials. Here, we present a method for determination of phthalates in breast milk using on-line sample processing and selective determination by HPLC-MS. A restrictive access reverse phase type column has been tested for sample clean-up.

Materials and Methods

Chemicals

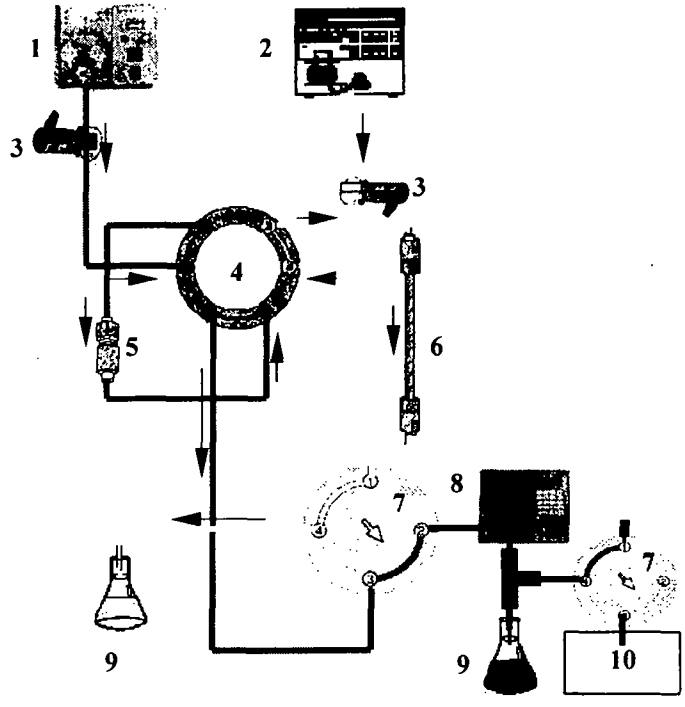
Breast milk was obtained as generous gift from a colleague. All standards both, analytical grade substances and a standard solution of phthalate ester mixture were purchased from Aldrich-Supelco. All solvents used were of HPLC grades.

Sample preparation

1 ml of breast milk sampled into 2 ml microcentrifuge tube (Corning) was deluted with 2-propanol in amount to get 30 – 50 % (v/v) content of the solvent in the sample. The sample was spiked with internal standard, a methanol solution of *n*-dioctyl phthalate and with a mixture of dialkyl phthalates at two levels 500 and 50 ng/ml. Before analysis, the sample

was centrifuged at 5000 rpm for 10 min. 100 µl of the sample was injected on the pre-column.

On-line sample processing and determination of phthalates in milk
A set-up in loading stage is shown in Scheme 1. The sample was loaded on LiChrospher RP-8 ADS pre-column (25 µm; 25 x 4 mm) (Merck), a restricted access reverse phase type column[5], using Rheodyne 7125 injector and methanol/water (5/95) mobile phase. The mobile phase was delivered by isocratic pump (Waters) at a flow of 1.0 ml/min. Washing out of proteins was monitored by variable UV detector (Hewlett-Packard 1050) at 254 nm connected to the pre-column through six-port switching valve (Valco). The purified sample was transferred onto an analytical column Waters Symmetry RP 18 (5 µm; 2.1 x 150 mm) by backflash elution with acetonitrile/water (15/85), as mobile phase, delivered by gradient pump (Perkin-Elmer) at a flow of 0.5 ml/min. Analytes were separated using acetonitrile/water (90/10) mobile phase fortified with 2% (v/v) formic acid at 0.2 ml/min and detected by VG Platform quadruple mass spectrometer (Fisons Instruments) equipped with an atmospheric pressure chemical ionisation source in positive mode. Ions selective for the determined compounds were monitored. The set-up was further equipped with second Rheodyne 7125 injector enabling direct injection of standards on analytical column and with second four-port switching valve enabling switching between MS detector and waste.



Scheme 1. Instrument set-up adjusted for loading of sample
1 - isocratic pump, 2 - gradient pump, 3 - injector, 4 – six-port switching valve, 5 – pre-column, 6 – analytical column, 7 – four-port switching valve, 8 - UV detector, 9 – waste, 10 – APCI-MS

Extensive washing of pre-column with 2-propanol/water (4:1) was performed during the separation of solutes on analytical column in order to prepare the pre-column for the next injection.

Results and Discussion

Phthalates exhibit very broad range of hydrophobicity that makes their sample clean-up and analysis difficult. Five phthalates were included in this study: dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), di(2-ethyl-hexyl) phthalate (DEHP) and butylbenzyl phthalate (BBP). Di-*n*-octyl phthalate (DOP) was used as internal standard.

Sample preparation

Breast milk must be centrifuged prior to injection on pre-column. Because of high lipid content, centrifugation resulted in a separated layer of fat on the top of the milk sample. In order to increase solubility of lipids addition of different amounts of 2-propanol was tested. Homogenous sample solution was first obtained with more then 40% 2-propanol in sample.

Sample clean-up

Alkyl-diol silica adsorbent used for sample clean-up in the pre-column permits adsorption of low molecular weight solutes, while proteins are excluded from pores. Conditions were matched to get full uptake of DMP and simultaneously prohibit precipitation of DEHP in mobile phase with low organic content. Several mobile phases based either on methanol or acetonitril were tested for sample loading. Methanol/water (1:19) was found to be an optimal mobile phase composition with respect to both washing out of proteins and retention of analytes. The conditions resulted in about 15 min breakthrough time for DMP. 5 min was selected as loading time. Uptake of DEHP by the pre-column was enhanced by increasing amount of 2-propanol in sample as shown in Table 1.

Table 1 Effect of 2-propanol content in sample for diethyhexyl phtalate (3.88 µg/ml) recoveries.

Sample matrix	Water		Breast milk	
2-propanol content	40%	50%	40%	50%
DEHP recovery	87%	100%*	26%	85%

Response value set arbitrarily to 100%

Monitoring of this step was achieved using variable UV detector. Thus introducing sample matrix into the MS was avoided.

Increasing column retention power (octyl-silica - octadecyl-silica) resulted in solute focusing on analytical column. Mobile phase consisting in acetonitril/water (15:85) worked well for transfer of DMP, the other phthalates were transfer by gradient mobile phase composition up to acetonitril/water (9:1).

Analytical separation and MS detection

Good separation was achieved with step gradient in mobile phase composition. Sensitivity of MS detection was increased about 3 times by fortifying mobile phase with 2% formic acid.

As soon as solutes were transferred on the analytical column the flow through the pre-column was reversed to loading mode and the pre-column was extensively washed with 2-propanol/water (4:1). Thus, low blank levels of the selected dialkylphthalates were obtained.

Determination of dialkylphthalates in breast milk samples
Levels of dialkylphthalates determined in breast milk spiked at two levels as well as in non-spiked breast milk and in procedural blank are shown in Table 2.

Table 2. Phthalate levels determined in spiked and non-spiked breast milk

	Spiking level	Solute concentration [ng/ml]			
	[ng/ml]	**DEP**	**BBP**	**DBP**	**DEHP**
Milk	500	324 ± 58	452 ± 35	512 ±29	329 ± 110
Milk	50	---	38 ± 7	45 ±8	27 ± 7
Milk	0	---	0	14 ± 2	19 ± 4
Blank	0	---	0	8 ± 2	10 ± 3

Uptake of phthalates from spiked breast milk was good for BBP and DBP at both high and low level spiking. APCI MS resulted in good sensitivity for these solutes. Sensitivity for other solutes was much lower. It was not possible to determine DMP at both spiking levels and DEP at low level. Precision of determination of DEHP was rather low. However, the mass spectrometric detection has not yet been optimised. The possibility of increasing sensitivity by injection of about 5 to 10 time larger sample should be as well investigated. The blank levels were acceptably low.
Low levels of DBP and of DEHP were found in the breast milk sample.
In conclusion, the presented method is applicable for analysis of dialkyl phthalates in breast milk. Good sensitivity was obtained for analysis of DBP and BBP. Mass spectrometric detection of other phthalates should be further optimised. DBP and DEHP may accumulate in breast milk.

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