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Automated on-line liquid chromatography–photodiode array–mass spectrometry method with dilution line for the determination of bisphenol A and 4-octylphenol in serum

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Abstract

A novel on-line liquid chromatography–photodiode array detection–mass spectrometry (LC–DAD–MS) system was established with restricted-access media (RAM) pre-column and dilution line combined with a column-switching valve. The serum samples were injected directly onto pre-column under diluted condition by dilution line. After elution of proteins in the serum, the analytes were backflushed onto an ODS analytical column using a six-port column-switching device. The influence of the composition of the mobile phase, for instance, organic modifer, ionic strength, pH, dilution times and the rotation time of the switching valve have been investigated using bisphenol A (BPA) and 4-octyphenol (4-OP) as analytes. The evaluations for peak responses and sensitivity were conducted by MS, and proteins were removed by RAM-column with DAD monitoring at 280 nm. The peak shape was improved by adding a dilution line, especially in the case of large volume injection (LVI), which increased the sensitivity of the analysis. The selective and sensitive quantification of BPA and 4-OP in serum sample could be finished within 25 min. The method had linearity in the range 0.1–500 ng/mL with a limit of quantification for BPA and 4-OP of 0.1 and 0.5 ng/mL, respectively. The recoveries were in the range of 80–101% with less than 9.0% RSDs. This on-line LC–MS method demonstrates potential application to evaluating the exposure and risk of BPA and 4-OP in human.

Keywords: On-line LC–DAD–MS; Dilution line; Bisphenol A; 4-Octyphenol

1. Introduction

Conventional biological matrix extraction methods such as protein precipitation, liquid–liquid extraction, and solid-phase extraction (SPE) are frequently laborious and time-consuming and they have relatively poor reproducibility [1]. As a result, these tedious processes become bottlenecks and often limit the throughput of analysis. In addition, off-line extraction procedures increase the risk of sample loss or contamination [2].

Significant effort has been done to develop automated sample pretreatment techniques. On-line sample extraction using column switching has become an increasingly popular method for high throughput bioanalysis [3]. Simple process of sample preparation also reduces the risk of handling the potentially bio-hazardous material. On-line technique offers a fast and rugged extraction; however, when this is done with a regular reversed-phase packing material, the life-time of the extraction pre-column is reduced drastically due to increasing back pressure caused by protein precipitation [4].

A new pre-column packed with RAM was introduced. The samples were cleaned up based on two simultaneous chromatographic separation mechanisms: the outer surface of these porous RAM particles contains hydrophilic, electroneutral diol groups or methylcellulose and internal surface reversed-phase supports, such as butyl (C4), octyl (C8) or octadecyl (C18) groups are bonded. By size exclusion principle, macromolecules, such as protein, are excluded and flushed away to avoid the adsorption on the inner surface. While small target molecules can penetrate into the pores and be retained due to

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hydrophobic interaction. The retained analytes are subsequently eluted from the extraction column using an organic mobile phase onto an analytical column for the chromatographic separation. Such an automated system can be achieved by coupling a RAM to conventional liquid chromatography via column switching. This approach has been applied widely and successfully to the analysis of small molecular drug such as indomethacin [5] and pollutant such as 1-hydroxypyrene [6] in different biological matrices [7,8]. Due to trace amount chemicals in biological matrices, highly sensitive and accurate methods are required for the detection and measurement of them; while LC–MS with RAM column just satisfies the requirement of analysis and become an attractive technique for the fast and sensitive determination of analytes in biological fluids.

A typical reason of sensitivity loss in LC–MS analysis of biological samples is the so-called matrix effect [9]. In addition to posing challenges to quantification, the matrix effect also results in the suppression of the analyte signal and lower sensitivity. Several approaches have been investigated to address matrix effects and improve sensitivity [10]. Large volume injection has also been demonstrated for environmental analysis [11]. However, direct LVI of complex matrix into LC–MS system may induce matrix signal suppression, column overload and also result in peak broadening [12]. To prevent a breakthrough of the analyte and ion suppression effect, Wyss and co-workers [13,14] took on-line dilution to accomplish large volume plasma injection after deprotein with ethanol for the determination of Ro 63–1908 in rat and cynomolgus monkey plasma. However, the on-line dilution system was very complicated including many valves and pumps.

RAM pre-column has been mainly used to medical analysis in bio-analysis [8]. With increasing of environmental pollution, environmental monitor with high throughput analysis is really required since kinds of pollutants enter into human body by exposure to the environment. Recently, analytical methods for the determination of phenols [15], naphthol [16], phthalate [17,18], polycyclic aromatic hydrocarbons (PAHs) and their metabolites [6,19,20] in biological matrices have been reported. Widespread human exposure to such endocrine disrupting chemicals (EDCs) has had a potential health risk. Bisphenol A (BPA), an estrogenic endocrine disrupting chemical, is widely used in the production of polycarbonate plastics and epoxy resins, which are used in dentistry, food packaging, and as lacquers to coat food containers, bottletops, and water pipes [21]. 4-Octylphenol (4-OP), which is one of alkylphenols and breakdown product of alkylphenol polyethoxylates (APEs), is one of popular non-ionic surfactants that are widely used in many detergent formulations and plastic products for industrial and domestic use [22]. For instance, BPA and 4-OP can enter into the body via the gastrointestinal tract as food contaminants. Only trace these chemicals usually remain in body, and the levels are different in bodies lived in different regions [23]. Thus, it is great important to develop a sensitive and selective method for the determination of BPA and 4-OP in biofluid to assess their exposure and danger for human. It has been reported to determine BPA in biofluid sample by LC [24,25], GC–MS [26,27] after derivatization to improve sensitivity. LC–MS is an excellent alterative method reported by Sajiki et al. [28]. Inoue published a series of methods using LC–MS [29–31] for the determination of BPA in human plasma, semen and urine. The methods of sample pretreatment included off-line SPE and on-line exaction using Oasis HLB pre-column [29] or RP-18 GP pre-column [31]. Ye et al. [3] developed a method using isotope dilution on-line solid-phase extraction coupled to high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) for the determination of nine environmental phenolic compounds in urine.

The purpose of the present research was to develop a new on-line automated pretreatment LC–DAD–MS system with RAM pre-column and dilution line, and achieve the determination of BPA and 4-OP in serum samples. In order to optimize the analytical condition, the composition of the extraction mobile phase, such as the content of organic modifier, ionic strength, pH and dilution times were studied. By monitoring the protein elution, the most suitable time for the rotation of switching valve were determined in order to remove effectively protein. A full validation was performed to assess the accuracy, precision, linearity, and limit of detection and quantification of the compounds, and the observed results presented demonstrate that the method was feasible to analyze BPA and 4-OP in human serum. It was subsequently used to analyze their concentrations in human serum samples. To our knowledge, it is the first report that on-line LC–MS with RAM pre-column and dilution line was established and used for the determination of BPA and 4-OP in human serum by direct injection.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade water was obtained by purification of de-ionized water through a Milli-Q system (Millipore, Bedford, MA, USA). Methanol and acetonitrile (HPLC grade) were purchased from Fisher (Fair Lawn, NJ, USA). BPA (lot GK01, purity 99%) was purchased from Kasei Kogyo (Tokyo, Japan) and 4-OP (lot 15108EB, purity 99%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate was purchased from Wako (Osaka, Japan). Stock solutions of BPA and 4-OP at 1.0 mg/mL were prepared in methanol and working standard solutions were made at various concentrations by appropriate dilution of the stock solutions. These solutions were stored at −25 °C.

2.2. Serum samples

Human serum samples were obtained from Pingdingshan People's Hospital (Henan, China) and stored at −25 °C. Before use, the serum samples were thawed at room temperature and centrifuged at 5000 rpm for 10 min. These serum samples were spiked with the diluted standard solutions daily.

2.3. Apparatus

The shimadzu LC–MS 2010EV system was applied. It is configured with two LC-20AD pumps, one LC-20AB gradi-

ent delivery pump, a DGU-20A5 degasser, a SIL-20AC autosampler, CTO-20AC equipped with two six-port valves, SPD-M20A photodiode array detector, CBM-20A communication base module and a single quadrupole MS analyzer equipped with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) ionization interfaces. Both ionization interfaces can be operated in either positive or negative ionization modes. DAD was set at 280 nm. Data acquisition and processing were performed with LC–MS solution Ver3.2 workstation. The chromatographic separation was performed on Intersil-ODS column (150 mm × 2.0 mm, 5 μm) purchased from GL Science (Tokyo, Japan). RAM pre-column, Shim-pack MAYI-ODS (10 mm × 4.6 mm, 50 μm) was obtained from Shimadzu (Kyoto, Japan).

2.4. Detection conditions of mass spectrometry

Operating conditions of the APCI interface in negative ionization mode: interface temperature, 400 °C; CDL temperature, 200 °C; block heater, 200 °C; nebulizer gas (N_2), 2.0 L/min; drying gas (N_2), 0.03 MPa; detector voltage, 1.5 kV; probe voltage, 4.5 kV.

Time-scheduled selected-ion monitoring (SIM) of the most abundant ions $[M–H]^-$ (227 for BPA and 205 for 4-OP) of each compound was used for quantification.

2.5. On-line sample clean-up and chromatographic conditions

A diagram of LC–DAD–MS system with on-line automated pretreatment was presented in Fig. 1. At position A of the valve, the serum samples were injected automatically onto RAM extraction column with dilution ratio of one-seven delivered by pumps C and D. Pump C was used as the carrier of sample, while pump D was used for the dilution line. Both mobile phases were methanol–water (5:95, v/v) containing 10 mM ammonium acetate (pH 6.67) at the flow-rate of 0.3 and 2.1 mL/min, respectively. Following removal of serum proteins and other matrix components for 5 min, the serum proteins were washed away to the waste while analytes were retained on the RAM pre-column. During this step, the analytical column was re-equilibrated with methanol–water (30:70, v/v) at a flow-rate of 0.5 mL/min delivered by pump AB. The analytes were then eluted with the back-flush mode by switching the valve to position B, transferred to analytical column and separated under a gradient condition at the flow rate of 0.5 mL/min. The gradient program for the mobile phase driven by pump AB (methanol–water) was as follows: first, 30% (v/v) methanol hold for 3 min, then, the content of methanol was increased linearly from 30 to 100% in 4 min; and the content of methanol was hold at 100% for 9 min. Finally, the content of methanol was decreased linearly to 30% from 16 to 21 min. At 21:00 min, the switching valve was returned to its original position. Extraction column was re-equilibrated with the mobile phases (5% methanol containing 10 mM ammonium acetate) and analytical column was re-equilibrated with the mobile phases (30% methanol) for 4 min, respectively. The total analytical time for a single analytical run was 25 min. Both columns were kept at 40 °C. Injection volume was set at 100 μL.

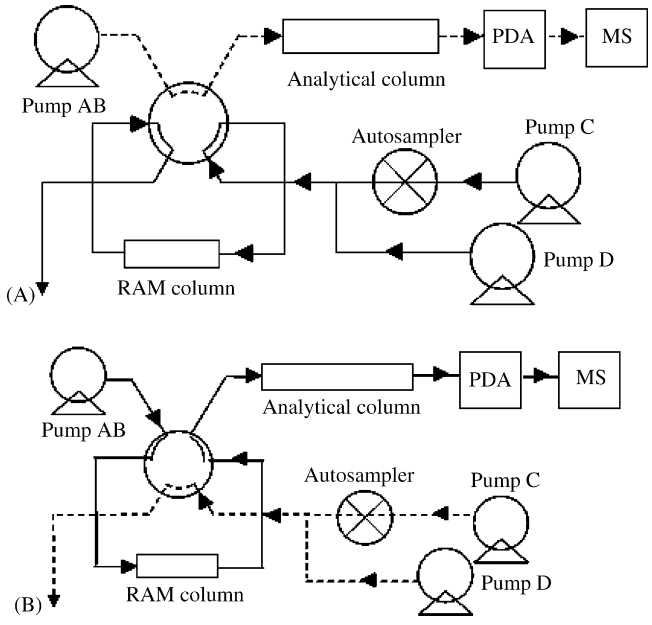

Fig. 1. Schematic diagram of the on-line LC–DAD–MS. (A) Serum sample was injected into the RAM precolumn. Analytes were retained on the precolumn and proteins were removed from precolumn. (B) The analytes were eluted from precolumn in backflushing mode to analytical column and separated.

2.6. Validation study

The linear dynamic range, precision, recovery, limits of detection (LODs) and quantification (LOQs) were evaluated for the analytical methodology developed. The linearity was determined by calibration curve created with spiked serum samples of 0.1, 0.5, 1, 5, 10, 50, 100, 500 ng/mL. Recovery and precision were tested with serum samples spiked at three levels 0.5, 50 and 500 ng/mL. Five replicates were analyzed at each spiked level. LODs and LOQs for each analyte were estimated at the signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. The recoveries were calculated by comparing the peak areas obtained from spiked serum samples with those obtained from standard solutions.

3. Results and discussion

3.1. Optimization of on-line LC–DAD–MS conditions

For analyzing small molecules in serum sample, the analytes should first be released from protein because most of the analytes in the serum are usually bound to or aggregated with protein by hydrophobic or electrostatic interaction. The removal of protein usually takes an off-line and an on-line method. Although the on-line method has some advantages, several challenges are encountered, especially when coupled with LC–MS [32]. Since ion suppression often occurs due to matrix components, the ion intensity of the analytes becomes weak and the reproducibility is affected [33]. The challenge is especially true when the extraction mobile phase varies from the analytical mobile phase.

Therefore, during deproteinization, optimization of the composition of the extraction mobile phase, such as pH or ionic strength and the addition of organic solvent, may be required to remove protein and improve the ion intensity of the analytes. In addition, the rotation time of the switching valve and dilution times were optimized. In order to monitor the optimization conditions, the eluent from analytical column was taken directly to a DAD detector measuring at 280 nm, which would detect most proteins, and then tandem-connected with MS [33]. Thus, the profile of protein elution and the ionic signal intensity of analytes were measured by DAD and MS, respectively.

In the column-switching systems coupling RAM to LC, the addition of a limited amount of organic modifier, such as methanol or acetonitrile, to the extraction mobile phase is important to enhance the extraction selectivity, to release the analyte reversibly bound to serum proteins, and to obtain high recoveries [8,34,35]. Therefore, the influence of methanol and acetonitrile in the extraction mobile phase was tested. Methanol was more efficient for obtaining high recovery, which is most likely due to the fact that acetonitrile is more liable to precipitate protein which may adduct the analytes of interest. In the same way, a high concentration of methanol also caused protein precipitation, low recovery, the blocking of the tubes and rapid degeneration of the precolumn and analytical column. In order to avoid protein precipitation, the concentration of methanol in the extraction mobile phase was less than 20% (v/v) [36]. After experiments, it was found that slightly greater signal of analytes was obtained using 10% methanol as extraction mobile phase compared with 5% methanol. The reason could be that higher content of organic solvent weakened the hydrophobic interaction between protein and analytes. However, the column life was shortened dramatically due to protein precipitation when 10% methanol was used as the extraction mobile phase. Five percent methanol was taken.

It was well known that protein was eluted easily in acidic or basic solution far from isoelectric point (p*I*) of protein. However, on the one hand, a basic mobile phase would damage silica column; on the other hand, with a decrease of pH of the extraction mobile phase, the sensitivity reduced quickly since acidic ionization condition would suppress their ionization dramatically when BPA and 4-OP were detected in the negative mode. To avoid compromising sensitivity, we had no choice but to select a neutral extraction mobile phase.

Ionic strength of the mobile phase also plays an important role in protein elution. Usually, proteins are more soluble in solution with high ionic strength. We selected ammonium acetate to adjust the ionic strength by changing its concentration. The concentrations of ammonium acetate in mobile phase from 1 to 15 mM were investigated. Fig. 2 gave chromatograms eluted from analytical column after using different extraction mobile phases to elute protein. The absorbance indicated that the residual proteins on RAM were eluted by analytical mobile phase, entered into analytical column and detected by DAD at 280 nm. It could be seen that most proteins were removed, and only little protein entered into analytical column, when the concentration of ammonium acetate was increased to 10 mM. The signal of absorbance decreased slightly when ionic strength was increased

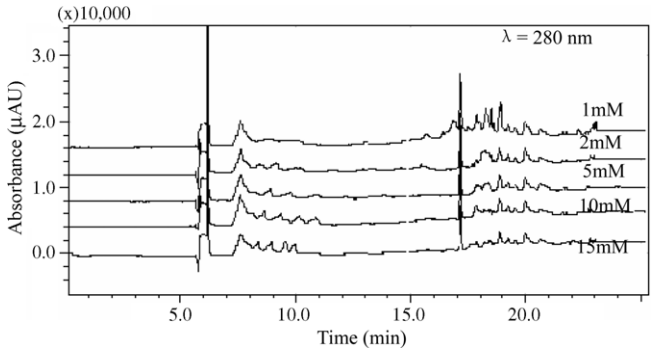

Fig. 2. Profile of protein absorbance after washed by different ionic strengths of NH_4Ac in extraction mobile phase. Detection: DAD at 280 nm. The concentrations of analytes were 50 ng/mL and injection volumes were 50 μL.

from 10 to 15 mM. Considering that more salt would liable to damage the MS instrument, 10 mM ammonium acetate was selected.

The most suitable time for the rotation of the switching valve was determined in order to clean-up effectively sample and transfer the analytes completely from pre-column to analytical column. The optimal time for washing proteins and retaining analytes was 5 min; otherwise, the sensitivity decreased whether shortening or prolonging time.

As shown in Fig. 3A, the serum sample enters into RAM column, proteins are removed due to size exclusion interaction, and small analytes would be retained by hydrophobic inner surface. Usually, on-line extraction usually takes a single line to load and

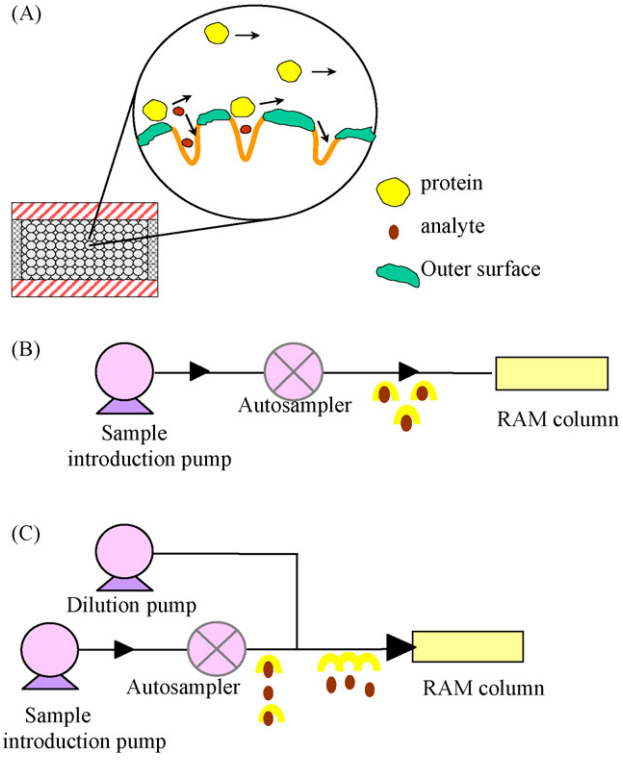

Fig. 3. The profile of RAM pre-column and sketch principle of sample loading and protein elution from RAM pre-column. (A) Configuration of RAM pre-column and principle of protein elution; sample was loaded and protein was eluted without dilution line (B) and with dilution line (C).

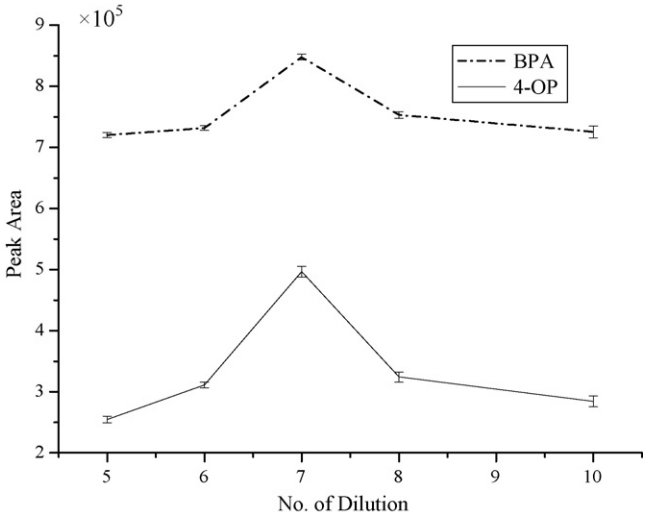

Fig. 4. The signal of analytes under different dilution times by dilution line during the extraction process. The condition was the same as Fig. 2.

elute sample as shown in Fig. 3B. Analytes adducted with protein in serum enter into RAM column with sample introduction mobile phase, thus, analytes liable to wash out of pretreatment column together with protein. When injection volume gets large, peak shape is deteriorated and recovery is decreased. The problem could be effective resolved by the use of a dilution line as shown in Fig. 3C. By addition of the dilution line, analytes could be released from protein due to impulsion by dilution mobile phase at large flow-rate. Thus, more analytes would enter into RAM column with free form which increased the retainable ability and column capacity of RAM. Even with LVI, the peak shape was symmetrical. Therefore, dilution times had a critical effect on the process, and it was optimized from 5 to 10 times as shown in Fig. 4. The optimum result was achieved using seven multiple volumes of the mobile phase to dilute sample. Proteins did not release the analytes completely at low dilution times, while analtyes especially relatively polar BPA, were washed out of the RAM pre-column and lost at high dilution times.

3.2. *Performance of on-line LC–DAD–MS method and comparison of traditional on-line system*

Employing the optimized extraction mobile phase, column switching time and dilution times, typical chromatograms with injection of 100 μL samples was represented in Fig. 5A–C. As shown in Fig. 5B, the successful extraction, elution and separation of BPA and 4-OP were achieved by on-line LC–DAD–MS with a dilution line system using a RAM pre-column as extraction column. Comparison of the serum sample with a standard sample (Fig. 5A), it could be seen that most complex matrix components were removed and no endogenous peaks existed in SIM mode. Thus, credible determination of BPA and 4-OP could be achieved. In addition, the dilution line system provided a good peak shape compared with that obtained by the traditional system [6]. Chromatograms obtained with and without the use of dilution line were shown in Fig. 5B and C; it was obvious that the former had more symmetrical and sharper peaks especially

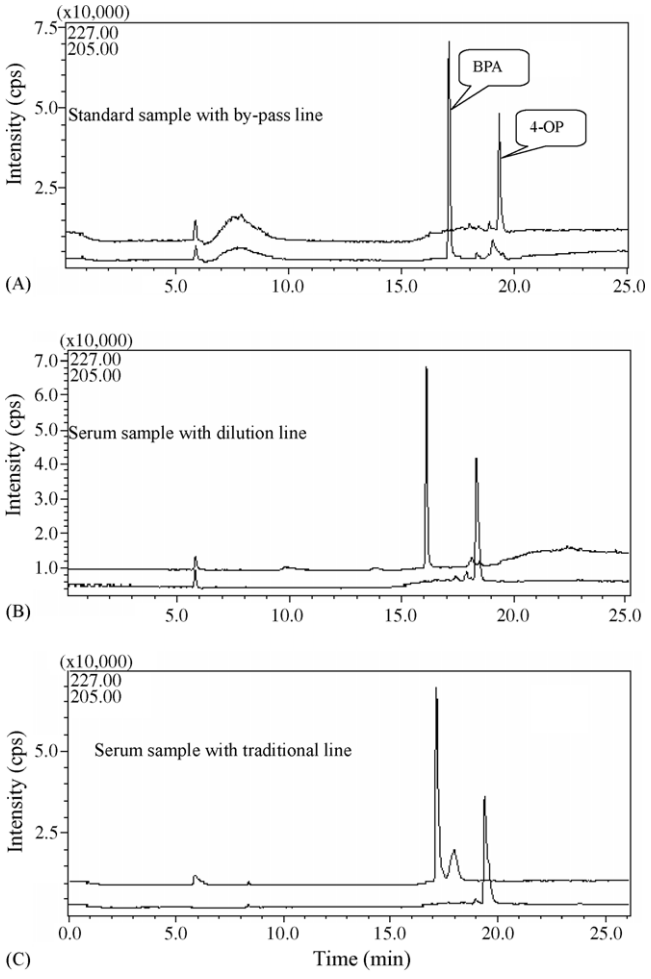

Fig. 5. Typical chromatograms obtained from standard sample (A) and serum sample (B) with dilution line, and traditional line (C) injected serum sample. The concentrations of analytes were 50 ng/mL.

for 4-OP. It may show that on-line pretreatment with dilution line is more suitable for LVI.

In order to prove further the idea, serum samples of different volume were injected into the system. Generally, the increase of detectors response is not exactly proportional to the increase of the volume injected probably due to the complex interactions among analytes, extraction sorbent and serum interferences. More than 100 μL injection sample would lead to lower recovery and signal suppression when traditional sorbent and enrichment method were used [36]. Samples spiked 100 ng/mL were selected to investigate the linearity relation between peak area and injection volume. However, using dilution line, a linear relationship (linear equation and R^2) between injection volume and peak area was obtained as shown in Fig. 6A. It demonstrated that no significant signal suppression was observed by using the on-line RAM with dilution line. The UV absorbance spectra of protein were obtained by injection different volume samples (Fig. 6B). The UV chromatograms of the protein elution showed that matrix interference and background level were still low and almost independent of the injection volumes. Despite increasing injection volume to 200 μL, this method allowed to clean effectively up and remove most serum proteins. As a result, sen-

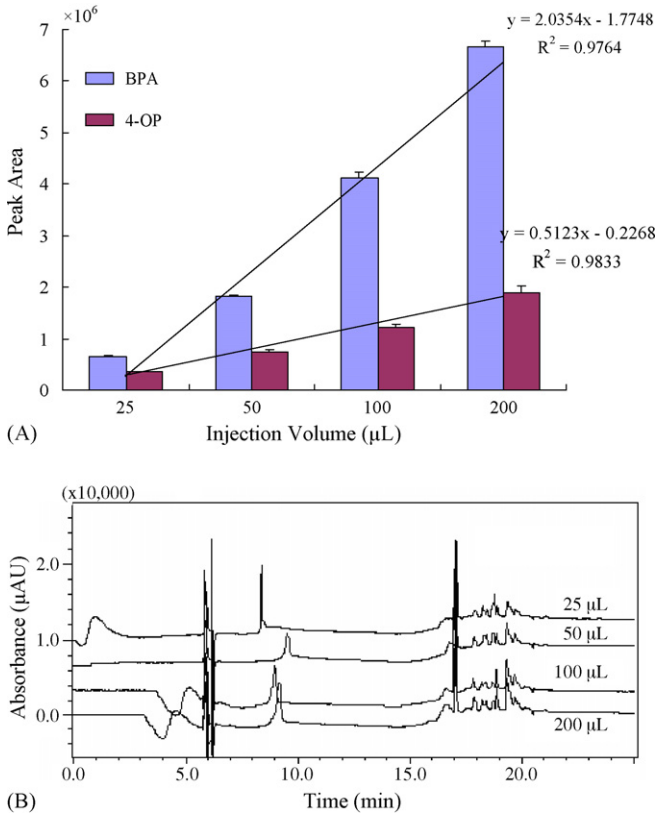

Fig. 6. Peak areas (A) and UV absorbance of protein (B) against injection volumes, respectively. The experimental condition is the same as Fig. 4 except the concentration of analytes is 100 ng/mL.

sitivity, S/N and LOD increased with LVI. This was the other foundation for improvement of sensitivity and LOD using LVI in combination with on-line RAM pre-column. These results indicated that, with LVI, the analytes in serum could be concentrated and recovered from protein.

3.3. Validation study

Both calibration curves were linear within the given concentration range (0.1, 0.5, 1, 5, 10, 50, 100, 500 ng/mL) and obtained linear correlation coefficients over 0.99. This wide working range was enough to cover the variable levels of BPA and 4-OP in human serum. The intraday accuracy and precision of the assay were evaluated by analyzing serum spiked with 0.5, 50 and 500 ng/mL of each analyte. After the initial analysis, the samples with the low and high concentration levels were left on the autosampler for 24 h and then reinjected, respectively. As presented in Table 1, the recovery was between 80.9 and 101% and the precision ranged from 1.72 to 8.90%. The data of recovery and precision were acceptable, which implied that the matrix effect was not significant. LOD and LOQ for BPA in serum were 0.05 and 0.1 ng/mL, respectively while those of 4-OP were 0.1 and 0.5 ng/mL, respectively. The data of LODs and LOQs is equal to the results having been reported [24]. Although different methods using GC–MS [37], LC with electrochemical detector [25,28], fluorescence detection [24,25] and

Table 1
Recovery and RSD of spiked serum samples ($n = 5$)

		Concentration (ng/mL)				
		Intra-day			Inter-day	
		0.5	50	500	0.5	500
BPA	Recovery (%)	87.7	101	89.8	80.9	94.0
	RSD (%)	6.53	2.95	2.16	6.61	5.66
4-OP	Recovery (%)	83.4	99.7	86.0	89.8	86.7
	RSD (%)	7.54	4.93	1.72	8.93	5.28

mass spectrometry [24,29] have been taken to determinate BPA and alkylphenols in different matrices such as serum, plasma and urine, all LODs were about sub-ppb level.

LODs and LOQs could be lowered further by increasing the sample injection volume from 100 to 200 μL or even more. It has been reported that larger injection volumes could be achieved lower LOQs. However, by utilizing this approach, the lifetime of the extraction and analytical columns may be reduced [36]. Considering the method has relatively high sensitivity, only 100 μL serum sample was injected.

3.4. Application to the real samples

Ten serum samples collected from healthy human were analyzed using the method established. For most samples, BPA and 4-OP could not be detected. Only three samples found trace amount BPA with concentration of 0.17, 0.23 and 0.28 ng/mL, respectively; while 4-OP was detected in only two samples, and their concentration were just above LOD about 0.2 ng/mL. The level of BPA was almost similar to values reported in the literatures [25,28]. Typical chromatograms were represented in Fig. 7.

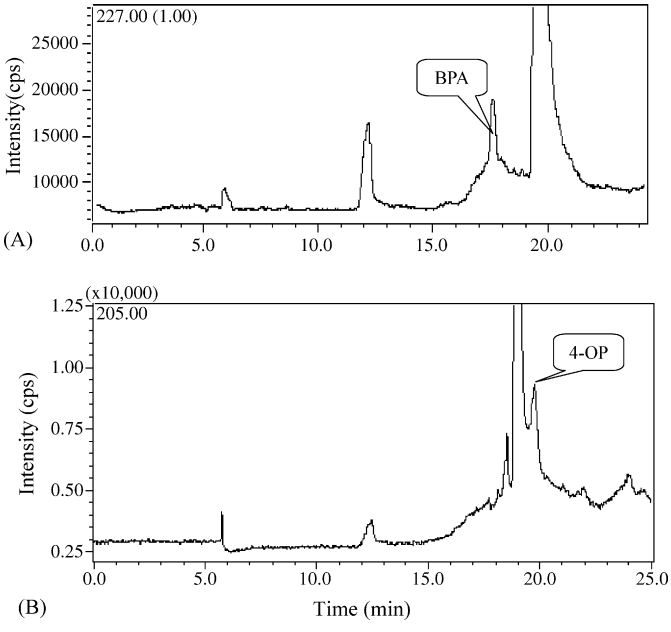

Fig. 7. Typical chromatograms of real serum samples. (A) BPA; (B) 4-OP.

4. Conclusions

An automated on-line LC–DAD–MS method was established by addition of a special dilution line with a RAM pre-column. Appropriate composition of the extraction mobile phase was selected and the most suitable time for the rotation of switching valve was optimized to effectively remove serum proteins without compromising either sensitivity or reproducibility of MS. The resulting dynamic range, lower limit of quantification, recovery and precision were well within the acceptable range. This method has been applied successfully to determine BPA and 4-OP in the serum of healthy human. Compared with traditional on-line extraction system, the method is more suitable for using LVI to improve sensitivity and reduce LODs. At the same time, it increased the capacity of pre-column, thus avoided the breakthrough of high concentration analytes without compromising chromatographic peak shape. Therefore, the method is more suitable for the determination of the levels of analytes over a wide rang of concentrations. It may act as a simple and effective method for the improvement of conventional, on-line high throughput sample preparation and analysis of compounds in serum and other complex matrices. As a result, the proposed method is, therefore, attractive for routine analysis and potentially sensitive enough to determine environmental as well as occupational exposure. Further studies on LVI with system are in progress in our laboratory.

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