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Original Research Article

Measurement of Bisphenol-A in Human Urine by Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry

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Abstract: A simple and rapid ultra-performance liquid chromatographic tandem mass spectrometric assay for the measurement of bisphenol-A (BPA) in human urine was developed and validated. Sample preparation involved addition of 4-nitrophenol (as an internal standard, IS) to 1.0 ml human urine sample containing BPA, and extraction with ethyl acetate and hexane (6:4,v:v), evaporation, and dissolving the residue in the mobile phase. Analytes separation was performed using a reversed phase Atlantis dC18 column (2.1x 100 mm, 3 µm) protected by guard pre-filter and a mobile phase that consisted of 5.0 mM ammonium acetate: acetonitrile (20:80, v:v) and was delivered at a flow rate of 0.30 ml/min. The analytes were quantified in negative ion mode, using electrospray ionization (ESI) set at transitions of mass to charge (m/z): 226.87 → 212.01 and 137.84 → 107.86 for BPA and IS, respectively. The relationship between BPA concentration and peak area ratio (BPA /IS) was linear in the range 0.2 - 20 ng/ml. Mean extraction recovery of BPA and the IS was ≥ 90% and 97%, respectively. The method was validated in respect to accuracy, precision, linearity, and specificity. Stability of BPA in urine was determined under conditions generally encountered in the clinical laboratories. The method was successfully applied to determine BPA level in urine samples obtained from healthy volunteers.
Keywords: Bisphenol-A, 4-Nitrophenol, Urine, LC-MS/MS

INTRODUCTION

Bisphenol-A (BPA, CAS: 80-05-7) is one of the most commonly used chemical as a precursor and/or starting material in manufacturing of several polymeric products, including polycarbonate plastic and epoxy resins [1]. It is also found in the epoxy lining of metal food cans and in plastic food containers, including some baby feeders, microwave ovenware and eating utensils [2]. BPA containing products especially when exposed to heat or acidity can leach into canned foods, infant formula, and other food products [3, 4].

BPA is considered a potential endocrine disruptor that may alter normal hormonal function. Several biomonitoring studies confirmed the presence of free and/or conjugated BPA in blood and urine in populations unintentionally exposed to BPA [5, 6].

Analytical methods used to monitor BPA levels in blood or urine include high performance liquid chromatography (HPLC) with electrochemical [7] or florescence detector [8], gas chromatography/mass spectrometry (GC-MS) [9], and liquid chromatography/mass spectrometry (LCMS/MS) [10-12]. Most of the reported LCMS/MS methods used bisphenol A d_{16} as internal standard, which may not be readily available in many laboratories [11, 12].

In the present paper, we describe a precise and rapid LCMS/MS assay for determination of BPA level in human urine using 4-nitrophenol as internal standard. The method involves liquid/liquid extraction, and requires 1.0 ml human urine. It was used to determine BPA levels in human urine samples obtained from healthy volunteers. Further, it was used to assess the BPA stability under various clinical laboratory conditions.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. BPA and ß-glucuronidase-helix pomatia, were purchased from Sigma-Aldrich Co., St. Louis, MO, USA, and 4-nitrophenol (IS) from Spectrosol, BDH Chemical ltd. Pool, England, respectively. Acetonitrile (HPLC grade), sodium acetate, acetic acid, hydrochloric acid, ethyl acetate, hexane, and ammonium acetate were purchased from Fisher Scientific, NJ. USA. Water for HPLC was prepared by reverse osmosis and further purified by using synergy water purification system (Millipore,

Bedford, MA, USA). Urine samples were collected from volunteers after obtaining the approval of the Research Ethics Committee, King Faisal Specialist Hospital & Research Centre (FSHRC) Riyadh, Saudi Arabia, (RAC# 2141021).

Instrument and chromatographic conditions

The liquid chromatograph tandem mass spectrometer (LC-MS/MS) consists of XEVO-TQD equipped with Z-spray, an atmospheric pressure ionization (API) interface, Acquity UPLC H-Class system, and integrated solvent and sample manager (Waters Corporation, Milford, MA, USA). Analysis was performed at room temperature using a reversed phase Atlantis dC18 column (2.1x 100 mm, 3 µm) steel column was protected by a column guard on-line filter, (0.2 µm x 2 mm). The mobile phase was composed of 5.0 mM of ammonium acetate and acetonitrile (20:80, v:v), and was delivered at a flow rate of 0.30 ml/min. The electrospray ionization (ESI) source was operated in the negative ion mode at a capillary voltage of 2.85 kV and a cone voltage of 47 V. Nitrogen was used as the nebulizing and desolvation gas at a flow rate 900 L/hr. Argon was used as the collision gas at flow rate 50 L/hr maintaining cell pressure at 3.7775 E^{-003} mbar. An optimum collision energy 20 eV was applied for both BPA and IS. The ion source and the desolvation temperatures were maintained at 150 ºC and 500 ºC, respectively. BPA and IS were detected and quantified in the negative ion mode, product ion response was measured at set transitions mass to charge (m/z): 226.87 → 212.01 and 137.84 → 107.86. Masslynx Ver 4.1 (Waters Corporation, Milford, MA, USA) software working under Microsoft Window XP professional environment was used to control the instrumental parameters, data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements.

Preparation of standard and control solutions

BPA and IS stock solutions were prepared in methanol (1.0 µg/ml). Calibration standards at eight concentrations (0.2-20 ng/ml) and quality controls at four concentrations: (0.2, 0.6, 10, and 18 ng/ml) were prepared in human urine. The IS working solution was prepared in mobile phase (1.0 ng/ml). Standard and control solutions were vortexed for one minute and 1.0 ml aliquots were transferred into 7 ml glass culture tubes and stored at -20 °C until used.

Preparation of samples

150 µl of the IS working solution was added to 1.0 ml calibration standard, urine samples or quality control samples in a 7 ml glass culture tubes and vortexed for 30 seconds. 4.0 ml extraction solvent mixture of ethyl acetate and hexane (6:4,v:v) was added to each tube, vortexed for one minute, and centrifuged at 6000 rpm for 10 minutes at room temperature. The clear supernatant layer was transferred to a clean borosilicate culture tube and dried under gentle steam of nitrogen at 40 °C. The residue was reconstituted in 100 µl mobile phase and 10 µl of the clear solution was injected into the LC-MS/MS system.

The urine sample of a healthy volunteer was prepared (before adding the IS) as follows: 1.0 ml urine was mixed with 60 µl of 2 M sodium acetate (pH 5.0, adjusted with acetic acid) and 30 µl ß-glucuronidase aqueous solution was added, vortexed, and subjected to continuous mixing at 37 ºC for 3 hours. Then 200 µl of 2 N hydrochloric acid was added and processed.

Stability Studies

Two QC samples (0.6 and 18 ng/ml) were used for stability studies. Five aliquots of each sample were extracted and immediately analyzed (baseline). Five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed, five aliquots were stored at -20 ºC for 12 weeks before being processed and analysis, and five aliquots were processed and stored at room temperature for 24 hours or at -20 ºC for 48 hours before analysis. Fifteen aliquots were stored at −20 °C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were analyzed and the rest stored at to -20 ºC for another 24 hours. The cycle was repeated three times.

RESULTS AND DISCUSSION

Method Development

Mass spectra: Precursor and product ion of BPA and IS were determined using configured Intellistart program during the infusion of standard solutions into the mass spectrometer. The product ion transitions were quantitatively measured as peak area response at m/z 226.87 → 212.01 for BPA and 137.84 → 107.86 for IS in multiple reaction mode. Chemical structures and MS spectra of BPA and IS are shown in Fig. 1

Selection of internal standard and optimization of LC conditions: We tried various derivatives of phenol as internal standards and selected 4-nitrophenol. Detection and quantification of BPA were optimized using a mobile phase composed of 5.0 mM ammonium acetate and acetonitrile (20:80, v:v) and a flow rate 0.3 ml/min. The high proportion of acetonitrile facilitated shorter run time (< 2.0 minutes).

Extraction recovery: The absolute recovery of BPA was assessed by comparing absolute peak area of spiked human urine and mobile phase samples, using five replicates for each of four concentrations (0.2, 0.6, 10 and 18 ng/ml). Similarly, the recovery of the IS was determined by comparing the peak area of the IS in 5 aliquots of human urine spiked with 1.0 ng/ml IS with the peak area of equivalent samples prepared in mobile phase. Mean extraction recovery was 90-98% for BPA

and 97% for the IS. The results are presented in [table 1].

Method Validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [13]. The validation parameter included: specificity, recovery, linearity, accuracy and precision.

Specificity: In order to evaluate specificity, we screened six batches of blank urine and eight frequently used medications (aspirin, acetaminophen, ascorbic acid, ibuprofen, caffeine, nicotinic acid, omeprazole, and ranitidine) for potential interference. No interference was found by urine components and none of the drugs co-eluted with BPA or the IS. Fig. 2 depicts a representative chromatogram of BPA-free human urine that was used in preparation of calibration curve and quality control samples.

Linearity and limit of quantification: Linearity was evaluated by analyzing a series of calibration standards at eight concentrations in the range of 0.2 – 20 ng/ml. Corresponding peak area ratios and concentrations were subjected to regression analysis. The mean equation obtained from eight standard curves was y= 0.085-0.009x, with R^2 (SD) = 0.9940 (0.0038). Fig. 3 depicts representative calibration curve used in measuring BPA levels. BPA detection and quantification limits were as 0.1 ng/ml and 0.2 ng/ml, respectively.

Accuracy and precision: Accuracy and precision were determined by measuring levels of BPA in four spiked quality control samples (0.2, 0.6, 10 and 18 ng/ml). The intra-and inter-day precision and accuracy were determined over three different days. Intra-day (n=10) precision and accuracy were in range of 6.4 – 11.7% and 92 – 104%, respectively. Inter-day (n=20) precision and accuracy were in range of were 6.8 – 12.2% and 96 – 104%, respectively [table 2]. Fig. 4 depicts LCMS/MS chromatograms of control urine samples spiked with 1.0 ng/ml IS and BPA at four concentrations (0.2, 0.6, 10 and 18 ng/ml), respectively.

Matrix Effect

Matrix effect is defined as the effect of co-eluting residual matrix components on the ionization of the target analytes that results suppression or enhancement of analyte response. It is calculated as: (peak area response in absence of matrix – peak area response in presence of matrix) divided by peak response in absence of matrix x 100. In the present study, the matrix effect was measured at three concentrations (0.6, 10 and 18 ng/ml). It was 10.0%, 3.1% and 9.9%, respectively.

Stability

It is necessary to perform stability studies of the analyte and IS to determine the range of appropriate conditions and times of storage. The availability of stability studies for extended period is essential for study planning and results interpretation. In the present study, BPA and IS stability in processed and unprocessed urine samples was investigated. BPA (0.6 and 18 ng/ml) in processed samples was stable for 24 hours at room temperature (≥95%) and 48 hours at -20 ºC (≥96%); in unprocessed samples for at least 24 hours at room temperature (≥97%), twelve weeks at -20 ºC (≥95 %), and after three freeze-and thaw cycles (≥93%). The data are summarized in [table 3]. Further, no significant change in chromatographic behavior of BPA or the IS was observed under any of the above conditions.

Application to volunteer samples

The assay was used to measure BPA level in a urine sample from a healthy volunteer. Fig. 5 depicts the chromatogram, indicating presence of 1.13 ng/ml BPA.

Table-1: Extraction recovery of bisphenol-A and 4-nitrophenol (IS)

Bisphenol-A (ng/ml)	Human Urine *Mean (SD)	Mobile Phase *Mean (SD)	†Recovery (%)
0.2	59 (7)	60 (6)	98
0.6	240 (22)	267 (25)	90
10	2567 (149)	2782 994)	92
18	4810 (227)	5112 (66)	94
IS: 1.0	832 (14)	807 (44)	97

*Mean peak area of 5 replicate. †Mean peak area of BPA in urine divided by mean peak area in mobile phase x 100. SD, standard deviation.

Table-2: Intra-and inter-run precision and accuracy of BPA assay

Nominal level (ng/ml)	Intra-day (n=10) Measured level			Inter-day (n=20) Measured level		
	Mean (SD)	CV (%)	Accuracy (%)	Mean (SD)	CV (%)	Accuracy (%)
0.2	0.19 (0.02)	11.7	98	0.21 (0.02)	12.2	104
0.6	0.55 (0.05)	9.2	92	0.58 (0.06)	9.8	96
10	9.79 (0.73)	7.4	98	9.78 (0.67)	6.8	98
18	18.81 (1.20)	6.4	104	18.45 (1.30)	7.0	103

SD, standard deviation. CV, coefficient of variation = standard deviation divided by mean measured concentration x 100. Accuracy = measured level divided by nominal level x 100.

Table-3: Stability of BPA in different conditions

Stability (%)									
Nominal Level (ng/ml)	Urine samples							Stock Solution	
	Unprocessed		Processed		Freeze-Thaw				
	24 hrs. RT	12 wks (-20 ºC)	24 hrs. RT	48 hrs (-20 ºC)	Cycle-1	Cycle-2	Cycle-3	24 hrs. RT	6 wks (-20 ºC)
0.6	97	95	104	96	104	94	98	98	105
1.8	102	100	95	110	98	93	96		

Stability (%) = mean measured level at the indicated time divided by mean measured level at base line X 100 (n=5). RT, room temperature (21 ºC, ±2 ºC). FT, Freeze-thaw cycle; samples were frozen at -20 ºC and thaw at RT.

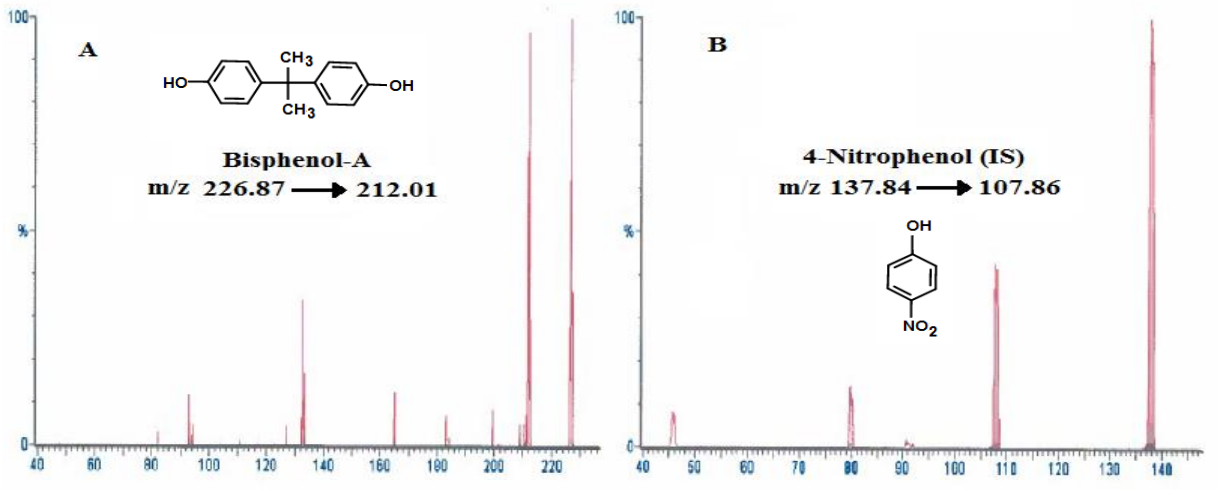

Fig-1: Chemical structures and MS-MS Spectra of: A) Bisphenol-A (BPA) and B) 4-Nitrophenol (IS)

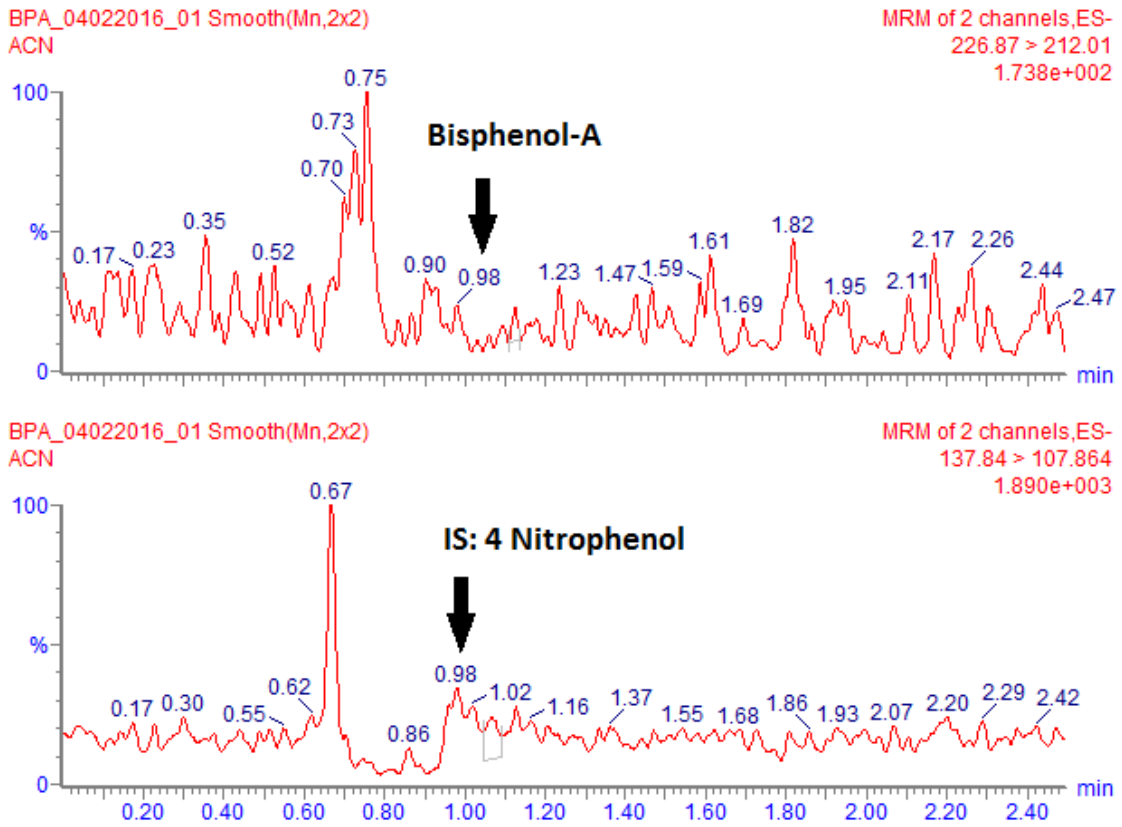

Fig-2: MRM chromatogram of BPA-free urine sample used in calibration standards and quality control samples preparation

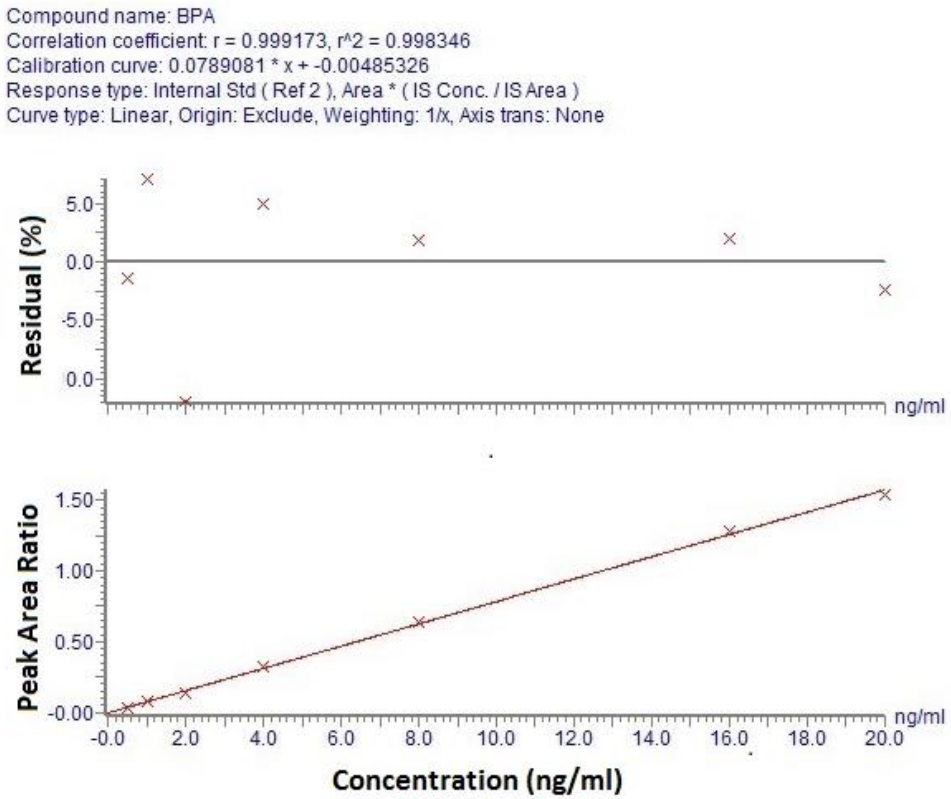

Fig-3: Representative calibration curve of BPA concentration (0.2 -20 ng/ml) using peak area ratio as a response

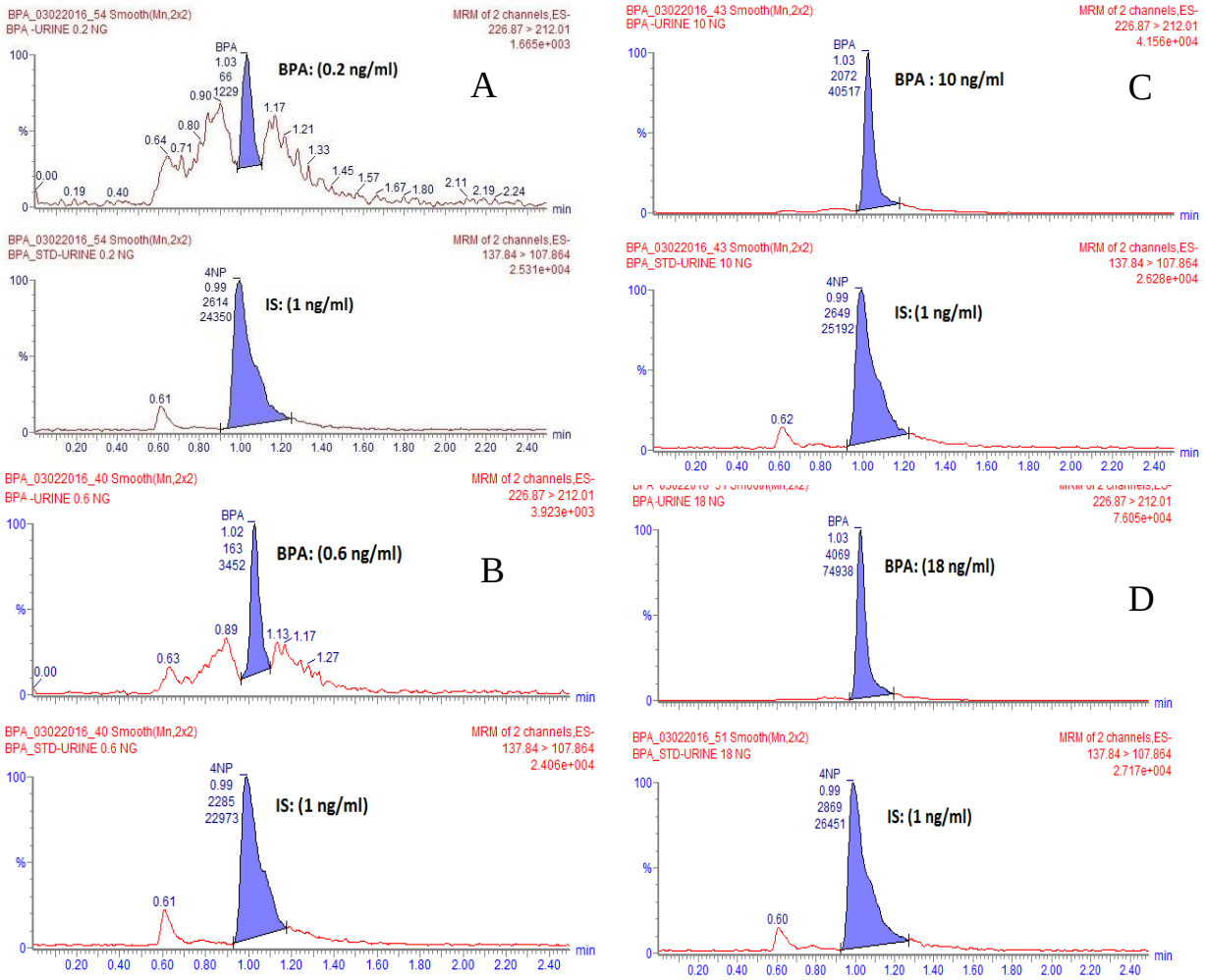

Fig-4: Quality control samples of human urine spiked with BPA at four concentrations (0.2, 0.6, 10 and 18 ng/ml)

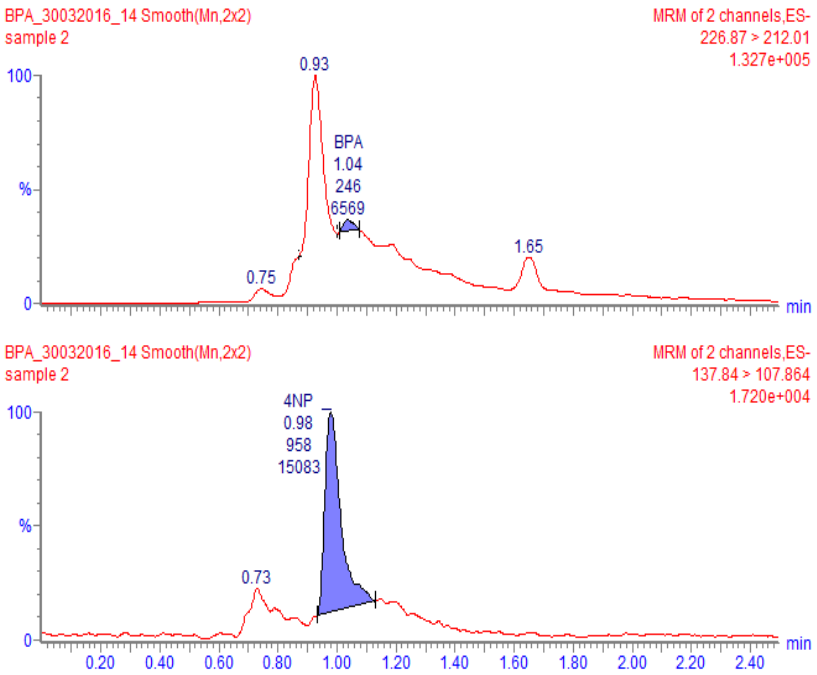

Fig-5: MRM chromatograms of urine sample collected from a healthy volunteer indicating the presence of 1.13 ng/ml BPA

CONCLUSION

The proposed method is simple, precise, and accurate for rapid measurement of BPA level in human urine samples. The method was fully validated and successfully used in stability studies and in determining BPA level in a urine sample from a healthy volunteer.

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