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Development and Validation of A Reversed Phase-HPLC-DAD Method for Determination of Bisphenol-A in Artificial Saliva

FATMA DEMIRKAYA and YUCEL KADIOGLU*
Department of Analytical Chemistry, Faculty of Pharmacy,
Ataturk University, 25240 Erzurum, Turkey
Fax: (90)(442)2360962; Tel: (90)(442)2311536
E-mail: yucel@atauni.edu.tr; yucelkadi@gmail.com

Bisphenol-A is one of estrogenic xenobiotics present as an impurity in dental sealants and composites based on dimethacrylate monomers. In this study, bisphenol-A in artificial saliva was analyzed by simple, accurate, precise and sensitive reversed phase HPLC-DAD method requiring simple sample preparation. Bisphenol-A separated by the reversed-phase C_{18} column using simple the mobile phase containing isocratic mixture of acetonitrile-Milli-Q grade water (50:50 v/v) at flow rate of 1 mL min^{-1}. Linearity has been obtained in the range of 0.5-30 μg mL^{-1}, whereas limit of detection and limit of quantification are 0.01 and 0.02 μg mL^{-1}, respectively. Both intra day and inter day precision showed acceptable RSD values were lower than < 6.3 % and < 9.5 %, respectively. The recovery of developed method was also between 96.8-105.9 %. It was suggested that proposed method has been successfully applied in routine analysis of bisphenol-A in saliva after dental treatment.

Key Words: Bisphenol-A, Reversed phase HPLC-DAD method, Validation.

INTRODUCTION

Dental sealants and composites based on dimethacrylate monomers like bisphenol-A diglycidyl dimethacrylate (bis-GMA) and bisphenol-A dimethacrylate (bis-DMA) used as an alternative to the dental amalgams play a significant role in preventing tooth decay an in maintaining healthy dental[1,2]. But bisphenol-A (4,4'-isopropylidenediphenol) (Fig. 1) present as an impurity in some resins (bis-GMA) and as a degradation product in others (bis-DMA and bis-GMA)[1]. Bisphenol-A is one of estrogenic xenobiotics and causes various diseases such as human infertility and genital tract malformations[3]. Bisphenol-A is used not only as a component of resin materials but also to form derivatives for industrial use[4].

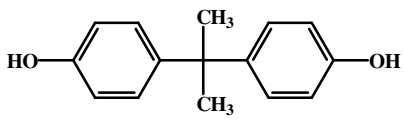

Fig. 1. Chemical structure of bisphenol-A

Bisphenol-A in saliva was determined so far been determined by using a variety of extraction procedures and analytical techniques. Olea *et al.*[5] reported the determination of bisphenol-A by HPLC/UV method. In a similar study, Arenholt-Bindslev *et al.*[6] applied with HPLC/UV and Fung *et al.*[7] with HPLC/FD, Zarfa *et al.*[2] with GC/MS, Sasaki *et al.*[8] with ELISA and Joskow *et al.*[9] with GC/MS applied for determination of bisphenol-A in saliva. While Fung *et al.*[7] and Joskow *et al.*[9] extracted bisphenol-A from saliva with solid phase extraction. Zafra *et al.*[2] used exhaustive liquid-liquid extraction procedure. Olea *et al.*[5] and Arenholt-Bindslev *et al.*[6] also analyzed by diluting saliva with ethanol. Comparing to the literature methods for the determination of bisphenol-A in saliva, our reversed phase HPLC-DAD method uses a simple mobile phase composition and sample preparation.

For determination of bisphenol-A in saliva, this study describes developed and completely validated reversed phase HPLC-DAD method. The validation of method was carried out by establishing specificity, linearity, recovery values, limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day precision and accuracy according to International Conference on Harmonization guidelines for validation of analytical procedures[10]. Herein, the determination of bisphenol-A without derivatization in saliva is reported.

EXPERIMENTAL

Bisphenol-A as using reference material was purchased from Merck (Germany). All other chemicals were obtained from commercial sources and were of analytical grade and HPLC grade. Artificial saliva was prepared by Department of Biochemistry, Faculty of Medicine, Atatürk University, Erzurum, Turkey.

Chromatographic conditions (reversed-phase HPLC-DAD): The HPLC system consisting of a pump, a column oven, an autosampler and a photodiode array detector (DAD) was Thermoquest spectra system P 1500. The column oven was controlled at 40 °C. Separation of compounds was achieved using a phenomenex bondolone reversed-phase C_{18} column (150 × 3.9 mm, 10 μm) (USA). Isocratic mixture of acetonitrile-Milli-Q grade water (50:50 v/v) was used as the mobile phase. The mobile phase mixture was filtered through a 0.47 μm nylon membrane filter and degassed ultrasonically before use. The flow-rate was 1 mL min^{-1} and the injection volume was 20 μL. The detector was set to scan from 200 to 800 nm. The bisphenol-A was detected with UV detector at a wavelength of 270 nm, which was the wavelength used for quantification, with a total run time of 10 min.

Preparation of standard and saliva solutions: Stock solution of bisphenol-A (100 μg mL^{-1}) was prepared in acetonitrile and stored at -20 °C. The stock solutions were stable for at least 2 weeks when stored at -20 °C. The eight standard solutions from 0.5 to 30 μg mL^{-1} in acetonitrile were made by a serial dilution. Saliva working solutions were prepared by spiking into drug-free artificial saliva with different standard solutions, which were to give final concentrations of between 0.5-30 μg

mL^{-1} (0.5, 1, 3, 5, 7, 10, 20 and 30 μg mL^{-1}) of bisphenol-A for the calibration curve. In addition, saliva quality control samples were prepared in the same way from stock solution at concentrations of 2, 7 and 30 μg mL^{-1}.

Extraction procedure from saliva: After testing different solvents (methanol, ethanol and acetonitrile) and times (1, 3, 5 and 10 min), the best results were obtained with the following conditions: a volume of 1 mL of saliva was mixed with 0.5 mL of acetonitrile (deproteinizing solution) and placed in the shaker and then again mixed for extraction at room temperature for 15 min. The mixture centrifuged at 1200 g for 10 min. The supernatant layer was removed, filtered through a Phenomenex membrane of 0.45 μm pore size, transferred into autosampler vials and injected (10 μL) into the HPLC system.

RESULTS AND DISCUSSION

Optimization of conditions and chromatograms: During the development stage, the individual or different mixture use of methanol, acetonitrile and water as the mobile phase were tested. At the reported mobile phase composition (acetonitrile-Milli Q grade water (50:50 v/v)), the tailing factors (0.83) were within the acceptable limit resulting in good peak symmetry and resolution. In the optimized conditions, the mobile phase flow rate and maximum absorption wavelength was set at 1 mL min^{-1} and 270 nm, respectively. Acceptable retention times 6.92 min was determined for the peak of bisphenol-A using a reversed-phase C_{18} analytical column. In addition, system suitability parameters were calculated: capacity factor (k') 1.30, plate count (N) 957.7 and plate height (H) 1.56×10^{-2} cm. The parameters were compared against that recommended by the Centre for Drug Evaluation and Research (CDER)[11].

Specifity and selectivity: The selectivity of the method was also tested by observing potential interferences with bisphenol-A peak arising from the saliva components or the deproteinizing solution. No interfering peaks and ghost peak were observed at the retention times of bisphenol-A in the blank saliva and spiked saliva samples. A typical chromatogram corresponding to a blank saliva sample is shown in Fig. 2C. Fig. 2A-B shows also the overlay of typical chromatogram obtained from the analysis of a working saliva solutions. Peak purities for bisphenol-A were further confirmed by means of a photo-DAD system.

Linearity: Eight-level calibration series with six analyses at each concentration level were measured. The calibration curves obtained by plotting the peak area against the concentrations of bisphenol-A spiked to the drug-free artificial saliva were highly linear over the range 0.5-30 μg mL^{-1}. These curves were linear over the concentration range studied, with determination coefficients (r) being greater than 0.999 for all curves. The regression equation calculated from calibration curves given with the standard deviations of slope (Sb) and intercept (Sa) on the ordinate are given in Table-1.

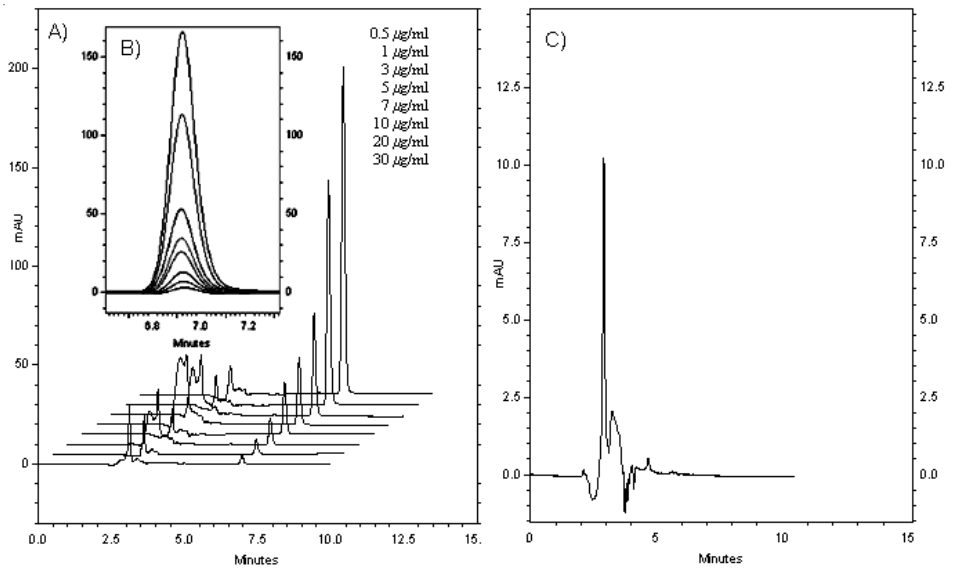

Fig. 2. (A) HPLC chromatogram of obtained concentration in calibration graph of bisphenol-A spiked in human saliva (B) zooming HPLC chromatogram (C) HPLC chromatogram obtained from drug-free saliva

TABLE-1
RESULTS OF REGRESSION ANALYSIS OF DATA FOR THE QUANTITATION OF BISPHENOL-A BY REVERSED PHASE HPLC-DAD METHOD

λ (nm)	Range (µg mL^{-1})	LR[a]	Sa	Sb	R	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
270	0.5-30	Y=54906x-2011.6	6080.5	1754.3	0.9994	0.01	0.02

λ = Wavelength, [a]Based on six calibration curves LR: Linear regression Sa: Standard deviation of intercept of regression line, Sb: Standard deviation of slope of regression line R: Coefficient of correlation x: bisphenol-A concentration (µg mL^{-1}), LOD: Limit of detection, LOQ: Limit of quantification.

Precision and accuracy: The precision of the methods as the relative standard deviation (RSD % = 100x standard deviation/mean) and the accuracy of this methods as per cent of mean deviation from known concentration [relative error %; (concentration found-known concentration) × 100/known concentration] were evaluated with intra-day and inter-day measurement at three different concentrations (2, 7 and 30 µg mL^{-1}) of bisphenol-A extracted from saliva.

The results of precision and accuracy for bisphenol-A in saliva are shown in Table-2. Precision and accuracy studies in saliva showed acceptable RSD % values and the relative errors %. Intra-day (repeatability) and inter day (intermediate precision) precision, RSD % values, were lower than 6.3 and 9.5 %, respectively. Intra-day and inter day accuracy, relative error %, values were also lower than 6.0 and 5.9 %, respectively. These data indicated that the developed reversed phase HPLC-DAD method for determination of bisphenol-A had a good precision and accuracy.

TABLE-2
PRECISION AND ACCURACY OF REVERSED PHASE HPLC-DAD METHOD

λ (nm)	Added (μg mL^{-1})	Intra-day			Inter-day		
		Found±SD (μg mL^{-1})	Precision RSD %[a]	Accuracy	Found±SD (μg mL^{-1})	Precision RSD %[a]	Accuracy
270	2.0	2.12±0.14	6.37	6.05	2.12±0.20	9.53	5.92
	7.0	7.39±0.13	1.70	5.59	7.41±0.32	4.33	5.84
	30.0	30.03±0.99	3.31	0.01	29.06±1.58	5.43	-3.12

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation, [a]Average of six replicate determinations, Accuracy: (relative error %) (found-added)/ added × 100.

Limit of detection (LOD) and limit of quantification (LOQ): In this work, LOD and LOQ were determined by an empirical method that consisted of analyzing series of extracted saliva solutions which contain decreased amounts of bisphenol-A. LOD was defined as a signal-to-noise ratio 3 and LOQ was defined as a signal-to-noise ratio 8 were 0.01 μg mL^{-1} and 0.02 μg mL^{-1}, respectively. Furthermore, these values were in acceptable precision and accuracy values.

Recovery: The extraction recovery of bisphenol-A in saliva was determined at three different concentrations (2, 7 and 30 μg mL^{-1}) of bisphenol-A by comparing the data obtained by the direct injection of standard aqueous solutions to those obtained after the extraction procedure. The percentage recovery of bisphenol-A was calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous standard solutions of the same concentrations. The recovery of developed method was between 96.8-105.9 % (Table-3). These results showed that these methods were precise, accurate and selective.

TABLE-3
RECOVERY VALUES OF BISPHENOL-A IN PROPOSED METHOD

Saliva pools (1 mL)[a]	Added (μg mL^{-1})	Intra-day		Inter-day	
		Recovery (%)	RSD %[b]	Recovery (%)	RSD %[b]
	2.0	106.0	6.75	105.9	10.09
	7.0	105.5	1.79	105.8	4.59
	30.0	100.0	3.31	96.9	5.26

[a]Saliva volume, RSD: Relative standard deviation, [b]Average of six replicate determinations.

Conclusion

A reversed phase HPLC-DAD method for the determination of the dentistry materials bisphenol-A in saliva samples has been developed. Acetonitrile was used both deproteinezed and solvent of bisphenol-A in saliva samples. Only one-step sample preparation before HPLC-DAD analysis was fairly rapid (no requiring time-consuming) and advantage (no requiring evaporation step in liquid-liquid extraction and solid phase extraction cartridge) than others reported previously[2,7,9] for analysis

of bisphenol-A in saliva. Specificity, linearity, LOD, LOQ values, intra- and inter-day precision, recovery and accuracy of the proposed method was obtained during the validation studies.

We have developed a reversed phase HPLC-DAD method that has satisfactory linearity, recovery, precision, sensitivity and accuracy for determination of bisphenol-A in human plasma. This method can be directly and easily applied in saliva samples and very useful and an alternate to performing the studies in the determination of bisphenol-A in plasma for clinical use. Additionally, the short analysis time and low costs are the other advantages of the method for routine analysis.

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