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ANALYTICAL METHOD VALIDATION OF BENZENE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN BEVERAGE CONTAINING SODIUM BENZOATE AND ASCORBIC ACID

MELANIA PERWITASARI[1], ENDANG LUKITANINGSIH[2], SUDIBYO MARTONO[2]

[1]**Faculty of Pharmacy, Sanata Dharma University**
[2]**Faculty of Pharmacy, Gadjah Mada University**
Email correspondence: *melania@usd.ac.id*

Abstract: *Several countries reported discovering benzene in beverages containing benzoic acid and ascorbic acid. Benzoic acid decarboxylation by ascorbic acid will form benzene. American Beverage Association (ABA) recommended the use of accelerated testing to test benzene in beverages. High Performance Liquid Chromatography (HPLC) is one of several methods to analyze benzene in a wide variety of samples, but there is no much information provided regarding the validation of analysis method of benzene. Therefore, developing analysis method of benzene and its validation becomes a current need. The HPLC system consists of Hitachi L-2130 pump, sample injector with 20 µL sample loop, and UV detector L-2420 operating at 205 nm. The analytical column is a LiChrosorb® Phenomenex RP-18 (250 x 4 mm, 10 µm, 100 Å), the mobile phase is acetonitrile:aquabidest (60:40 v/v) and pumped at a flow rate of 0,8 mL/min. Benzene separated from the matrix and follows the validation requirements. The developed analytical method showed that resolution was 8.37, r = 0.995 with LOD and LOQ 6.52 ppb and 19.75 ppb, with a precise of ≤ 11% and recovery of 80-110%. Accelerated testing indicated that benzene levels increased with increasing of the temperature. Beverages containing 400 mg/mL of ascorbic acid and benzoic acid formed benzene which was detected as 699.38 ppb at 25 ° C, 799.61 ppb at 40 ° C, and 808.94 ppb at 60 ° C in 48 hours. In conclusion, the method was fully validated and can be utilized to analyze benzene in beverages with the accelerated testing at 60 ° C in 48 hours, so that benefits the producers and consumers in the end.*

Keywords *: benzene, HPLC, validation method, ascorbic acid, benzoic acid*

1. Introduction

Consuming foods and drinks containing benzene for long term, even though only in a low level can cause damage to blood cells, such as anemia and leukemia, immune system disorders, and cancer (Anonym, 2011). Since the discovery of benzene in food products in the early 1990s, a concern about the formation of benzene in food products has been increasing. The presence of benzene in food products can be generated from loss of benzene from the packaging material, water for production process which contains benzene, and the reaction between sodium benzoate and ascorbic acid (Barshick et al., 1995; Aprea et al., 2008).

American Beverage Association (ABA) recommends several steps that could be conducted by manufacturers to prevent the formation of benzene in beverages products, one of them is an accelerated testing. Accelerated test can be carried out by conditioning products at minimum temperatures of 40 and 60 °C for 24 hours or longer, depending on the product formulation (Anonym, 2006). Accelerated testing is expected the benzene formation reaction.

High Performance Liquid Chromatography (HPLC) is one of several methods that can be used to analyze benzene in various samples (Khan, 2006). Benzene was successfully analyzed using reversed-phase HPLC column by some previous researchers (Gardner and Lawrence, 1993; Zoccolillo et al., 2001). The chromatographic system gave linear response for benzene standards over a concentration range of 0-

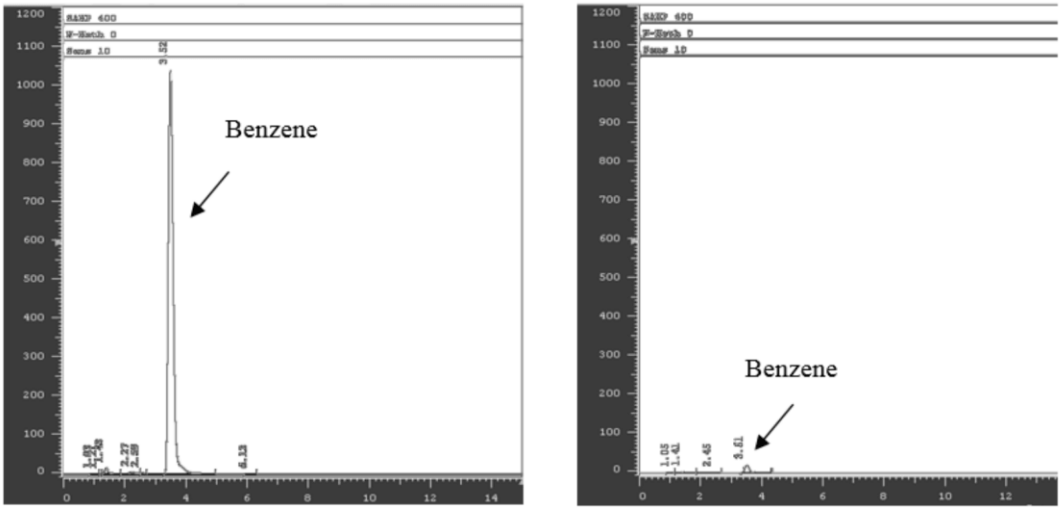

Figure 1. HPLC chromatogram of 100 ppm benzene at 205 nm (A) and 254 nm (B). Column: LiChrosorb® Phenomenex RP-18 (250 x 4 mm, 10 µm, 100 Å); mobile phase $CH_3CN:H_2O$ (75:25 v/v); λ 205 nm; flow rate 0.8 mL/min; sample 20 µL

Table I. Response (peak height) of UV detector for benzene (100 ppm in acetonitrile) at 254 and 205 nm.

Substance	Detector response (mV)	
	λ 254 nm	λ 205 nm
Benzene	19	1400

100 nM ($r^2 > 0.98$), with a retention time was approximately 8 minutes, LOD was approximately 1 nM in solution contain benzoic acid, ascorbic acid and transition metal (Gardner and Lawrence, 1993). Benzene was analyzed in gasoline and the validation of analytical method was not been reported (Zoccolillo et al., 2001).

The information regarding the development and validation of analytical methods of benzene in beverage using HPLC is not fully being reported furthermore the differences of sample and column will need different analytical method. It is a need to develop analytical method of benzene in simulation solution of drink products containing sodium benzoate and ascorbic acid, and further to validate it.

2. Material and Methods

2.1. Materials

Simulated solution containing sodium benzoate (Multi Kimia Raya, Indonesia), ascorbic acid (DSM, USA), and bottled water (R®, Indonesia).

2.2. Chemicals

Reference standard of benzene had purity 97% (Merck, Germany) and stored refrigerated in a capped container. All solvents used (acetonitrile and methanol) were of analytical grade (Merck, Germany). Aquabidest (Ikapharmindo Putramas, Indonesia) was used for mobile phase.

2.3. Sample Preparation

Beverage or simulation solution was prepared by dissolved 400 mg ascorbic acid and 400 mg sodium benzoate in 1L of bottled water. Solution represents beverage products in the market (Aprea et al., 2008). The solution were filtered through a 0.45 µm membrane filter (Phenex, NY).

2.4. Calibration

5.70 µL benzene was placed in 50 mL volumetric flask and dissolved in acetonitril. Beginning with this solution, calibration levels were prepared by a serial dilution with beverage. The range of concentrations injected was 200-1100 ppb.

2.5. Analytical Method

Method development and quantification studies were performed on a LaChrom Elite HPLC system (Merck-Hitachi, Tokyo, Japan), equipped with L-2420 UV-detector, a sample injector with a 20µL sample loop, and L-2130 pump. An optimum separation of benzene in sample was achieved on Phenomenex LiChrosorb® RP-18 column (250 x 4 mm, 10 µm, 100 Å) and mobile phase comprising a 60:40 mixture of acetonitril and aquabidest. Flow rate and injected sample volume were adjusted to 0,8 mL/min and 20 µL, respectively. Detection was performed at 205 nm.

2.6. Method Validation

The HPLC method was validated for accuracy, precision, selectivity, linearity, limit of quantification and detection regarding to USP (Anonym, 2009).

Selectivity were determined by optimized the wave length at 254 nm and 205 nm, composition mixture and flow rate of mobile phase (Anonym, 2005).

Limit of quantification (LOQ) and limit of detection (LOD) were determined by serial dilution of standard solutions (20-100 ppb) containing benzene and calibration curves were generated by linear regression based on peak height.

Accuracy and precision was confirmed by spiking placebo with three concentrations of the quantified standard compounds.

2.7. Accelerated testing

50 mL simulation solution in scoth duran bottle were subjected to the following accelerated testing conditions: 25, 40 and 60°C for 48 hours.

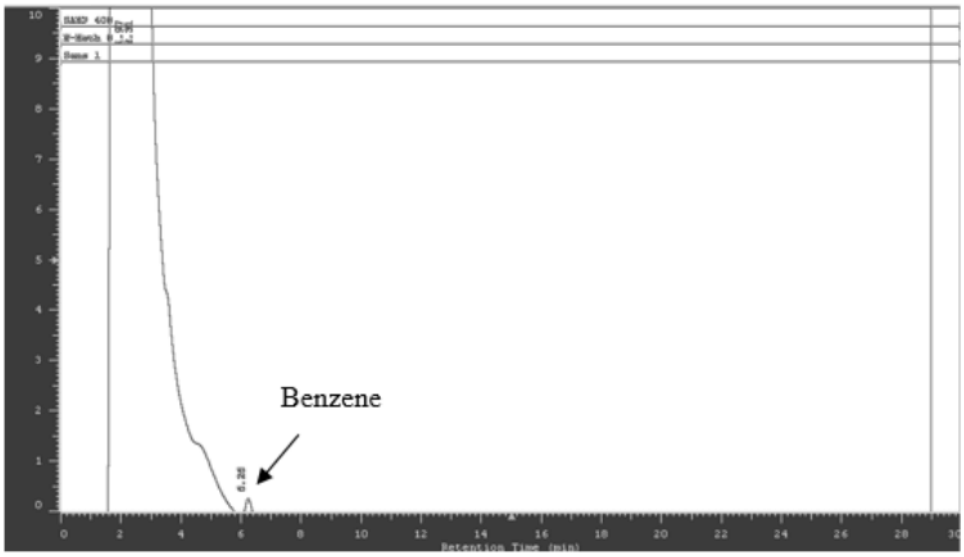

Figure 2. Chromatogram of 100 ppb benzene in beverages contain sodium benzoate and ascorbic acid (400 mg/L). Column: LiChrosorb® Phenomenex RP-18 (250 x 4 mm, 10 µm, 100 Å); mobile phase CH_3CN:H_2O (60:40 v/v); λ 205 nm; flow rate 0.8 mL/min; sample 20 µL.

Table II. Calibration data, regression equation, correlation coeficient (r), and limit of detection and quantitation (LOD and LOQ)

Benzene concentration (ppb)	Regression equation	r	LOD (ppb)	LOQ (ppb)
20-100	Y = 1.57X + 46.6	0.991	6.52	19.75
200-1100	Y = 2.54X -264.2	0.995		

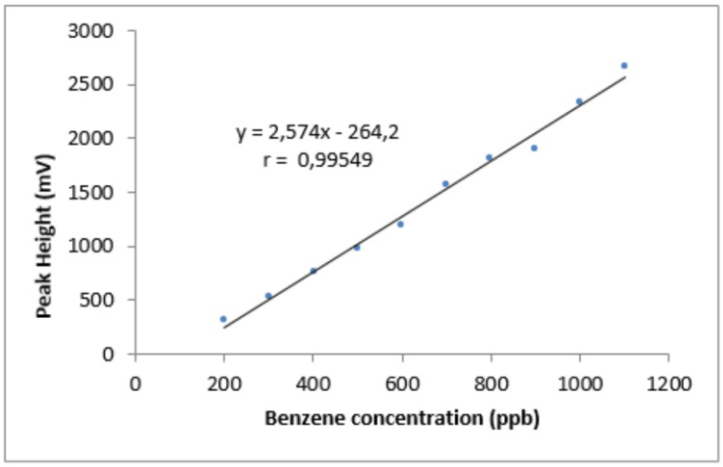

Figure 3. Calibration curve of benzene; Column: LiChrosorb® Phenomenex RP-18 (250 x 4 mm, 10 µm, 100 Å); mobile phase $CH_3CN:H_2O$ (60:40 v/v); λ 205 nm; flow rate 0.8 mL/min; sample 20 µL.

Table III. Accuracy and precision of test results determining levels of benzene in the sample by HPLC

Benzene concentration (ppb)	Replication	Rt (minutes)	Peak Height (mV)	Benzene concentration (ppb)	*% Recovery*
200	1	6.23	252	200.5	100.272
	2	6.23	305	221.1	110.5672
	3	6.20	259	203.3	101.6317
average		6.22	272	208.3	104.157
SD		0.017	28.792	11.2	5.593
RSD		0.278	10.585	5.4	5.370
500	1	6.20	847	431.7	86.340
	2	6.21	910	45.2	91.235
	3	6.19	899	451.9	90.381
average		6.195	885.333	446.6	89.319
SD		0.007	33.650	13.1	2.615
RSD		0.114	3.800	2.9	2.927
1100	1	6.19	2407	1037.8	94.342
	2	6.18	2775	1180.7	107.339
	3	6.18	2512	95	98.050
average		6.183	2564.667	1099	99.910
SD		0.006	189.569	73.6	6.695
RSD		0.093	7.392	6.7	6.701

3. Results and discussion

To select working wavelength of benzene analysis were carefully investigated. Using 25% aqueous acetonitrile (Gardner and Lawrence, 1993) and 100 ppm benzene, a typical chromatogram obtained for benzene is shown in **Figure 1**. This figure shows chromatogram of benzene at 254 and 205 nm. These two wave length were preferred because benzene has absorption in UV area around 250 and 205 nm (Suzuki, 1967; Zoccolillo et al., 2001). The detector response at 205 nm was greater 74 times than at 254 nm, it shown in **Table 1**. Wavelength at 205 nm was chosen as working wavelength.

The HPLC assay had to be optimized in order to give satisfactory results. Concerning the mobile phase, out of a number of different composition mixture of acetonitrile : aquabidest (60:40, 75:25, 80:20 v/v) and flow rate (0.6; 0.8; 1.0 mL/minutes) tested, the best result were obtained with 60:40 v/v

Table IV. Analysis of benzene in simulation solution at temperature 25, 40 and 60 °C for 48 hours

Replication	Benzene conentration (ppb)				
	Ascorbic acid	Sodium benzoate	25 °C	40 °C	60 °C
1	ND	ND	591.4	871.5	777.5
2	ND	ND	776.3	635.3	883.9
3	ND	ND	730.5	892.1	765.4
average	-	-	699.4	799.6	808.9
SD	-	-	96.3	142.7	65.2
RSD (%)	-	-	13.8	17.8	8.1

ND : not detected

of composition mixture and 0.8 mL/minutes of flow rate. Using this optimization, 100 ppb of benzene has the best separated and eluted from the column at 6.27 minutes (**Figure 2**), ascorbic acid and sodium benzoate had the same retention time around 2.3 minutes. The result of resolution was 8.37.

To confirm that an analytical method is suitable for its intended use, it has to be validated. In the present case these investigations were performed according to ICH guidelines (Anonym, 2005). Data presented in **Table 2** indicated the linearity of the assay within the tested range (200-1100 ppb), combined with sensitivity and accuracy. The latter was confirmed by spiking placebo at three concentration levels with standard compounds (**Table 3**).

The linearity of benzene obtained from sample preparation was studied by evaluating the calibration curve at ten level of concentrations. The result shown a good linearity with correlation coefficient (r) > 0.98. See **Figure 3** for calibration curve.

The limit of detection (LOD) of the method was 6.52 ppb and the limit of quantification (LOQ) was 19.75 ppb (**Table 2**). Accuracy and precision of test results determining levels of benzene in the sample by HPLC, shown at **Table 3**, represented that

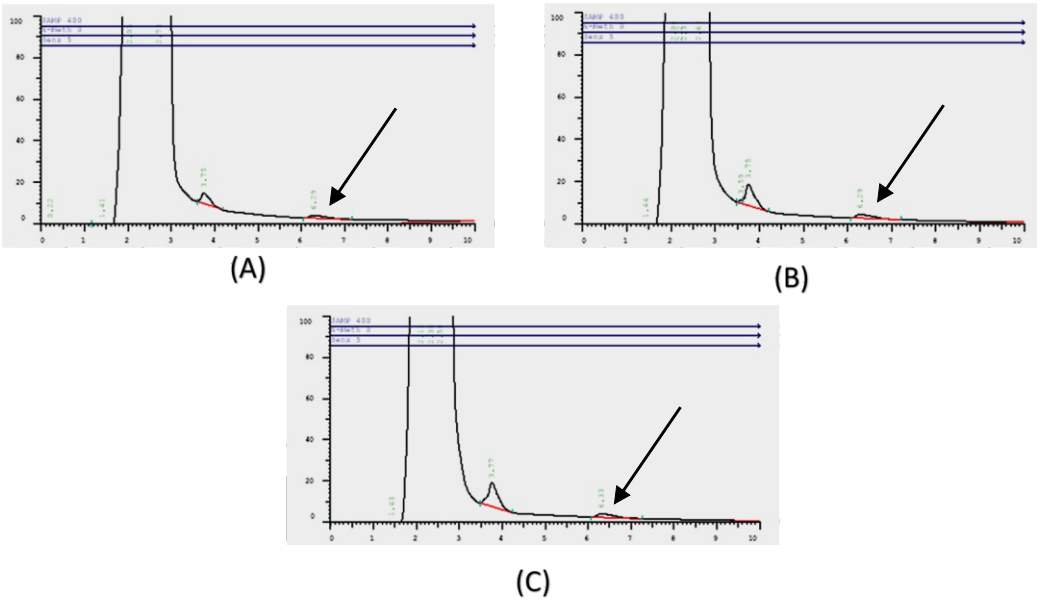

Figure 4. Chromatogram of benzene production in beverages contain sodium benzoate and ascorbic acid (400 mg/L) at 25 (A), 40 (B) and 60 (C) °C storage temperature for 48 hours. Column: LiChrosorb® Phenomenex RP-18 (250 x 4 mm, 10 µm, 100 Å); mobile phase CH_3CN:H_2O (60:40 v/v); λ 205 nm; flow rate 0.8 mL/min; sample 20 µL.

the method used was accurate and precise. The accuracy and precision of three concentration (200, 500, 1100 ppb) of benzene were meet the requirement, 80-110% recovery for the accuracy and RSD (Relative Standard Deviation) < 11% for the precision.

The sample which was stored for 48 hours at temperature of 25, 40, and 60 ° C detected benzene (**Figure 4**) by 699.38 ± 96.30 ppb (RSD 13.8%), 799.61 ± 142.69 ppb (RSD 17.8%), 808.93 ± 65.21 (RSD 8.1%), respectively (**Table 4**). It showed that higher the storage temperature, higher benzene which was formed. Levels of benzene obtained in this experiment was higher than some previous studies. Aprea et al. (2008) detected 118 ppb benzene in the simulation solution with levels of ascorbic acid and sodium benzoate 400 mg/mL which were stored in a temperature 45 ° C for 24 hours. A study conducted by Nyman et al. (2010), stated that cranberry juice containing 0.04% ascorbic acid and benzoate formed 1.3 ppb of benzene that storage at room temperature (25 ° C). In addition, storage at 40 ° C and 60 ° C for 24 hours detected the existence of benzene in a row at 2.2 and 5.8 ppb. Higher benzene level was generated with the increasing of storage temperature.

4. Conclusions

HPLC with UV detector has been successfully developed and validated for benzene in beverages. Evaluation of analytical method parameters including selectivity, linearity, accuracy, precision and sensitivity showed acceptable result. The developed method can also be used to quantitative analysis of benzene in beverages samples available in market with accelerated testing.

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