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HPLC Determination of Patulin in Apple Juice: A Single Center Study of Southwest Area of Iran

^{1,2}A. Jalali, ¹Z.N. Khorasgani, ¹M. Goudarzi and ³N. Khoshlesan

¹Department of Pharmacology and Toxicology, Faculty of Pharmacy,

²Cellular and Molecular Research Center,

Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran

³Department of Food Control, Ahvaz Jundishapour University, Ahvaz, Iran

Abstract: The aim the present study was the quantitative analysis of a potential fungal toxin, patulin in various samples of juice supplied from a commercial apple juice factory in Southwest area of Iran. For this purpose 150 samples of apple juice (local product) from the Southwest reign of Iran, were taken. Ten milliliter of each sample was dissolved in 5 mL buffer. The samples were treated by C₁₈-SPE cartridge and analyzed by high-performance liquid chromatography (HPLC) using UV detector. Results showed level of patulin were higher than 10 µg L⁻¹ in 90% of apple juices. Overall 13/3% of the apple juice samples had levels higher than 50 µg L⁻¹ with maximum level of 106/01 µg L⁻¹. The mean concentration of patulin in apple juice was 26/92 µg L⁻¹. The results of this study showed not significant proportion of the patulin exceeding the 50 mg L⁻¹ limit for apple juice set by WHO and certain European countries. However, constant surveillance of the patulin is strongly recommended because the available data is limited and the hot weather conditions in this area, incidence and level of this toxin could change according to factors like weather conditions, pH, type and stage of apple maturation.

Key words: Patulin, HPLC, apple juice, Iran, Ahvaz

INTRODUCTION

Patulin is a mycotoxin produced by a wide range of fungal species of the genera *Penicillium* and *Aspergillus*, principally *Penicillium expanses* on fruits such as apples and apple products (Gokmen and Acar, 1998; Leggott and Shephard, 2001; Wu, 1992). It has also been determined in oranges, peaches, apricots and tomatoes and its products. Acute symptoms of patulin consumption result in agitation, convulsions, edema, ulceration, intestinal disturbance and vomiting (Speijers, 2004). Chronic health effects of patulin include genotoxicity, immunotoxicity and neurotoxicity in rodents, while its effects on humans are not clear yet (Wouters and Speijers, 1996). Patulin has also become important to apple processors as a method for monitoring the quality of apple juices and its concentrates (Li *et al.*, 2007). Due to its mutagenic, teratogenic nature and possible health risks to consumers, many countries have regulations to reduce the level of patulin in apple products to as low as practically possible. Thus, the determination of Patulin is very important for food controls.

Corresponding Author: A. Jalali, Department of Pharmacology and Toxicology,
Faculty of Pharmacy, Ahvaz Jundishapour University of Medical Sciences,
Ahvaz, Iran Tel: 0098-611-3738380 Fax: 0098-611-3738381

Keeping in view the human health and the possibility of using patulin as a quality indicator in foods, the World Health Organization (1995) has established a maximum recommended concentration of $50 \mu\text{g L}^{-1}$ in apple juice. The International Agency for Research on Cancer has evaluated the toxicity data and classified patulin a Group three carcinogen or a compound for which there is not enough data to allow its classification (IARC, 1986). The Joint Food and Agricultural Organization and World Health Organization Expert Committee on Food Additives (JECFA) altered the provisional tolerable weekly intake (PTWI) of $7 \mu\text{g/kg b.wt./week}$ to a provisional maximum tolerable daily intake (PMTDI) of $0.4 \mu\text{g kg}^{-1}$, based on a No Observable Effect Level (NOEL) of $43 \mu\text{g/kg b.wt./day}$ and a safety factor of 100 $\mu\text{g kg}^{-1}$ (World Health Organization, 1995).

The natural contamination with patulin in apple products, including juice has been reported in different studies carried out in several countries (Funes and Resnik, 2009; Iha and Sabino, 2006; Spadaro *et al.*, 2007; Boonzaaijer *et al.*, 2005; Leggott and Shephard, 2001). Traditional extraction and clean up methods for patulin analysis are based most frequently on liquid-liquid extraction (Iha and Sabino, 2006; Boonzaaijer *et al.*, 2005; MacDonald *et al.*, 2000; Trucksess and Tang, 1999) and more recently implemented solid-phase extraction by Li *et al.* (2007).

Khuzestan Province is located in the southwest of Iran. Its center (Ahwaz city) has the highest temperature among most cities during summer and sometimes its maximum temperature exceeds 50°C . The warm season usually starts at the beginning of spring and continues until November. While apple juice consuming is a popular beverage among people of this part of Iran and patulin monitoring remain important for the quality of apple juice products in Jundishapour University of Ahwaz, so this study was designed to screen apple juice producing in Ahwaz City for patulin content.

MATERIALS AND METHODS

Materials and Chemicals

One hundred and fifty samples of apple juice were evaluated for the presence of patulin (February 2009-June 2009). Apple juice samples provided from Himalia Fruit Juice Company located in Ahwaz city, Khuzestan Province, Iran. Patulin standard (pure crystalline) was obtained from Sigma. A stock standard solution of patulin was prepared by dissolving 5 mg of pure crystalline patulin in 50 mL of double distilled water (pH 4.0) acidified with acetic acid. Working standard solutions were prepared by appropriate dilution of this solution with water (pH 4.0).

Acetic Acid Buffer

Acetate buffer was prepared by adding 0.45 mL acetic acid glacial to 40 mL of H_2O , then dissolving the 0.245 g $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in the above solution, followed by adjusting the pH to 4.0 with acetic acid glacial. The volumes were adjusted to 50 mL with H_2O after the pH titration procedure. The buffer solution was stored in a brown bottle.

Ethyl acetate, methanol, hexane, acetone (analysis pure); acetonitrile (HPLC grade) and acetic acid were also used (all from Sigma). Used water in all the experiments were double distilled and deionized.

Apparatus

High-Performance Liquid Chromatography (HPLC), equipped with UV detector was used (Shimadzu, Japan).

Material for Solid-Phase Extraction (SPE) was ODS- C_{18} Cartridges made in BM TRADA of Japan.

SPE and Samples Procedure

Solid-Phase Extraction (SPE) pretreatment method was used, as previously described by Li *et al.* (2007). The C₁₈-SPE cartridges were pre-washed with 10 mL methanol, 3 mL 10% methanol and 10 mL water in file before using. A 10 mL volume of sample apple juice was mixed with 5 mL of acetic acid buffer solution. The elution and the column walls washing were completed with 5 mL hexane. The column packing dried with a strong stream of air for 15 min. These elutes were discarded and then the receiver replaced by a small pear shaped flask (with screw cap). The column was eluted with 3×5 mL grade eluting solvents (hexane: ethyl acetate: acetone = 1:5:4, 1:4:5, 1:3:6, respectively). In order to allow the sufficient contact time of solvent with the column packing, the flow of each portion being stopped for approximately 1 min. The added combined solution to one drop of acetic acid glacial was evaporated in a water bath at 40°C under a gentle stream of nitrogen.

As earlier described, the residue was immediately dissolved in 1 mL of acetic acid buffer solution and injected into the HPLC system (Li *et al.*, 2007).

Analytical Procedure

The final solution was analyzed under the following conditions: the analytical column was from BM TRADA (250×4.6 mm ID, 5 μm C₁₈ stationary phase) Mobile phase was acetonitrile-water (1:10, v/v), with flow rate at 1.0 mL min⁻¹; UV detector wavelength set at 276 nm; sample injection was 20 μL.

Calculation of Results

The amount of Patulin in the final solution was determined by using a calibration graph of concentration vs. peak height. The patulin content C of the apple juice was found by using the equation:

$$C (\mu\text{g L}^{-1}) = 1000 C_{sp} V/m$$

where, C_{sp} is the concentration of patulin in the final solution (μg mL⁻¹), V is the total volume of the final solution (mL) and m is the volume of apple juice taken from extraction (mL).

Statistical Analysis

The results were presented as Mean±SEM. In order to compare proportions, the chi-squar test was used; differences were considered significant with less than 5% of the associated probabilities as previously described by Li *et al.* (2007).

RESULTS

Chromatography of Patulin Standard Solution

Figure 1 shows the HPLC chromatogram of the 100 ng mL⁻¹ (ppb) patulin standard solution. The chromatographic resolution was satisfactory. The retention time of patulin was 9.7 min.

Linearity Studies

The linearity was studied for the patulin over a range of spiking levels in the Limit of Detection (LOD) from 5 to 100 ppb on the 5 matrices. The concentration coefficient is 0.9994 and fulfils the requirement for a linear method.

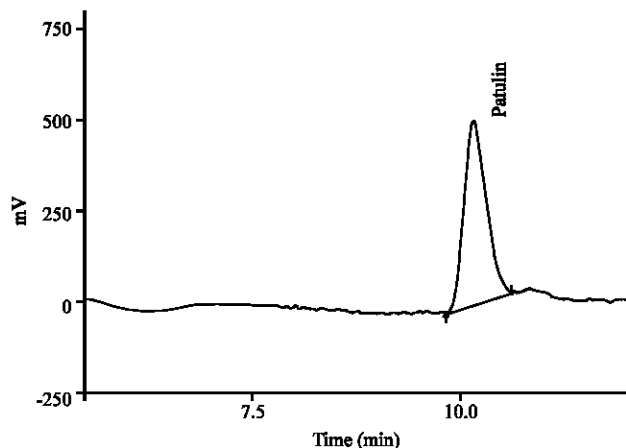


Fig. 1: HPLC chromatogram of 100 ng mL⁻¹ (ppb) patulin standard solution

Table 1: Recoveries of patulin from spiking into the samples

Spiking level (ppb)	Recovery (%)	SD (%) (n = 6)	CV
0.1	13.33	78	0.0104
0.5	5.90	83	0.0245
1.0	5.11	89	0.0455

Table 2: Results of survey for patulin contaminations of apple juice samples

Sample type	No.	Patulin concentration				Mean±SD
		<10 µg L ⁻¹	<10-50 µg L ⁻¹	<50 µg L ⁻¹	Max. (µg L ⁻¹)	
Apple juice	150	15 (10)	115 (76.66)	20 (13.33)	106.01	26.92±20.84393

Values in brackets indicate percentage

Recovery Studies

Recovery studies were carried out by spiking fresh samples (suitable amount depending on the extraction procedure) with known volumes of the appropriate working standard solution. Table 1 presents the results of spiking samples with patulin. Average recoveries were 78-89% and CV ranged from 5.11-13.33%.

Limit of Detection (LOD)

The limits of detection were calculated as the lowest concentration giving a response of three times the average of the baseline noise defined from three unfortified samples. The LOD for patulin were 3 ppb.

Analytical Parameters

Results from the survey of apple juice for patulin contamination are presented in Table 2. Results showed level of patulin were higher than 10 µg L⁻¹ in 90% of apple juices. Overall 13/3% of the apple juice samples had levels higher than 50 µg L⁻¹ with maximum level of 106/01 µg L⁻¹. Figure 2 shows the HPLC chromatograms of patulin and 5-HMF in apple juice. Retention time of patulin was 9.781 min.

Patulin could be resolved from 5-hydroxymethylfurfural (5-HMF), which is the most important interfering co-extractive of apple juice.

Patulin was detected in all of the 150 samples of apple juice at concentrations ranging from 6 to 106 µg L⁻¹. Patulin levels exceeded 50 µg L⁻¹ in 13.3% of all analyzed samples.

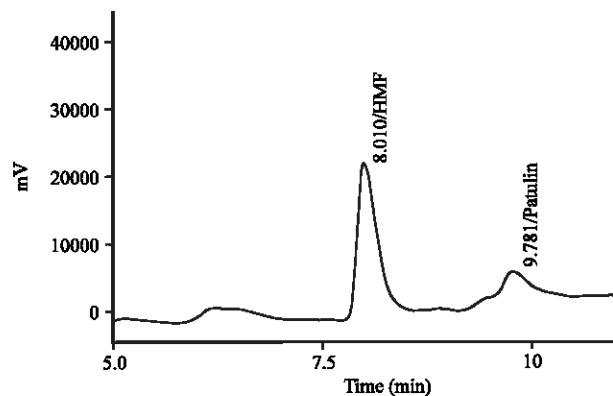


Fig. 2: HPLC chromatogram of apple juice containing patulin and 5-HMF. The retention time of patulin is 9.781 min. The retention time of 5-HMF is 8.010 min

DISCUSSION

Patulin, an important contaminant of apple products and other fruits, is a toxic secondary metabolite produced by a wide range of *Aspergillus* and *Penicillium* fungi. Mold growth and subsequent production of patulin occurs mainly where the surface tissue of fruit has been damaged.

Numerous surveys of patulin in apples and apple products have been published in the literature over the past years. Worldwide interest in patulin contamination dramatically increased over the last few years following a series of articles, which published the results of several surveys conducted in different countries.

Different studies indicated varying ranges of patulin contamination. Twenty-three percent of 328 fruit product samples surveyed between 1989 and 1990 in UK was contaminated and 22% of these samples contained levels between 51 and 1130 $\mu\text{g L}^{-1}$ (Burda, 1992). Among 100 apple juice samples surveyed in Spain, 82 samples were contaminated with a mean of 13.8 $\mu\text{g L}^{-1}$ and 8% of these samples had patulin levels above 50 $\mu\text{g L}^{-1}$ (Prieta *et al.*, 1994). Two hundred and fifteen samples of apple juice concentrates were analyzed in Turkey had contamination ranging from seven to 376 $\mu\text{g L}^{-1}$ and 45% contained amounts above 50 $\mu\text{g L}^{-1}$ (Gokmen and Acar, 1998). In earlier study conducted in Tehran, Iran, 42 samples of processed fruit juices were analyzed. Thirty-three percent of the samples had levels higher than 50 with maximum level of 285 $\mu\text{g L}^{-1}$ (Cheraghali *et al.*, 2005). A survey performed during 1980 showed that patulin was in 70% of tested samples at concentrations ranging from 1 to 38 $\mu\text{g L}^{-1}$. A New Zealand survey reported that 15% of apple juices sampled contained patulin levels ranging from 106 to 216 $\mu\text{g L}^{-1}$ (Wilson, 1981).

In a recent study, 150 samples of apple juice were surveyed for patulin contamination. The concentration of patulin was found to exceed 10 $\mu\text{g L}^{-1}$ in 90% of samples. In general, 13.3% of the apple juice samples had patulin levels higher than 50 $\mu\text{g L}^{-1}$ with maximum level of 106.01 $\mu\text{g L}^{-1}$.

The incidence of patulin in apple juice concentrate in Ahwaz City, Khuzestan Province was remarkably less than other study, which carried out on apple juice, produced such as in Tehran (Cheraghali *et al.*, 2005). The results show only 13.3% of samples has patulin higher than the maximum level recommended by the codex Alimentarius (50 $\mu\text{g L}^{-1}$) which is less than other studies such as Burda (1992), Gokmen and Acar (1998) and Prieta *et al.* (1994).

In Fact, the level of patulin in this study was higher than studies such as De Sylos and Rodriguez-Amaya (1999), Spadaro *et al.* (2007) and Iha and Sabino (2006) studies.

The presence of high amounts of patulin indicates that moldy apples were possibly used in the production of the juices. The contamination of apple juice products may sometimes be as high as 8000 $\mu\text{g L}^{-1}$ in apple juices made from partly rotten apples (Brackett and Marth, 1979). The control of patulin in fruit juice and fruit products could be achieved by using healthy fruit, hygienic storage, sorting damaged and rotten fruits, trimming off rotten tissue, filtration through activated charcoal, pasteurization and addition of sulfur dioxide or ascorbate (BSDA, 2001). Additional removal of rotten and damaged fruit prior to further processing significantly reduced the mean patulin level in the juice (Sydenham *et al.*, 1995).

CONCLUSION

The results of this study that patulin does not seem to be a problem in apple juice drink commercialized in Ahwaz, Iran with insignificant proportion of the products exceeding the 50 mg L^{-1} limit for apple juice set by WHO and certain European countries. However, constant surveillance of the patulin is strongly recommended because the available data is limited and the hot weather conditions in this area, incidence and level of this toxin could change according to factors like weather conditions, pH, type and stage of apple maturation (Druschs and Ragab, 2003).

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