

Research Note

Incidence of Patulin in Apple Juices Marketed in Turkey

TÜRKAN YURDUN,* GÜLDEN ZEHRA OMURTAG, AND ÖMER ERSOY

Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 81010 Haydarpaşa, Istanbul, Turkey

MS 01-94: Received 9 March 2001/Accepted 14 May 2001

ABSTRACT

The purpose of this study was to investigate the patulin contamination of apple juices consumed by the Turkish population. Patulin was detected using high-performance liquid chromatography (HPLC) with a UV detector at 280 nm, and the identification of patulin was further confirmed by thin-layer chromatography (TLC). Using HPLC, the recoveries were $79.9 \pm 6.7\%$ and $83.7 \pm 4.6\%$, and the coefficients of variation were 8.4 and 5.5% for apple juices spiked with the known amounts of patulin (60 and 120 $\mu\text{g}/\text{liter}$, respectively). The minimum patulin level detected was 5 ng in a standard solution and 5 $\mu\text{g}/\text{liter}$ in apple juices. The TLC method was used only to confirm patulin levels higher than 20 $\mu\text{g}/\text{liter}$ (100 ng/spot) in apple juices. The total number of samples was 45. Patulin was present in detectable levels in 60% of apple juices at concentrations ranging from 19.1 to 732.8 $\mu\text{g}/\text{liter}$. Forty-four percent of the apple juice samples had patulin contamination levels higher than 50 $\mu\text{g}/\text{liter}$, which is the allowable upper limit in Turkey.

Patulin [4-hydroxy-4H-furo (3,2c) pyran-2(6H)-one] is a mycotoxin produced by several species of molds (*Penicillium*, *Aspergillus*, and *Byssoschlamus*). *Penicillium expansum* is the most common mold-producing patulin in apples, pears, grapes, peaches, apricots, and cherries. *Penicillium patulum* (20), *Aspergillus clavatus*, and *Aspergillus giganteus* are also known to produce patulin (13). Patulin was first synthesized by Woodward and Singh (32), biosynthesized by Tanenbaum and Bassett (28), and isolated by Suzuki et al. (26). Scott et al. (23) first reported the occurrence of patulin in apple juices. Patulin contamination is primarily associated with areas of rotten tissue (29). However, levels of patulin in fruit products can be reduced by removing rotten tissue from the fruit. Optimum conditions for patulin production are a temperature of 25°C and a pH of 3 to 6.5 (8). Synonyms for patulin appearing in the literature are clavacin, clavatin, claviformin, expansin, gigantic acid, leucopin, mycoin c, penicidin, and tercinin (1). Patulin is highly toxic to animals, and the median lethal dose by oral administration to mice is 35 mg/kg (4). Dickens and Jones (6) found that patulin caused tumors in rats when given by subcutaneous injection. Patulin has also been implicated in teratogen (5). Although no data are available about the toxicity of patulin in humans, some governments such as those in Switzerland, Sweden, Belgium, Russian Federation, and Norway have established a maximum permitted concentration of 50 $\mu\text{g}/\text{liter}$ in apple juices (1). Many countries regulate patulin in juices at levels ranging from 20 to 50 $\mu\text{g}/\text{liter}$. The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives

established a provisional maximum tolerable daily intake for patulin of 0.4 $\mu\text{g}/\text{kg}$ of body weight/d (29).

Many different methods have been used for the estimation of patulin based on thin-layer chromatography (TLC) (9, 14, 23), gas liquid chromatography (9, 23), gas chromatography/mass spectrometry (16, 21), high-performance liquid chromatography (HPLC) (1, 7, 14, 17–19, 29), and the polarographic (31), dansitometric (24), and colorimetric methods (25). The purpose of this investigation was to determine the patulin contamination of apple juices in Turkey, especially the ready-to-drink products on the shelves for the consumer. The chromatographic methods used in the analysis were HPLC and TLC. The HPLC method with a UV detector at 280 nm used in this study was modified from Moller and Josefsson (17). The samples were collected from local markets in Istanbul and originated from different parts of Turkey.

MATERIALS AND METHODS

Chemicals. A patulin standard was obtained from Sigma Chemical Co., St. Louis, Mo. (P-1639). Chemicals were Merck products. All solvents used were HPLC grade. High-purity water obtained through a Milli Q-RG water purification system (Millipore, Bedford, Mass.) was used in all procedures.

Preparation of stock standard solutions. To prepare a stock standard solution at the concentration of 200 $\mu\text{g}/\text{ml}$, patulin was dissolved in chloroform. A 500- μl volume of this solution was transferred to a 10-ml volumetric flask and evaporated just to dryness under a stream of nitrogen at room temperature. The residue was immediately dissolved in water/acetonitrile (1/1, vol/vol). Working standard solutions were prepared by appropriate dilution of this solution with water/acetonitrile (1/1, vol/vol) (1 or 5 ng patulin/ μl). The stock standard solution was stored in the freezer at -20°C , and the working solutions kept at 2 to 5°C were stable. Working standard solutions were prepared weekly.

* Author for correspondence. Tel: +90 (0) 216-418 81 90; Fax: +90 (0) 216-345 29 52.

Extraction procedure. Samples (5 ml) were extracted two times with ethylacetate (10 ml) by shaking vigorously for 1 min. Organic phases were combined and cleaned up by extraction with 2 ml of 1.5% sodium carbonate by shaking for 1 min. The aqueous phase was separated and immediately extracted with 5 ml ethylacetate by shaking vigorously for 1 min. The combined organic solutions were dried over 3 g of anhydrous sodium sulphate and filtered. Then, the extract was evaporated just to dryness in a water bath at 40°C under a gentle stream of nitrogen (3, 17). The residue was dissolved in 150 μ l of water/acetonitrile (1/1, vol/vol). Ten microliters and/or 50 μ l was injected into the HPLC apparatus. Samples were prepared and analyzed in triplicate. The final solutions were stored in a freezer at -20°C if liquid chromatogram analysis was delayed.

Apparatus. HPLC (Waters Corp., Milford, Mass.) was a combination of a model 600 pump multisolvent delivery system with a model 481 variable-wavelength UV detector. The injector was a Rheodyne (Canada) 7725 sample injector with a 200- μ l accessory. A reverse-phase Kromasil 100-5 C₁₈, 250- by 4.6-mm I.D. column (catalog no. KR100-5 C₁₈-250A, Hichrom), protected by a guard column filled with the same material, was used. The data station was a Unicam 4880 Chromatography Data Handling System (Unicam Ltd., UK).

Operating conditions. Mobile phase was 0.02 M ammonium acetate/acetonitrile (9/1, vol/vol) (pH 6.9), flow rate was 1.1 ml/min, chart speed was 0.25 cm/min, and column temperature was ambient. The determination of patulin was performed at 280 nm by the external standard method. The mobile phase was filtered through a Millex HV Millipore (0.45 μ m) and degassed by an in-line degasser (Waters). The standard curves of patulin were linear in the concentration ranges of 5, 10, 20, 25, 50, and 100 ng. The linear regression line for patulin was found to be $y = 6.9373x - 0.2878$ ($r^2 = 0.9994$). The standard curve was prepared by plotting peak area versus concentration of patulin working standard solutions. The retention time of patulin was 8.83 ± 0.014 min, with a relative standard deviation of 0.162%, indicating reproducible results.

RESULTS AND DISCUSSION

In this study, 45 apple juice samples provided from Istanbul markets were studied, and patulin was detected in 60% of them. Patulin was detected in 27 samples at an average contamination level of 139.9 ± 114.6 μ g/liter (19.1 to 732.8 μ g/liter). According to our findings, patulin content was higher than 50 μ g/liter in 44% of the apple juice samples. The detection limits of patulin for the HPLC and TLC methods were 5 and 100 ng/spot, respectively. The HPLC method detection limit was about 5 μ g/liter. Our detector could discern 5 ng of patulin with a signal/noise ratio of 3/1. Apple juices were spiked with 60 and 120 μ g/liter; percentage recoveries of patulin were $79.9 \pm 6.7\%$ and $83.7 \pm 4.6\%$, respectively, and coefficients of variation were 8.4 and 5.5%, respectively. Five replicates were analyzed for each spiked level, and the recovery results were expressed as the mean of them. Confirmation was performed by TLC, according to the method prescribed by Fujimoto et al. (9). The R_f value of the patulin was 0.25. The limit of determination was approximately 20 μ g/liter (100 ng/spot) in apple juices for TLC.

Although the separation procedure of patulin and 5-hydroxymethylfurfural was, in general, achieved by using

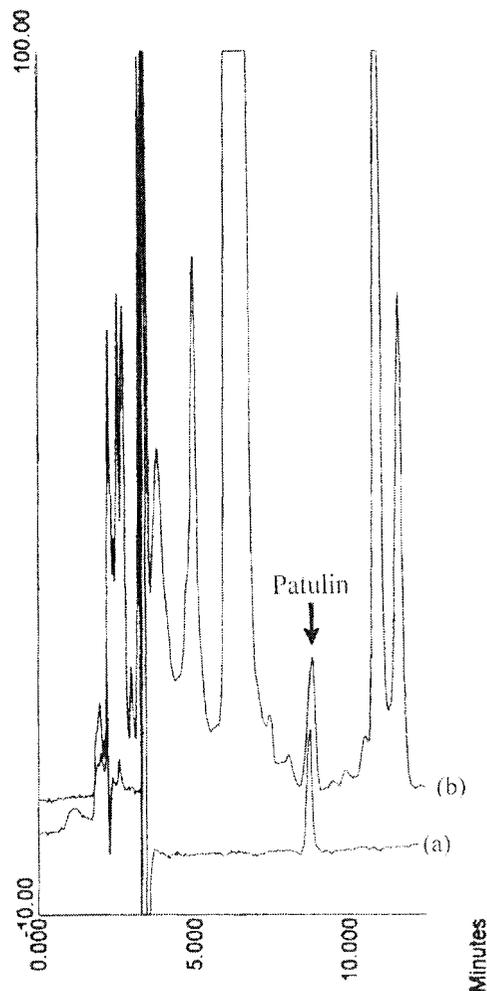


FIGURE 1. Liquid chromatogram of (a) a standard solution (25 ng) and (b) naturally contaminated apple juice (76.2 μ g/liter).

water (17), water/tetrahydrofuran (99/1) (19), and water/acetic acid (98/2) (1), in this study, we used 0.02 M ammonium acetate/acetonitrile (9/1, vol/vol). A typical chromatogram for the apple juices naturally containing patulin is shown in Figure 1. The selected mobile phase and the HPLC column were capable of yielding the maximum separation of patulin from interfering substances in apple juices. Patulin is as stable to heat in acidic medium as in apple juices; therefore, noticeable amounts of patulin remained in juices after processing (12). Because Scott and Kennedy (22) had reported that patulin was unstable if it remained as a thin film at the end of an evaporation, the residue was immediately dissolved in 150 μ l of water/acetonitrile (1/1, vol/vol) after evaporation of the eluates in order to avoid low recoveries in this study. The patulin levels obtained by HPLC in commercial apple juices purchased in Turkey are given in (Table 1).

In Washington, D.C., patulin was detected in concentrations ranging from 44 to 309 μ g/liter in 8 out of 13 apple juices (30). Patulin levels between 10 to 350 μ g/liter were found in 23% of 40 apple juice samples in Wisconsin (2). In 26% of commercial apple juices (with an average of 15 μ g/liter) and in 31% of homemade apple juices (up to 1 ppm), patulin was detected (15). In Brazil, 111 samples of

TABLE 1. Patulin levels by HPLC in commercial apple juices obtained from Turkey (samples obtained in 1998–2000)

Patulin level ($\mu\text{g/liter}$)	No. positive samples ^a	%	Range (avg. of positives), ($\mu\text{g/liter}$)	Average ($\mu\text{g/liter}$), standard deviation
<50	7	16	19.1–47.3	36.4 \pm 10.8
50–500	19	42	60.2–403.3	146.9 \pm 115.5
>500	1	2	732.8	732.8

^a Total number of samples was 45.

processed fruit juices (such as apple and grape) were analyzed for patulin by HPLC, and 3.33% were found positive at 17 $\mu\text{g/liter}$ (27). In Turkey in 1993, patulin was detected in 95% of 20 commercial apple juice samples from local markets in Ankara, and only two of them contained patulin at levels higher than 50 $\mu\text{g/liter}$ (10). From 1996 to 1997, the same researchers reported that the 50- $\mu\text{g/liter}$ patulin contamination level was exceeded in 43.5% of 215 samples (11). These results seem very similar to our findings, but it is noteworthy that their samples were apple juice concentrates. Noting that the limit for juices set by the World Health Organization is 50 $\mu\text{g/liter}$, the incidence of patulin detection in apple juices in Turkey is rather high. The level and incidence of patulin found in apple juice samples in Turkey were higher than those reported in other countries. This study indicates that mycotoxin is a potential contaminant of apple juices under the conditions of our country. Therefore, adequate attention must be given to the production and control phases of apple juices as well as to the most susceptible foods and food products.

ACKNOWLEDGMENT

This study was conducted as part of the research activities of the Research Fund of Marmara University, project no. 1997-SB-44.

REFERENCES

- Bartolome, B., M. L. Bengoechea, F. J. Perez-Illzarbe, T. Hernandez, I. Estrella, and C. Gomez-Cordoves. 1994. Determination of patulin in apple juice by high-performance liquid chromatography with diode-array detection. *J. Chromatogr.* 664:39–43.
- Brackett, R. E., and E. H. Marth. 1979. Patulin in apple juice from roadside stands in Wisconsin. *J. Food Prot.* 42:862–863.
- Brause, A. R., M. W. Trucksess, F. S. Thomas, and S. W. Page. 1996. Determination of patulin in apple juice by liquid chromatography: collaborative study. *AOAC Int.* 79:451–455.
- Broom, W. A., E. Bülbring, C. J. Chapman, J. W. F. Hampton, A. M. Thomson, J. Ungar, R. Wien, and G. Woolfe. 1944. The pharmacology of patulin. *Br. J. Exp. Pathol.* 25:195–207.
- Ciegler, A., A. C. Beckwith, and L. K. Jackson. 1976. Teratogenicity of patulin and patulin adducts formed with cysteine. *Appl. Environ. Microbiol.* 31:664–667.
- Dickens, F., and H. E. H. Jones. 1961. Carcinogenic activity of a series of reactive lactones and related substances. *Br. J. Cancer* 15: 85–100.
- Forbito, P. R., and N. E. Babsky. 1985. Rapid liquid chromatographic determination of patulin in apple juice. *J. AOAC* 68:950–951.
- Frank, H. K. 1974. Toxische Stoffwechselprodukte von Schimmelpilzen. *Alimenta* 13:98–101.
- Fujimoto, Y., T. Suzuki, and Y. Hoshino. 1975. Determination of penicillic acid and patulin by gas-liquid chromatography with an electron-capture detector. *J. Chromatogr.* 105:99–106.
- Gökmen, V., and J. Acar. 1996. Rapid reversed-phase liquid chromatographic determination of patulin in apple juice. *J. Chromatogr.* 730:53–58.
- Gökmen, V., and J. Acar. 1998. Incidence of patulin in apple juice concentrates produced in Turkey. *J. Chromatogr. A* 815:99–102.
- Gökmen V., and J. Acar. 1999. Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography. *J. Chromatogr. A* 847:69–74.
- Katzman, P. A., E. E. Hays, C. K. Cain, J. J. van Wyk, F. J. Reithel, S. A. Thayer, E. A. Doisy, W. L. Gaby, C. J. Carroll, R. D. Muir, L. R. Jones, and N. J. Wade. 1944. Clavacin, an antibiotic substance from *Aspergillus clavatus*. *J. Biol. Chem.* 154:475–486.
- Leuenerberger, U., R. Gauch, and E. Baumgartner. 1978. Neue Bestimmungsmethode des Mykotoxins Patulin mit Hilfe der Dünnschicht- und Hochdruckflüssigchromatographie. *J. Chromatogr.* 161: 303–309.
- Lindroth, S. 1980. Occurrence, formation and detoxification of patulin mycotoxin. Academic dissertation. Espoo Finland, ISBN 951-38-1096-8.
- Llovera, M., R. Viladrich, M. Torres, and R. Canela. 1999. Analysis of underivatized patulin by a GC-MS technique. *J. Food Prot.* 62: 202–205.
- Moller, T., and E. Josefsson. 1980. Rapid high pressure liquid chromatography of patulin in apple juice. *J. AOAC* 63:1055–1056.
- Priest, J. W., and R. J. Light. 1990. Applications of high-performance liquid chromatography to quantitation of metabolites and enzymes of the patulin pathway from *Penicillium patulum*. *J. Chromatogr.* 513:237–246.
- Prieta, J., M. A. Moreno, J. Bayo, S. Diaz, G. Suarez, and L. Dominguez. 1993. Determination of patulin by reversed-phase high-performance liquid chromatography with extraction by diphasic dialysis. *Analyst* 118:171–173.
- Raistrick, H., J. H. Birkshaw, A. Bracken, S. E. Michael, W. E. Gye, and W. A. Hopkins. 1943. Patulin in common cold. Collaborative research on a derivative of *Penicillium patulin* Bainier. *Lancet* 245: 625–635.
- Rupp, H. S., and S. B. Turnipseed. 2000. Confirmation of patulin and 5-hydroxymethylfurfural in apple juice by gas chromatography/mass spectrometry. *AOAC Int.* 83:612–620.
- Scott, P. M., and B. P. C. Kennedy. 1973. Improved method for the thin layer chromatographic determination of patulin in apple juice. *J. AOAC* 56:813–816.
- Scott, P. M., W. F. Miles, P. Toft, and J. G. Dube. 1972. Occurrence of patulin in apple juice. *J. Agric. Food Chem.* 20:450–451.
- Siriwardana, M. G., and P. Lafont. 1979. Dansitometric method for the determination of patulin in fruit juices. *J. Chromatogr.* 173:425–428.
- Subramanian, T. 1982. Colorimetric determination of patulin produced by *Penicillium patulum*. *J. AOAC* 65:5–7.
- Suzuki, T., M. Takeda, and H. Tanabe. 1971. A new mycotoxin produced by *Aspergillus clavatus*. *Chem. Pharm. Bull.* 19:1786–1788.
- Sylos, C. M., and D. B. Rodriguez-Amaya. 1999. Incidence of patulin in fruits and fruit juices marketed in Campinas, Brazil. *Food Addit. Contam.* 16:71–74.
- Tanenbaum, S. W., and E. W. Bassett. 1959. The biosynthesis of patulin. III. Evidence for a molecular rearrangement of the aromatic ring. *J. Biol. Chem.* 234:1861–1866.
- Trucksess, M. W., and Y. Tang. 1999. Solid-phase extraction method for patulin in apple juice and unfiltered apple juice. *AOAC Int.* 82: 1109–1113.
- Ware, G. M., C. W. Thorpe, and A. E. Pohland. 1974. Liquid chromatographic method for determination of patulin in apple juice. *J. AOAC* 57:1111–1113.
- Wasicki, P., F. Scholz, and K. D. Schleinitz. 1986. Polarographic determination of patulin. *Pharmazie* 41:843–844.
- Woodward, R. B., and G. Singh. 1950. The synthesis of patulin. *J. Am. Chem. Soc.* 72:1428.