Analysis of Wine for Penicillin

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Abstract

This study addresses the question of whether the antibiotic Penicillin, which is produced by the common mold *Penicillium notatum*, could possibly become a contaminate of wine during the fermentation process. The significance of this study is related to the potential health effects this agent might produce in those consumers who have an allergic response to Penicillin. It has been estimated that between 6% and 8% of the American population is subject to this type of allergic response. A method is developed for the detection of penicillin in wines using high-pressure liquid chromatography. We demonstrate that penicillin G hydrolyzes rapidly in wine with first-order kinetics, and the half-life of this antibiotic is 147 min in a typical commercial wine. An analysis of a number of commercial wines shows no evidence of the presence of penicillin, which should negate the question of any allergic response associated with this potential contaminate.

Introduction

Today, the wine industry is quite concerned with the consequences of trace amounts of materials or contaminates such as sulfites, pesticides, and even ethylene glycol in wine. Certain of these possible contaminates require adequate labeling on wine for consumer satisfaction and protection. Some of the potential contaminates are the result of viticultural practices including the use of pesticides and herbicides in the vineyard, and others may arise during the fermentation process. The question has arisen as to whether wines prepared from natural yeast or even those prepared using commercial yeast might contain trace amounts of penicillin. Because the penicillin mold might be easily adaptable to most winery conditions and thus enter the fermentation process either directly or indirectly, the question of its possible contamination of commercial wines would seem to be relevant. This is particularly significant in view of the number of consumers that might be at risk to allergic responses to this antibiotic.

Native yeast is the natural yeast found on the skins of nearly all grapes at the time of harvest. These yeasts are typically removed with a sulfite spray prior to fermentation and do not present a problem in later stages of the wine production. *Penicillium* mold is ubiquitous and may be present on the surface of fermentation tanks, barrels, and any other porous surfaces used in the production of most commercial wines. Poorly maintained equipment may allow for the infiltration of the *Penicillium* mold and the subsequent production of the penicillin antibiotic in the finished wine (1). It was suspected that penicillin may have been a contaminate in certain ciders produced in the 18th century when the liquid broth from the fermentation was used medicinally to treat slaves stricken with a deadly fever (2).

A method has been developed and tested in our laboratory using high-pressure liquid chromatography (HPLC) to detect the presence and survival of penicillin G in commercial wines. Wine samples spiked with penicillin G were analyzed by HPLC over a measured period of time to determine whether penicillin G was stable in wine. It is generally accepted that penicillin readily undergoes rapid hydrolysis under acidic conditions, but the question of its survival or persistence in wine has not been addressed and should be of interest to consumers who wish to avoid potential allergic responses. It should be noted that the pH range for most wines is between 3.0 and 3.5. White wines typically have lower pH values than red wines because of the skin and juice contact in the fermentation of red wines (1).

Experimental

Development of an HPLC analytical method for penicillin detection in wine

The optimum wavelength to monitor penicillin was determined to be 230 nm using a spectrophotometer (Varian CARY 3-Bio, Varian Inc., Walnut Creek, CA). A standard sample of sodium penicillin G (Sigma Chemical Co., St. Louis, MO) was then analyzed on an HPLC (168 diode-array detector with a 125 pump unit, Beckman) using a gradient changing from a 100:1 to a 70:30 ratio of 50mM sodium phosphate buffer (pH 7.0)–acetonitrile at a flow rate of 1 mL/min. This allowed for the determination of the optimum ratio of the mobile phase to elute the penicillin G from the column. An isocratic solvent system with a ratio of 75:25 for the 50mM sodium phosphate buffer (pH 7.0)–acetonitrile was selected based on the results of the gradient

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study. This solvent ratio eluted the penicillin G from the column most rapidly and with a retention time (RT) that did not overlap with any of the other peaks found in the wine sample. The optimum elution time for this analysis was found to be 20 min at a flow rate of 0.8 mL/min. Twenty-microliter samples of wine were analyzed with a C18-aq reverse-phase (S-5 µm-120 octadecylsilane) column (YMC, Morris Plain, NJ) fitted with a C18-aq guard column (YMC). All samples were analyzed using the same conditions outlined in this section.

The detection limit for penicillin G using the Beckman 168 diode-array detector was determined to be 100 ppm using the formula:

$$\text{mean of blank} + 2.6x \qquad \text{Eq. 1}$$

where x is the standard deviation of the mean of 20 blank runs using the method developed by Buttnen (3). It is generally accepted that the level or amount of penicillin that is required to initiate a penicillin sensitivity response or allergic reaction is extremely small, perhaps in the microgram range. Penicillin sensitivity is not assumed to be dose dependent, and even if a lower detection level of penicillin could be achieved using HPLC, the present data suggests that penicillin does not survive the rapid hydrolysis observed in wine.

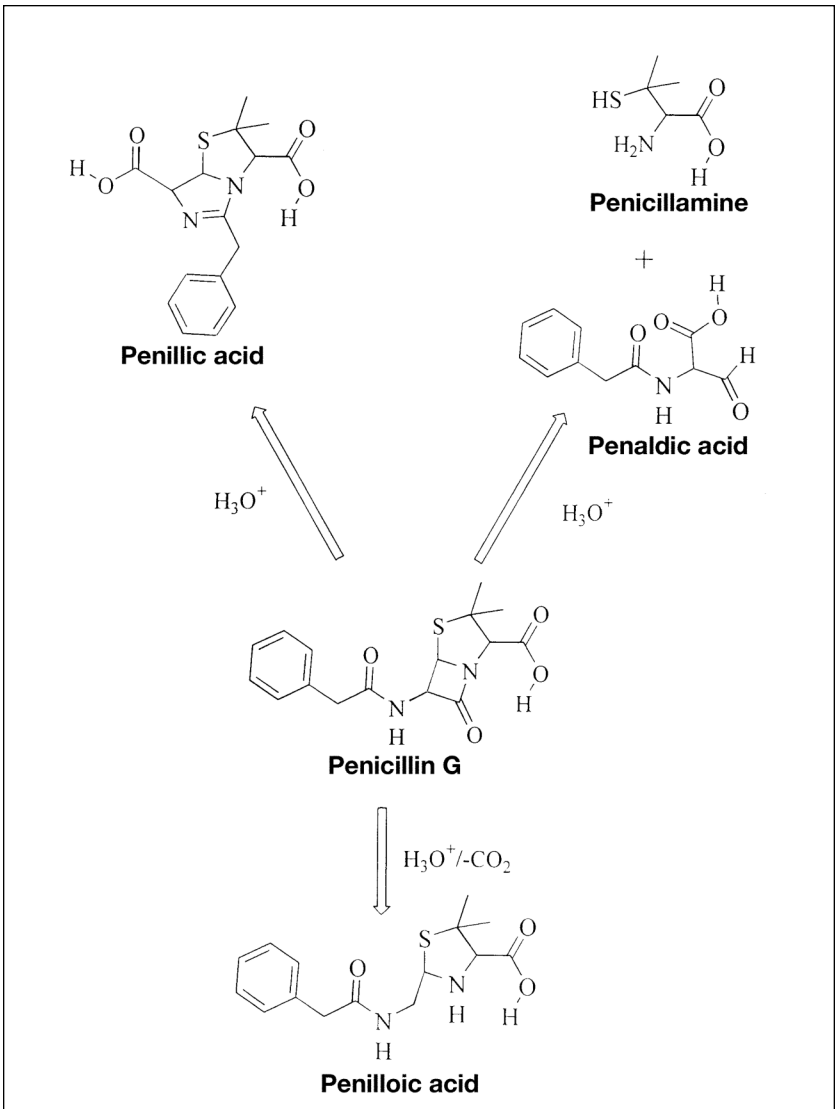

Figure 1. Penicillin hydrolysis products.

Analysis of penicillin G in wine

The stability of penicillin G under the acidic conditions typically found in wine (pH range from 3.2 to 3.7) was followed over a 24-h period. Penicillin G (Sigma Chemical Co.) was added to a red wine (pH 3.60), a white wine (pH 3.20), and an aqueous sulfuric acid solution that was 5.0×10^{-4} molar (pH 3.30). The wine samples were prepared by adding 50.0 mg of penicillin G to 750 mL wine. The bottles were purged with nitrogen and resealed. Aqueous sulfuric acid standards were prepared by adding 50 mg of penicillin G to 750 mL deionized water, and the pH was adjusted to 3.3 with concentrated sulfuric acid. The final concentration of the penicillin G in all three samples was 0.19mM. All three samples were analyzed by HPLC before, immediately after, and 24 h after the addition of the penicillin G. Upon evaporation of the aqueous acid solution we isolated a solid hydrolysis product, which was identified as penillic acid (Figure 1) by infrared (IR) analysis and a melting point of 180°C, (literature melting point between 181°C and 182°C) (5).

A number of different commercial wines (red and white) were then analyzed by this HPLC method for the presence of penicillin G. All analyses were done by the direct injection of 20-µL samples of each wine following the protocol previously described.

Kinetics of penicillin G hydrolysis in wine

The kinetics of the hydrolysis of penicillin G in wine was examined in order to support the results observed in the stability study of penicillin G in wine. A 3.4mM penicillin G sample was prepared in a white wine (pH 3.28) that had been analyzed by HPLC prior to the addition of penicillin. The wine was then analyzed immediately following the addition of the penicillin ($t_1 = 0$) and then at 20 min ($t_2 = 20$), 40 min ($t_3 = 40$), 60 min ($t_4 = 60$), 300 min ($t_5 = 300$), and 1185 min ($t_6 = 1185$). The concentrations of penicillin G at t_i were used in the plots of a zero-, first-, and second-order reaction in order to determine the kinetics of the penicillin hydrolysis at room temperature (22°C). The order of the reaction was determined from the plot in which the values fit linearly, the slope of which provided the rate constant (k).

Results and Discussion

The stability study showed that when penicillin G was added to the white wine (pH 3.20) and red wine (pH 3.60) it was quickly hydrolyzed and disappeared completely within 24 h. The same rapid

disappearance of penicillin G was observed in the sulfuric acid controls (pH 3.30). This would indicate that penicillin is not stable in wine at these low pHs and that its instability is most likely because of rapid hydrolysis. This would also suggest that penicillin G should not be present in wine after fermentation and bottle aging. Although the instability of penicillin in an acidic medium has been documented (Figure 1) (4), it was important to determine whether penicillin G would be stable in the natural environment and low pH of commercial wines. The initial peak for penicillin G in the wine sample (RT = 11.17 min) disappeared and a new peak appeared much earlier in the chromatogram (RT = 4.33 min) (Figure 2). It is known that penicillin G undergoes rapid hydrolysis of the β-lactam ring in the presence of certain enzymes and is subsequently converted to penillic acid. The mechanism of the acid hydrolysis of penicillin G is through a similar nucleophilic addition reaction to the strained carbonyl group of the β-lactam ring (Figure 1). Penillic acid was shown to be the hydrolysis product in our control studies with sulfuric acid and was identified by IR analysis and the melting point of the product. The RT of this product was identical with the RT of the peak observed in the reaction of penicillin G in both red and white wines (data not presented).

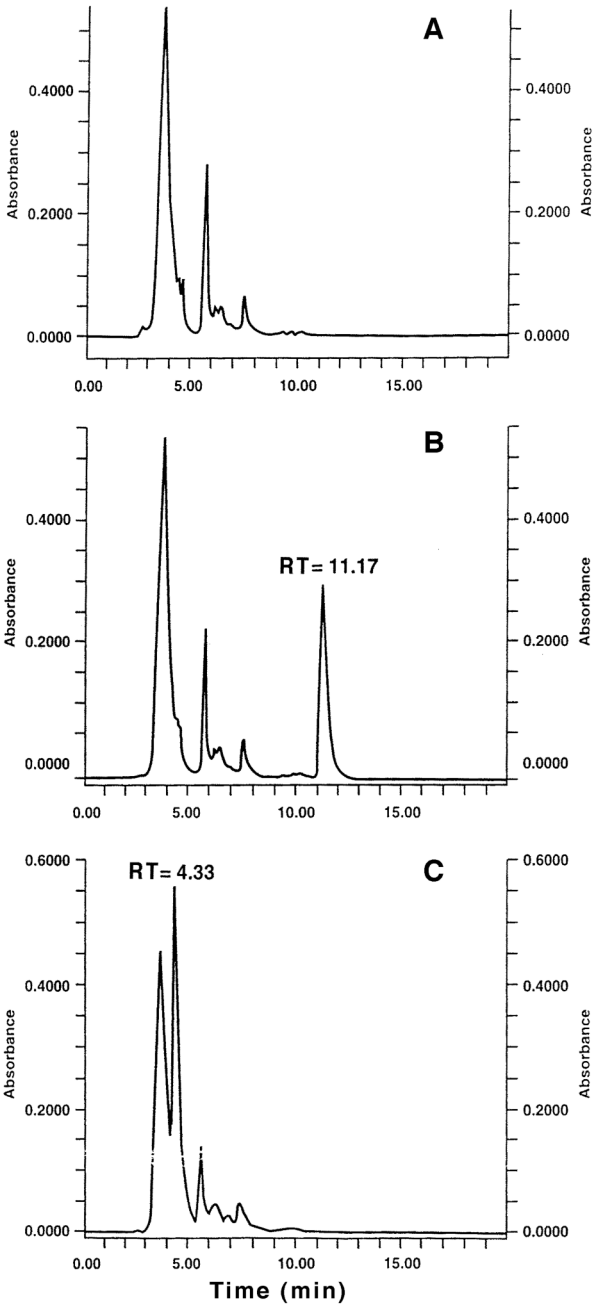

Figure 2. Chromatograms of (A) untreated wine, (B) wine treated with penicillin (3.4mM) at t = 0 min, and (C) a treated wine sample at t = 1185 min.

The kinetics of hydrolysis of the penicillin G was established in white wine at a pH of 3.28. The initial concentration of penicillin G in the wine was 3.4mM, and it can be seen from a plot of penicillin concentration versus time (Figure 3) that the concentration of penicillin G decreases in an exponential manner. The data from this study is in agreement with a first-order reaction (Figure 4). This implies that penicillin G is rate limiting to the reaction and it is not hydrolyzed through a catalytic mechanism. From this plot the reaction k value of the hydrolysis of penicillin G in wine at room temperature (22°C) was calculated to be 7.83×10^{-5} s^{-1}. This number is in good agreement with the k value calculated from the experimental values determined by Pawelczyk et al. (6) for the hydrolysis of the β-lactam ring of the penicillin analog dicloxacillin (6.40×10^{-5} s^{-1} at 22°C, pH 3.28).

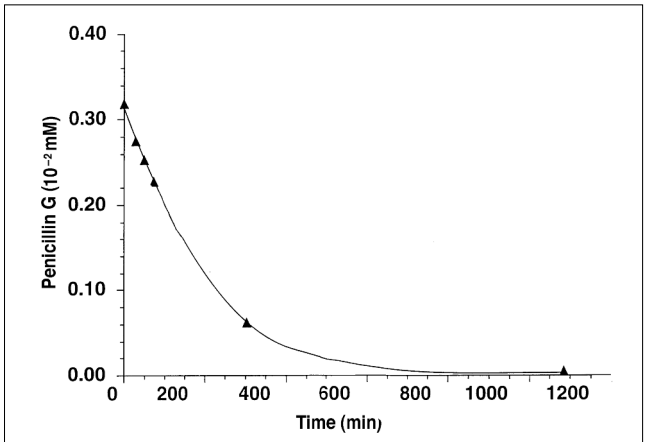

Figure 3. Time study of penicillin G in wine (pH 3.28).

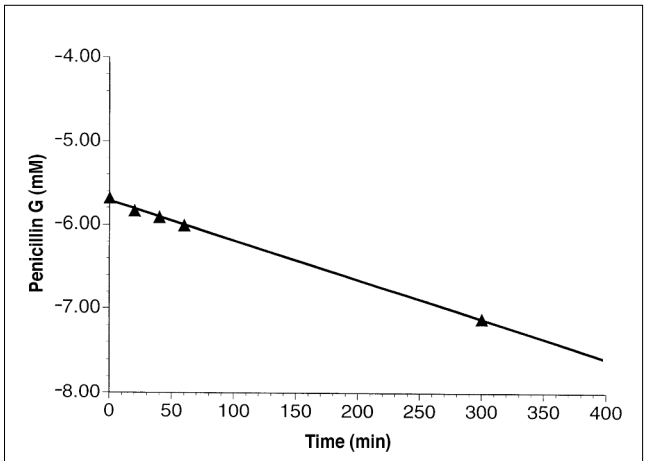

Figure 4. Kinetics of penicillin G in wine (pH 3.28), (the k value was 0.0047 min^{-1} for five independent experiments, which included those in aqueous sulfuric acid and in red and white wines).

A first-order reaction has a half-life that is independent of the initial concentration of the reactant. It was calculated (using the *k* value measured in this experiment) that it will take 147.0 min for penicillin G to reach half of its initial concentration through hydrolysis. This is in good agreement with the plot in Figure 3.

Throughout this study no penicillin G was ever detected in the commercial wines that were analyzed. The hydrolysis product of penicillin G (penillic acid) could not be detected in the analysis of the untreated wine samples because of the complexity of the chromatogram in the region of RT = 4.33 min. Most wines exhibit a large number of peaks in this region typical of a wide variety of aromatic components found in wine.

Conclusion

The amount of penicillin antibiotic that might contaminate a wine would depend on the amount of *Penicillium sp.* present on the grapes at the time of harvest or found in the fermentation tanks and bottling machinery. It is known that the presence of yeast and fermented liquid broth is favorable for the proliferation of *Penicillium notatum*. However, it has been shown in this study that the potential for trace amounts of penicillin G in wine is very unlikely. This is mainly because of the instability of penicillin G under the acidic conditions normally associated with commercial wine production.

When taken orally, penicillin G may be absorbed by the human gastrointestinal walls. However, approximately 50% of the drug is destroyed in the stomach within 20 min of ingestion because of the low pH levels present in the stomach (pH range from 1.0 to 2.0). Because of this, only approximately one-third of the penicillin taken orally is absorbed into the circulatory system (4). Based on our kinetics study of penicillin G in wine and the length of time it would be in contact with the relatively low pH of wine, it is highly unlikely that significant levels of penicillin would be present in commercially available wines, and the threat of an allergic response seems rather unlikely.

The one remaining question with regard to the presence of penicillin G in wine revolves around the nature of any degradation products. Penicillin G is known to have several decomposition products that are biologically active (7). It is quite likely that penillic acid would be produced by the acid hydrolysis of penicillin G in wine, and the presence of this degradation product may be of some concern to consumers. However, there are no clinical studies or evidence that suggests that penillic acid is capable of initiating an allergic response. Further studies to identify penillic acid in wine are currently under way in our laboratory.

Acknowledgments

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