The Attempted Enrichment of Beer with Thiamine Alkyl Disulphides

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Summary

The Wernicke-Korsakoff syndrome, commonplace in Australia, might be prevented by the enrichment of alcoholic beverages with thiamine. The use of the well absorbed thiamine alkyl disulphides for the enrichment of the most relevant Australian beverage, namely beer, is examined. A liquid chromatographic method is described whereby thiamine tetrahydrofurfuryl disulphide and thiamine propyl disulphide can be detected in beer in concentrations down to 125 ng/ml. It is concluded that the thiamine alkyl disulphides offer no special advantage because their disulphide bonds are reduced by substances in beer, yielding free thiamine.

Key Words: Wernicke-Korsakoff syndrome, Australia, vitamin enrichment, prevention, thiamine, thiamine alkyl disulphides, thiamine tetrahydrofurfuryl disulphide, thiamine propyl disulphide, liquid chromatography

In Australia the Wernicke-Korsakoff (W-K) syndrome, a condition seen almost entirely in alcoholics after they have become acutely thiamine deficient, is so common that it does not, as elsewhere in the world, constitute a rarity (1, 2). For example, in the largest mental hospital in the Australian State of Queensland, Price and Theodoros (1) found 170 cases of the Korsakoff syndrome out of a total inpatient population of approximately 1,100. The W-K syndrome comprises an acute encephalopathy (Wernicke's encephalopathy) followed in the majority of cases by a variably severe but often permanent impairment of short-term memory.

Price and Theodoros supported Centerwall and Criqui's assertion (3), based on a U.S. study, that it would be cost-beneficial to supplement alcoholic beverages with thiamine in order to prevent the W-K syndrome occurring. They saw it as necessary to determine what beverages were being consumed in the critical period prior to the onset of Wernicke's encephalopathy, arguing that only the supplementation of these beverages could be justified. In Queensland, where their study was carried out, the beverage most frequently consumed during the critical period was beer, this beverage being all or part of what had been consumed in over half the cases where reliable information was available. The acute thiamine depletion leading to the W-K
syndrome related to the absence of significant amounts of thiamine from alcoholic beverages plus the fact that patients virtually stopped eating during the critical period.

Malabsorption of thiamine may occur in alcoholics (4, 5) and a deficiency of the folic acid necessary for the transport of water-soluble thiamines such as thiamine hydrochloride may also occur (6, 7). Furthermore, the absorption of these vitamins may be interfered with even in non-alcoholic subjects if they are taken with alcohol (4, 8). Hence there would appear to be merit in supplementing alcoholic beverages not with water-soluble derivatives of thiamine, but with one of the much more readily absorbed lipid-soluble alkyl disulphides (9) or related homologues (10).

Price and Theodoros (1) demonstrated the stability of thiamine hydrochloride in beer where its presence can be recognised by utilising the thiochrome reaction; this reaction provides the most frequently used method for measuring water-soluble thiamines in foodstuffs. The question arises whether the thiamine alkyl disulphides and their homologues are similarly stable in beer. However, the thiamine alkyl disulphides studied here, thiamine tetrahydrofurfuryl disulphide hydrochloride (TTFD HCl) and thiamine propyl disulphide (TPD) do not give a thiochrome reaction. Hence it became necessary to develop an alternative method for analysing these substances in which the presence of the thiamine alkyl disulphide would not be masked by interfering substances contained within beer, a highly complex chemical mixture.

**METHODS**

Their long-term stability in beer under various storage conditions was investigated by adding TTFD HCl and TPD as a methanolic solution at a concentration of 1.2 mg/ml in volumes of 30 μl, 50 μl or 100 μl to ice-cold 120 ml clear glass screw cap bottles under sterile conditions. Ice-cold beer was opened and used to rapidly fill these bottles and a gas gun (millipore) was used to add sterile high purity nitrogen gas to replace air in the top of the bottles immediately before they were crown sealed. The bottles were stored at 8°C in the dark, or at ambient temperature under light or dark conditions. Sterile disposable plastic syringes fitted with 25-gauge needles were used to withdraw beer samples for analysis from bottles thus fortified with 300 ng, 500 ng or 1,000 ng of both TTFD HCl and TPD per ml of beer.

For studies of immediate recovery and for calibration curve experiments, TTFD HCl and TPD plus an internal standard, nitrazepam (0.2 μg per assay tube), were added as methanolic solutions and evaporated to dryness before freshly opened beer was added. For studies of long-term stability, the internal standard nitrazepam was similarly added to each assay tube at the commencement of extraction.
THE EXTRACTION PROCEDURE

Beer (1 ml) mixed with 0.2 M trisodium orthophosphate (pH 13.5, 1 ml), anhydrous sodium sulphate (500 mg), methanol (1 ml) and chloroform (4 ml) was shaken in cetavalonized glass centrifuge tubes (15 cm × 15 mm) with teflon-lined screw caps on a Coulter mixer for 5 min. After centrifugation at 1,500 × g for 5 min the aqueous layer was discarded and the organic layer decanted into a cetavalonized glass centrifuge tube for an acid backwash by shaking for 5 min on a Coulter mixer with 0.2 M HCl (2 ml). After centrifugation at 1,500 × g for 5 min, the acid layer was transferred to another cetavalonized glass centrifuge tube. Trisodium orthophosphate (0.5 M, 2 ml), anhydrous sodium sulphate (1.0 g), methanol (1 ml) and chloroform (4 ml) were added and the mixture shaken on a Coulter mixer for 5 min. After centrifugation at 1,500 × g for 5 min the aqueous layer was aspirated and discarded. The organic layer was decanted into a 12 ml cetavalonized glass tapered tube. The tapered tube was placed in a water bath at 70°C and the organic phase removed by gentle evaporation under a stream of high purity nitrogen gas. Methanol (20 µl) was added to the tapered tube and the dried residues were dissolved by mixing on a vortex mixer for 1.5 min. Fifteen µl of extract were injected into a high pressure liquid chromatograph with a prepacked “µBondapak C₁₈” column (30 cm × 3.9 mm) with an average particle size of 10 µm (Waters Associates Inc.). The instrument consisted of a Model 6000 A Solvent Delivery System, a Model U6K Universal Injector and a Model 440 Absorbance Detector (Waters Associates Inc.). Peak heights at 254 nm were recorded with a 10-mV potentiometric chart recorder (Omniscribe). The mobile phase consisted of a 65% sodium phosphate buffer (0.1 M, pH 7.2) and 35% methanol. The mixture was filtered through a cellulose acetate filter (pore size 0.45 µm, Sartorius) and then degassed under reduced pressure. The flow rate was 1.8 ml/min.

RESULTS

The blank beer sample chromatogram contained a small peak which co-chromatographed with TTFD thus making direct measurement of TTFD in fortified beer impossible. A correction was made by subtracting the height of this peak from the combination peak seen in fortified beer. The chromatogram of a beer sample which has been fortified with TTFD HCl and TPD is shown in Fig. 1. Three other brands of beer which were fortified and extracted yielded very similar chromatograms to that depicted in Fig. 1. The retention times were 8.4 min for TTFD, 15 min for TPD and 20.1 min for the internal standard, nitrazepam. The detector responded to quantities of TTFD and TPD down to 50 ng.

Table 1 shows that when TTFD HCl or TPD, ranging in concentration from 125 to 1,000 ng per ml (2,000 ng per ml for TPD) were added to beer just prior to assay, there was a linear relationship between concentration and the peak height ratios of each compound to the internal standard.

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Fig. 1. Chromatogram of beer to which had been added, just prior to extraction, TTFD, TPD and nitrazepam (N).

Table 1. The relationship between the thiamine alkyl disulphide concentrations added to beer and peak height ratios.

| Concentration (ng/ml) | Peak height* ratios |
|----------------------|---------------------|
|                      | TTFDb               | TPDc               |
| 125                  | 0.155               | 0.081              |
| 250                  | 0.317               | 0.206              |
| 500                  | 0.739               | 0.458              |
| 1,000                | 1.401               | 0.825              |
| 2,000                | (2.303)d            | 1.608              |

*Peak height of each vitamin/peak height of the internal standard. b Linear regression line: Peak height ratio = Conc* (ng/ml) × 0.00143 − 0.0197; r = 0.9987. c Linear regression line: Peak height ratio = Conc* (ng/ml) × 0.00080 + 0.0133; r = 0.9989. d This value is above the limits of the linear relationship.

STABILITY

Based on Australian recommended daily allowances, to metabolize one ml of beer 300 ng of thiamine are required. The two thiamine alkyl disulphides added to beer in concentrations of up to 1,000 ng per ml disappeared from beer within 16 h.
when it was kept at ambient temperature in clear glass bottles or in the dark. When beer fortified with TTFD HCl or TPD in concentrations ranging from 300 to 1,000 ng per ml was kept at 8°C in the dark for 16 h, trace amounts of TTFD and TPD could be detected. By six days neither was detectable in these beers. In contrast, there was no loss of either compound added to 0.005 M NaH₂PO₄ buffer (pH 4.55) at a concentration of 500 ng/ml under any of these storage conditions.

The recovery of the thiamine alkyl disulphides added to beer 5 min prior to assay at concentrations between 125 and 2,000 ng per ml was 48.4 ± 5.16% for TTFD and 44.8 ± 4.95% for TPD, compared to their recovery from buffer (0.005 M NaH₂PO₄). The recovery of these compounds from buffer was over 90% of the theoretical maximum for each compound using the extraction procedure described. Taken together, these data suggest some immediate losses resulting from an interaction between compounds in the beer and the two compounds studied.

In order to explore further the breakdown of TTFD HCl and TPD in beer, the following experiments were performed. Each compound was added to separate specimens of beer in a concentration of 20 µg/ml and admixed thoroughly. Higher concentrations of TTFD HCl and TPD were used in this part of the study in an attempt to operate well within the sensitivity of the thiochrome method that was used for the estimation of free thiamine (II). Analysis for thiamine content was carried out 5 min after admixture and again 13 days later. In a further experiment, TTFD HCl and TPD were added at lower concentrations (1,000 ng/ml beer) and the beer analysed for free thiamine content at 1 h and 6 days. Storage of beer was at ambient temperature throughout. Unfortified beer contains no free thiamine when analysed by this method. The results depicted in Table 2 indicate the generation of free thiamine from the alkyl disulphides on a substantial scale.

|                   | Cone*  | Time interval* | Free thiamine content, expressed as % of amount added |
|-------------------|--------|----------------|------------------------------------------------------|
|                   | (ng/ml)|                |                                                      |
| TTFD HCl          | 20,000 | 5 min          | <D.L.                                                |
|                   | 20,000 | 13 days        | 42%                                                  |
| TPD               | 20,000 | 5 min          | <D.L.                                                |
|                   | 20,000 | 13 days        | 48%                                                  |
| TTFD HCl          | 1,000  | 1 h            | <D.L.                                                |
|                   | 1,000  | 6 days         | 68%                                                  |
| TPD               | 1,000  | 1 h            | <D.L.                                                |
|                   | 1,000  | 6 days         | 61%                                                  |

*The time interval is the time between adding the thiamine to beer and analysis. †The values obtained for specimens analysed at 5 min and 1 h were below the detection limits (D.L.) for the method used.
DISCUSSION

The method presented here appears to be specific and sufficiently sensitive to measure TTFD and TPD concentrations in beer well below the proposed fortification level of 300 ng per ml of beer.

The thiamine alkyl disulphides studied here are so unstable in beer that using them for fortification purposes would seem unlikely to achieve its aim. That they are stable in phosphate buffer of an equivalent pH to beer suggests that it is the beer per se which is responsible for their chemical destruction. The reaction rate is temperature-dependent but is insensitive to light.

Some preliminary experiments into the cause of destruction of TTFD and TPD suggested that the disulphide bond, common to both molecules, may undergo chemical reduction. For example, conversion to free thiamine could result from the action of cysteine, glutathione, other SH compounds or ascorbic acid contained in beer. It is common practice for breweries to add antioxidants to beer to enhance its stability (e.g. ascorbic acid has been added in concentrations ranging between 5 and 15 µg/ml of beer; 12). We found that L-ascorbic acid at a concentration of 80 µg/ml decreased the concentration of TTFD and TPD from 500 ng/ml (in 0.005 M sodium dihydrogen orthophosphate buffer, pH 4.55) to 383 and 334 ng/ml respectively. Although this ascorbate concentration is higher than that which has been added to beer, there are other compounds present capable of reducing the thiamine alkyl disulphides. A further example is sulphur dioxide, a byproduct of the beer fermentation process which is present in the free form in a concentration of about 5 mg per litre. Also, there may be reaction catalysts present like metal ions which could contribute to the breakdown of the alkyl disulphides. Considerable conversion to free thiamine is indicated by Table 2: over 60% in 6 days for each thiamine alkyl disulphide at the lower concentration studied.

Even though beer fortified with thiamine alkyl disulphides contains free thiamine after a period of storage and may contain other fragments or derivatives which possess biological activity as vitamins, the rationale for adding either of the two analogues studied here would be lost unless the fragments or derivatives retained lipid-solubility. It is lipid-solubility that determines that thiamine alkyl disulphides are better absorbed than water-soluble thiamines. These other fragments or derivatives would need to represent a substantial proportion of the breakdown products of TTFD HCl and TPD; to retain lipid-solubility; to possess biological activity as vitamins; and themselves to remain stable over time. It would indeed be surprising if all these requirements were met.

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