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Simultaneous Identification and Determination of Tetramine in Marine Snails by Proton Nuclear Magnetic Resonance Spectroscopy

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Tetramine (tetramethylammonium ion), the toxin from marine gastropod molluscs of the genus *Neptunea*, was determined, qualitatively as well as quantitatively, by ^{1}H NMR spectroscopy. The method was compared to the currently available methods of determination and found superior due to the inherent identification of the molecular species determined.

Poisonous substances in the commericially available carnivorous gastropod molluscs of the genus *Neptunea* recurringly cause serious problems for consumers (Asano, 1952; Asano and Ito, 1959, 1960; Emmelin and Fänge, 1958; Fänge, 1957, 1958, 1960; Anthoni et al., 1989b). Within 30 min after ingestion, severe headache, dizziness, seasickness, nausea, vomiting, and visual disturbances may occur. Current reviews (Baslow, 1969; Hashimoto, 1976; Anthoni et al., 1989a) conclude the active principle to be the tetramethylammonium ion (tetramine) occurring mainly in the salivary glands. However, other substances present in small amounts may have a synergistic action (Asano and Ito, 1959).

Several analytical methods have been proposed to characterize tetramine and to quantitate the content in *Neptunea* species, including thin-layer chromatography (Asano and Ito, 1959), ion chromatography (Saitoh et al., 1983), and bioassay techniques based upon dose–lethal time curves for mice (Saitoh et al., 1983; Kungsuwan et al., 1986) or killifish (Kungsuwan et al., 1986). Estimated amounts in the salivary glands of *Neptunea arthritica* vary from 0.2 to 7.5 mg of tetramine/g of gland. In view of the importance of determining the threshold concentration of tetramine giving rise to poisonings, the agreement in the current available methods leaves much to be desired.

This paper describes a simple, direct method for rapid identification and semiquantitative determination of tetramine in extracts from *Neptunea antiqua* by ^{1}H NMR spectroscopy.

EXPERIMENTAL SECTION

Sample Preparation. The salivary glands (60 g) were carefully excised from *N. antiqua* (30 snails, 1 kg without shells) and freeze-dried to constant weight (19.5 g). Repeated extraction with methanol at room temperature, filtration, and evaporation of the solvent gave a semicrystalline solid (2.7 g). Partitioning between ether and water served to produce a defatted aqueous extract, which after lyophilization left colorless crystals (2.6 g). These crystals were used for the NMR determinations.

The residual animal tissue (940 g) was freeze-dried to constant weight (240 g). Successive extractions with ethyl acetate (three times) and methanol (three times) gave a methanol extract, which after evaporation left a solid. Treatment as described above left 13.8 g of defatted material suitable for NMR analysis.

Measurements. The spectra were recorded at ambient temperature on a JEOL FX-90Q Fourier transform NMR spectrometer or on a Bruker WM-250 FT spectrometer. The experiment was performed on a Bruker AM-500 instrument to determine the effects of the superior resolving power of the higher field. The

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region around the two CH_3 signals was resolved farther in the 500-MHz spectrum, displaying five additional small signals estimated to contribute by 2–3% to the integrated intensity of the two signals arising from betaine and tetramine. Samples were prepared in deuterium oxide, functioning also as internal solvent lock to stabilize field/frequency. Chemical shifts are reported relative to the DOH signal since absolute chemical shift values are not needed in this method. The spectra were recorded at various spin rates in order to eliminate any interference from spinning side bands of other components from the crude extract. Optimized conditions for NMR spectroscopic quantitative determinations have been discussed (Bhattacharyya and Bankawala, 1978). The experimental parameters used in this work are pulse width (16.5 μs (90° pulse)), sweep width (2403 Hz), acquisition time (13.63 s), receiver delay (0 s), and interferogram (64 K). The integrated peak intensities used in the calculations are the means of the five best recordings.

RESULTS AND DISCUSSION

A weighted portion (44.6 mg) of defatted material from *N. antiqua* salivary glands (see the Experimental Section) was dissolved in 500 μL of D_2O with D_2SO_4 (100 μL, 96–98% D_2SO_4 in D_2O, pH <0). The NMR spectrum was recorded for the region extending from the solvent signals toward higher field as shown in Figure 1A. The four main signals observed in this region arise from tetramine, betaine, and homarine (Anthoni et al., 1989b), as indicated in Figure 1A. As the chemical shifts are pH dependent, they were related to the HOD signal at δ 5.5. Analytically pure tetramethylammonium chloride (6.7 mg), dried under vacuum over P_2O_5 for 48 h, was added to the solution in the NMR tube and the spectrum recorded again to give the trace shown in Figure 1B. Since the signal appearing at highest field increases in intensity, it is assigned to tetramine. Consecutive additions of tetramethylammonium chloride demonstrated linearity of the calibration curve for a given set of NMR parameters. The amount present in the extract can now be calculated with any well-separated peak as reference.

The results were analyzed by means of the following basic equations:

$$\frac{\text{mg tetramine (sample)}}{\text{ref}} = \frac{\text{integral area (sample)}}{\text{integral area (ref)}}$$

$$\frac{\text{mg tetramine (sample + addn)}}{\text{ref}} = \frac{\text{integral area (sample + addn)}}{\text{integral area (ref)}}$$

The glands of *N. antiqua* collected in the North Sea for the measurements were found to contain 8.5%, 1.1%, or 0.4% tetramine as chloride relative to the crystalline-defatted material, the freeze-dried gland, and the wet gland, respectively. The remaining parts of *N. antiqua* were similarly found to contain 1.8%, 0.1%, and 0.03%, respectively. An average fresh snail without shell weighs ca. 33 g and contains ca. 15 mg of tetramine as chloride. Measuring the effect on the isolated *Buccinum radula* muscle, Fänge (1960) estimated the wet gland of *N. antiqua* from the Swedish west coast to contain 0.9% tetramine, noting that this figure might be a little too high. Taking into account that bioassay methods measure the physiological activity not only of tetramine but also of other (synergistic) components present in the gland extract, the agreement is considered satisfactory.

Since the signals due to betaine and homarine are easily recognized, these compounds may be individually quantitated in the same way by the standard addition method.

Inspection of Figure 1 reveals the presence of several small signals in close vicinity to the tetramine signal. As

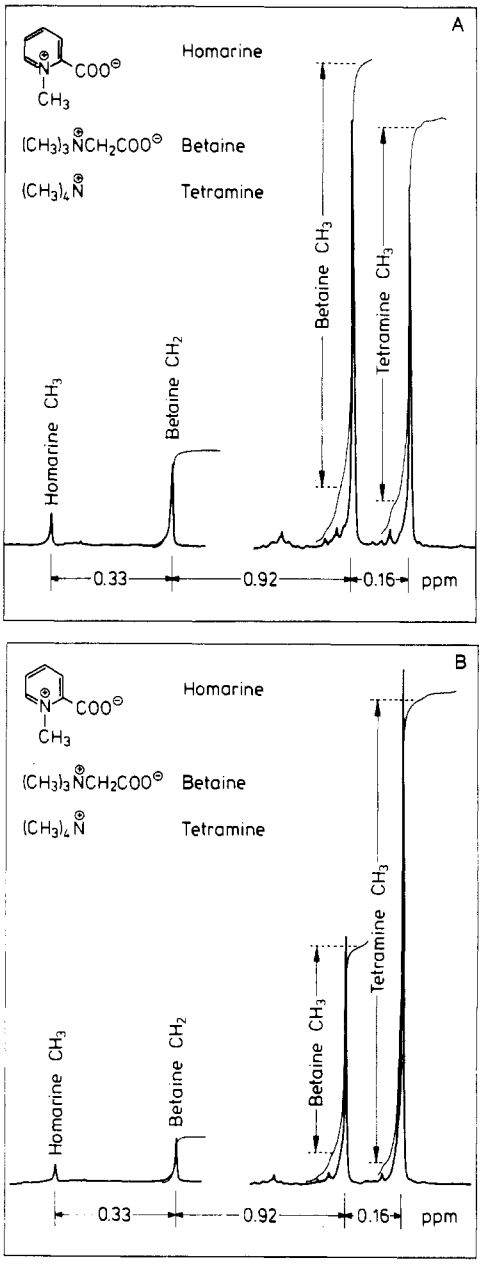

Figure 1. Proton magnetic resonance spectrum at 250 MHz of 44.6 mg of defatted aqueous extract from *N. antiqua* in D_2O/D_2SO_4 (pH <0) (A) and with 6.7 mg of added tetramethylammonium chloride (B).

described above, the improved resolution of the 500-MHz instrument may remove this difficulty. Alternatively, this complication was overcome by a change of pH, which leaves the tetramine signal almost unchanged but displaces signals due to compounds containing acidic or basic functions (e.g., betaine). The best results were obtained under strongly acidic conditions, e.g., pH <0. The integrated intensity of the tetramine methyl signal relative to the betaine methyl signal was determined as shown with dotted lines in Figure 1. The intensity of the betaine methylene signal, which is not disturbed by the presence of other peaks, may be used instead of the betaine methyl signal. Due to the ambiguity in the determination of the terminal points, an average of five integrations was used in all measurements. Nevertheless, an error estimated not to exceed 5% remains from this procedure.

The NMR analysis described above is much faster, less dependent on sample preparation, and less sensitive to the presence of other quaternary ammonium compounds than

the ion chromatographic (IC) method (Saitoh et al., 1983). One disadvantage associated with IC is the need to establish a calibration curve for tetramine relating peak height to concentration. This problem is circumvented in the NMR measurements where the increase in intensity of the tetramine signal on addition of a known amount allows calculation of the concentration. Another, more serious problem using IC is that the peak ascribed to tetramine is very broad and diminishes in intensity after the test solution is boiled. Since tetramine is inert to boiling, some other compound(s) must contribute to this peak. In this connection it may be noted that though choline was identified in the gland extract from *Neptunea intersculpta* and conditions were optimized to resolve this component, its presence could not be ascertained in the IC chromatogram used for estimating the tetramine content in this species. In contrast, the NMR method allows identification and quantitation of each component in the mixture by adding the authentic samples, one by one, and recording the intensity changes in each case. This procedure also has the additional advantage of internal check of integral accuracy.

The bioassay methods (Saitoh et al., 1983; Kungsuwan et al., 1986) are based upon the interperitoneal injection of defatted salivary gland extracts in mice and comparison of the dose–lethal curve to that measured for authentic tetramine. Obviously, use of this method involves the assumption that tetramine exerts the same poisonous effects alone or in admixture with other compounds present in the gland extracts. This is most unlikely in view of the fact (Saitoh et al., 1983) that boiling the test solution reduces the toxicity in mice. Rather, it can be concluded that the method is a valuable assay for the toxicity in mice of the mixture of compounds in the salivary gland extract. If the components are identified, the method is capable of revealing synergistic effects between tetramine and the remaining compounds.

In conclusion, the above results demonstrate that components in the extracts of *Neptunea* species can be rapidly demonstrated and quantitated by proton NMR analysis. The method offers many advantages of speed, specificity, and accuracy over previous analytical techniques. The techniques is applicable to extracts from natural sources where the components to be assayed are known and can be manipulated to give well-separated NMR signals.

Registry No. Tetramine, 51-92-3; betaine, 107-43-7; homarine, 445-30-7.

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