Ion-Pair High-Performance Liquid Chromatographic Determination of Strychnine Alkaloid in an Animal Tissue

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A method for the determination of strychnine alkaloid residues in animal tissues using ion-pair reversed-phase high-performance liquid chromatography is described. Following treatment with triethylamine, strychnine was extracted from whole-ground northern pocket gopher tissue with *n*-butyl chloride. The extraction solvent was evaporated and the sample reconstituted in the liquid chromatographic mobile phase containing the ion-pairing reagent heptanesulfonic acid. This method enables better than 80% strychnine recovery from tissue samples fortified with strychnine at concentrations ranging from 0.2 to 50 μg/g. Excellent precision is also obtained as demonstrated by standard deviations of replicate analyses of approximately 5%. The method limit of detection was 100 ng of strychnine/g of sample.

Keywords: *Strychnine; residue; animal tissue; ion-pair chromatography*

INTRODUCTION

Strychnine is one of the alkaloids derived from the seed of *Strychnos nux vomica*, a small tree native to India, northern Australia, and southeast Asia. Strychnine is an extremely toxic, fast-acting pesticide which acts on the central nervous system leading to convulsions and ultimately death via respiratory failure. Strychnine use dates to as early as 1640 when it was used in Europe as an agent for killing dogs, cats, and birds. More recently, it has been used for controlling vertebrates such as rats, skunks, coyotes, and pigeons. Currently its use is restricted to the below-ground application of strychnine-treated baits in burrow systems.

Administration of strychnine normally is achieved through use of a grain bait carrier. A variety of grains have been used for this purpose. The determination of strychnine residues in rodents exposed to strychnine baits is important in assessing the secondary hazard to nontarget species. This work was necessary to evaluate the secondary hazard associated with the below-ground application of strychnine baits for the control of the northern pocket gopher (*Thomomys talpoides*).

Chlorinated hydrocarbons are frequently used in the extraction of strychnine from various matrices. Samples are frequently made basic with hydroxide, carbonate, etc. followed by extraction with chloroform (Alliot et al., 1982; Crouch and Short, 1978; Hoogenboom and Rammell, 1985). Hunter and Creekmur (1984) described the use of methylene chloride as an extraction solvent. Ethyl acetate (Miller et al., 1982) and *n*-butyl chloride (Sharp, 1986) have also been used as extraction solvents. We report the use of triethylamine to ensure formation of strychnine as the free base followed by extraction with *n*-butyl chloride.

Several chromatographic techniques relating to the quantitative determination of strychnine at residue levels (low parts per million) have been published since 1980. Miller et al. (1982) described a gas chromatographic method for the determination of strychnine in alfalfa. A capillary gas chromatographic method for screening blood samples was also developed (Sharp, 1986). Methods have also been presented for the liquid chromatographic determination of strychnine in tissue extracts. A reversed-phase system was described by Egloff et al. (1982), while Hunter and Creekmur (1984) described a normal-phase chromatographic approach.

We desired a technique that would minimize the interferences associated with determining residues in animal tissue matrices. The reversed-phase separation of strychnine requires addition of a counterion due to the basic nature of the compound. Phosphate has been reported for this use (Crouch and Short, 1978). The U.S. Environmental Protection Agency made available methodology using heptanesulfonic acid as an ion-pairing reagent (Bontoyan, 1982). Octanesulfonic acid has also been reported as an ion-pairing reagent in the liquid chromatographic determination of strychnine (Hoogenboom and Rammell, 1985). We chose to use heptanesulfonic acid in the mobile phase as an ion-pairing reagent. This approach has allowed us to develop a simple technique for the quantitative determination of strychnine in animal tissue extracts.

EXPERIMENTAL SECTION

Apparatus. This work was performed using a Hewlett-Packard 1090M high-performance liquid chromatograph (HPLC) equipped with a diode array detector (Hewlett-Packard Co., Avondale, PA). A 5 μm octadecylsilane chromatographic column (4.6 mm × 25 cm; Alltech Associates, Deerfield, IL) was also used. The extraction was performed on a horizontal mechanical shaker with a 2-3/8 in. stroke (Eberbach, Ann Arbor, MI).

Reagents. Strychnine alkaloid was obtained from Aldrich Chemical Co. (Milwaukee, WI). A preformulated ion-pairing reagent of heptanesulfonic acid (IPC B7; Alltech Associates) was used in the preparation of the mobile phase which was prepared in two parts. The "aqueous" phase was prepared by the addition of 12.5 mL of the IPC B7 solution and 60 mL of methanol to 1 L of HPLC grade water. This resulted in a solution of about 2.4 mM heptanesulfonic acid and 5.6% (v/v) methanol in water. The addition of methanol to the mobile phase helped to prevent plugging of the analytical column when numerous injections of tissue sample extracts were made. After mixing by hand, the aqueous phase was filtered through a disposable 0.45 μm nylon membrane filter. Acetonitrile constituted the "organic" phase of the mobile phase. The

isocratic mobile phase consisted of 25% acetonitrile and 75% of the 2.4 mM heptanesulfonic acid in water containing 5.6% methanol.

The basic extraction reagent was prepared by combining 50 mL of triethylamine (TEA) and 15 mL of methanol. This mixture was then diluted with water to a final volume of 100 mL. This resulted in a solution that was 3.6 M TEA and 15% (v/v) methanol. Solutions of 1 N sodium carbonate and 2 N sodium hydroxide were also investigated for use as basic extraction reagents.

A concentrated strychnine standard solution was prepared in methanol at a concentration of 1.20 mg/mL. A 60 μg/mL intermediate strychnine solution was prepared in methanol from the concentrated solution. Calibration standards were prepared in mobile phase from the concentrated and intermediate standard solutions. Two sets of nine calibration standards were prepared ranging in concentration from 0.180 to 50.0 μg/mL. A working standard solution was also prepared in mobile phase from the concentrated standard solution at a concentration of 2.00 μg/mL for use in single-point calibrations.

Procedure. Whole ground pocket gopher tissue was prepared by removing the pelt and appendages of the animal and grinding the carcass in a household blender. Extraction of subsamples was performed by adding 1.5 mL of the basic extraction reagent to a 50-mL borosilicate glass screw-cap tube containing 2.5–3.5 g of accurately weighed tissue. The contents were mixed thoroughly with a vortex mixer and allowed to stand for 5–10 min to allow the tissue to be saturated with the extraction reagent. Solvent extraction was performed by the addition of 20 mL of *n*-butyl chloride, followed by vortex mixing and shaking on the horizontal mechanical shaker for 10 min at high speed. After centrifugation at 2500 rpm for 10 min, the *n*-butyl chloride was decanted into a clean 50-mL screw-cap tube. The extraction of the sample tissue with *n*-butyl chloride was repeated, and the two extracts were combined. The combined extracts were then evaporated to dryness under a gentle stream of nitrogen in a hot water bath at a temperature of approximately 75 °C. Samples were reconstituted with 3.00 mL of the mobile phase, followed by vigorous mixing and sonication for 15 min in a sonic water bath.

The working standard solution and sample extracts were filtered with disposable 0.45 μm Teflon filter units prior to injection into the HPLC. To assess response linearity, each of the 18 calibration standard solutions was filtered and injected in duplicate into the HPLC.

The chromatographic conditions were as follows: mobile phase flow rate, 1 mL/min; 25 μL injection volume; UV detection at 254 nm. An example of the chromatography obtained under these conditions is shown in Figure 1.

RESULTS AND DISCUSSION

Response Linearity. Eighteen strychnine calibration standard solutions (two sets of nine standards) ranging in concentration from 0.180 to 50.0 μg/mL were injected into the HPLC in duplicate. Linear regression analysis was performed on the data. Regression analysis produced the following statistics for peak response versus strychnine concentration: slope = 1.274, *y*-intercept = 0.252, and coefficient of determination = 0.9988. The *y*-intercept was not significantly different from zero ($p = 0.90$). Linear regression analysis of log[peak response] versus log[concentration] produced an equation of a line with slope = 1.0097, *y*-intercept = 0.106, and coefficient of determination = 0.9997.

Furthermore, the response factors obtained from all 18 calibration standard solutions were not statistically different. This was demonstrated by regression analysis of response factor versus concentration data. The null hypothesis that the slope was equal to zero could not be rejected ($p = 0.54$). These data demonstrate that a one:one linear relationship exists between chromatographic peak response and strychnine concentration.

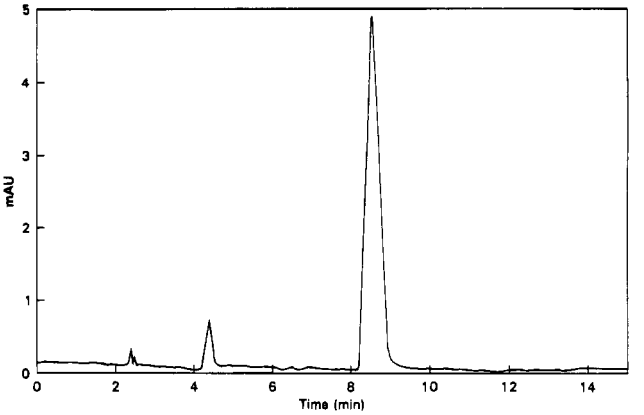

Figure 1. Chromatogram of a 5 μg/mL solution of strychnine in mobile phase. Chromatographic conditions: 75% 2.4 mM heptanesulfonic acid and 5.6% methanol in water/25% acetonitrile; flow, 1.0 mL/min; UV detection at 254 nm; 25 μL injection volume; 5 μm ODS, 4.6 × 250 mm analytical column.

Table 1. Fortification Level and Percent Recovery of Strychnine from Fortified Tissue Samples

	% recovery at fortification level of			
replicate	0.2 μg/g	0.5 μg/g	5.0 μg/g	50 μg/g
1	84.7	88.5	83.4	83.7
2	81.9	92.0	88.8	82.3
3	93.2	87.4	91.1	84.9
4	87.6	89.6	83.1	83.0
5	87.6	79.3	91.9	84.6
6	89.7	88.5	93.7	88.5
7	98.9	94.2	93.2	88.8
mean	89.1	88.5	89.3	85.1
SD	5.6	4.7	4.4	2.6
CV	6.3	5.3	4.9	3.0

Therefore, these data imply that a single-point calibration may be used for strychnine quantitation over the entire range. Clearly, since all response factors were not significantly different in this calibration, identical quantitative data should result from the use of any of the 18 calibration standards. The 2.00 μg/mL working standard solution was chosen to quantify extracts containing from 0.18 to 50 μg/mL strychnine.

Analyte Recovery and Repeatability. Replicate 3-g samples of control (containing no strychnine) whole ground pocket gopher tissue were fortified with strychnine at four concentrations. Syringes were used to deliver 10.0, 25.0, or 250 μL volumes of the intermediate strychnine standard to 3.0 g of tissue to prepare 0.20, 0.50, and 5.0 μg/g strychnine fortified samples. Similarly, 125 μL of the concentrated strychnine standard was used to prepare the 50 μg/g strychnine fortified samples. Fortified samples were mixed thoroughly with a vortex mixer. Seven fortified replicates and a control were prepared at each concentration and extracted immediately. The samples were extracted according to the described method using the TEA basic extraction reagent in the extraction step. Recovery data show consistent recovery of strychnine at each level and decreased variability at the higher concentration levels. Extraction of control samples demonstrated that no chromatographic interferences were present that may have been incorrectly identified as strychnine.

Strychnine recovery data from fortified tissue samples are presented in Table 1. Four fortification levels were investigated, and seven replicates were analyzed at each level. One-way analysis of variance of the data set confirmed that strychnine recovery did not vary over the range of fortification levels ($p = 0.28$).

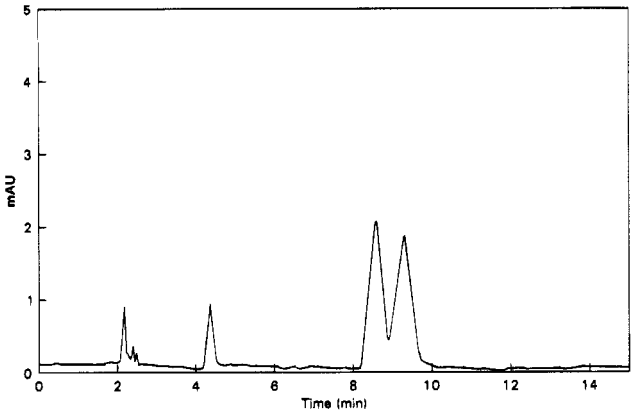

Figure 2. Chromatogram of tissue extract obtained using 1 N Na_2CO_3 as basic extraction reagent (chromatographic conditions as described for Figure 1).

Role of Basic Extraction Reagent. In addition to using the TEA solution reported here, 1 N sodium carbonate and 2 N sodium hydroxide were also investigated as basic extraction reagents. The addition of a base is necessary to produce the free base of strychnine, which is a dibasic alkaloid with reported pK_a values of 6.0 and 11.7 (*Merck Index*, 1983).

The extraction of strychnine from the matrix in this extraction scheme occurs in two phases. First, the basic solution saturates the matrix and solvates the free base as it is formed. The strychnine is then partitioned from the basic extraction reagent into the extraction solvent. Considering this mechanism, solubility of the alkaloid in the basic extraction reagent and the ability of the reagent to dehydrate both of strychnine's nitrogen atoms are limiting factors in the extraction process. The protonated form of strychnine may readily form a Lewis acid–base pair with any of a variety of organic acids present in the matrix. If the basic extraction reagent fails to dehydrate the nitrogen atoms in the initial step of the extraction, an ion pair will be partitioned into the organic phase rather than the free base. This may occur if the basic extraction reagent is not strong enough to promote formation of the free base or the compound is occluded in fats or lipids. Strychnine occluded in fats or lipids may be present either as the free base or as an ion pair. These fats or lipids may then be partitioned into the organic phase.

After elimination of the organic solvent through evaporation, reconstitution of the free base in the mobile phase containing heptanesulfonic acid is anticipated to form the desired ion pair. However, if a more thermodynamically stable ion pair was extracted from the matrix, it would be present rather than the expected strychnine/heptanesulfonic acid pair. This phenomenon was observed when Na_2CO_3 was used as the basic extraction reagent for the analysis of older tissue samples. Not only is the pH of 1 N carbonate not basic enough to promote complete dehydration of strychnine, but the aqueous nature of the basic extraction reagent precludes complete saturation of the matrix. Chromatographic analysis of tissue extracts obtained using the described procedure with 1 N Na_2CO_3 as the basic extraction reagent produced two distinct peak responses in some cases rather than the one expected for strychnine (Figure 2).

The appearance of the two peak responses was not systematic. Two peaks were observed unexpectedly after successfully using Na_2CO_3 as the basic extraction reagent for months in our laboratory. The "split peak" phenomenon was not observed in fresh samples but rather in quality control samples prepared from tissue that had been stored frozen for several months before fortification with strychnine. Therefore, sample degradation may be responsible for the appearance of the two peaks. Putrefaction of proteins present in the tissue can produce fatty acids by the Strickland reaction (Anglemier and Montgomery, 1976). The fatty acids produced as a result of tissue spoilage may then compete with heptanesulfonic acid in forming ion-pair products. The use of the TEA solution as the basic extraction reagent overcame this problem and consistently produced a single strychnine peak when stored tissue samples were fortified and analyzed.

Neither of the two peaks was present in tissue samples that had not been fortified with strychnine. The UV spectra obtained at the apex of the two peaks were identical and matched that of strychnine. Because ion pairing does not affect the conjugation that is responsible for strychnine's UV spectrum, the spectra were not expected to differ. However, the longer retention time of the second peak implies that the unknown ion-pair species is more hydrophobic than heptanesulfonic acid.

The use of 2 N NaOH as the basic extraction reagent also eliminated the unwanted ion-pair formation, as evidenced by only a single-peak response for strychnine extracted from fortified tissues that previously produced two peaks. However, the recovery data displayed much greater variability than that observed when using the TEA solution. Using 2 N NaOH as the basic extraction reagent, seven replicate extractions of 0.2 μg/g strychnine fortified tissue samples produced a mean recovery value comparable to that achieved when the TEA reagent was used, but the standard deviation of replicate analyses was 21%. This is likely a result of limited strychnine free base solubility in the aqueous basic extraction reagent. In addition to greater free base solubility in the TEA solution, the TEA solution more readily saturates the tissue matrix in comparison to aqueous bases.

Limit of Detection. The method limit of detection (LOD) was calculated from the strychnine responses obtained from the injection of seven extracts of tissue samples fortified with strychnine at 0.2 μg/g. The LOD was defined as the amount of strychnine required in the tissue to produce a chromatographic peak response corresponding to 3 times the peak-to-peak noise. The noise was measured in the controls at the retention time of strychnine. The LOD was determined to be 100 ng/g of strychnine in whole ground northern pocket gopher tissue.

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