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Note

Determination of 4-aminopyridine in horse plasma using gas–liquid chromatography

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4-Aminopyridine (4-AP) has been used clinically as an antagonist of the neuromuscular blocking action of non-depolarizing myoneural blocking agents[1–6], and for antagonism of ketamine-diazepam anesthesia[7], botulism[8], and myasthenia gravis[9]. More recently, 4-AP has been used alone and in combination with yohimbine hydrochloride for antagonism of xylazine sedation in dogs[10] and cattle[11], and xylazine-ketamine anesthesia in horses[12].

Existing gas chromatographic methods intended for determination of 4-AP in animal tissues[13] and in seeds[14] were reviewed and found to be inadequate for determination of 4-AP in body fluids. A recently developed gas chromatographic method for quantitating 4-AP in human plasma has several disadvantages, including a time-consuming purification step, low analytical recovery and an unexplained secondary peak in plasma 4-AP concentration between 20 and 60 min[15]. To study the pharmacokinetics of 4-AP in horse plasma, a new and sensitive gas–liquid chromatographic (GLC) method to quantitate plasma concentrations of 4-AP following intravenous (i.v.) injection was developed.

The method consists of extraction with ethyl acetate, derivatization with trifluoroacetic anhydride and quantitation by a nitrogen–phosphorous detector and proved to be rapid, sensitive and specific for 4-AP.

EXPERIMENTAL

Samples and chemicals

An analytical reference standard of 4-aminopyridine was purchased from Sigma (St. Louis, MO, U.S.A.). Trifluoroacetic anhydride was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was stored sealed at room temperature until ready for use. All other chemicals were reagent grade and solvents were commercially available pesticide analytical grade.

Blood samples were obtained from 6 horses by intravenous collection from the

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jugular veins. Samples were heparinized, centrifuged at 950 *g* for 10 min and the plasma was stored at −30°C until analysis was performed.

Chromatographic conditions

Gas chromatography was performed using a Tracor (Austin, TX, U.S.A.) Model 222 gas chromatograph equipped with a Tracor Model 702 nitrogen–phosphorous detector. A 2 m × 2 mm I.D. glass column packed with 10% FFAP on 80–100-mesh Chromosorb W HP (Supelco, Bellefonte, PA, U.S.A.) was operated at 180°C with a helium carrier flow of 35 ml/min. The inlet and detector temperatures were 250°C and 275°C, respectively.

Peak detection and integration were accomplished with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator set for optimum conditions using the peak height and external standard modes of operation.

Extraction

The plasma samples were brought to 37°C in a water bath. Control samples were fortified at 26, 52, 104, 208, and 832 ng/ml of 4-AP and carried through the extraction procedure along with the samples of interest. A 1.0-ml aliquot of each sample was transferred to a 15-ml culture tube and sealed with a PTFE-lined cap. After adjusting the pH to 13.0 (± 0.1) by addition of 0.1 ml of 5 *N* sodium hydroxide, 5.0 ml of ethyl acetate were added and the samples were mixed on a vortex for 30 sec. Samples were centrifuged to clarify the solvent layer and the ethyl acetate fraction was transferred to another 15-ml culture tube. The plasma was again extracted with 2.0 ml of ethyl acetate and combined extracts were placed in a water bath at 80°C and evaporated to dryness under a gentle stream of nitrogen.

The residue in each tube was redissolved in 1.0 ml of ethyl acetate to which 1.0 ml of trifluoroacetic anhydride was added. After heating the extracts for 15 min at 100°C in a sealed culture tube, they were again evaporated to dryness under a gentle stream of nitrogen. The residue was brought to a final volume of 0.20 ml with ethyl acetate and transferred to a 1.0-ml vial and sealed with a PTFE lined cap. Due to lack of interferences, no further sample cleanup was necessary.

Analysis

The chromatographic column was preconditioned for 24 h at 220°C. Several injections of derivatized control plasma extracts were made prior to sample analysis. This procedure resulted in sharper peaks and improved sensitivity for the trifluoroacetyl (TFA) derivative of 4-AP. Before analysis of each set of samples, the peak height response of four 1.0-μl injections of a standard (5.2 ng/μl) was averaged by the integrator in order to minimize minor changes in detector response. Quantitation was achieved by injection of 4.0 μl of derivatized extract and comparing peak height and retention time to that of the calibrated standard.

RESULTS AND DISCUSSION

The recovery at each fortification level is shown in Table I. Analysis of control samples indicated no matrix interference for the TFA derivative of 4-AP and a detection limit of 25 ng/ml as shown in Fig. 1. Detector response was shown to be

TABLE I

RECOVERY OF 4-AP FROM FORTIFIED CONTROL PLASMA

n (number of samples) = 5.

4-AP added (ng/ml)	*Recovery (±S.D.) (%)*
0	0.0 ± 0.0
26	99.2 ± 4.2
52	103.0 ± 3.4
104	89.0 ± 4.9
208	99.6 ± 4.1
832	99.6 ± 5.5

linear from 25 to 1000 ng/ml. A GC–mass spectrometry scan of the derivative contained a *m/e* 190 ion corresponding to TFA derivative of 4-AP and additional major ions at *m/e* 121 and 78.

In preliminary tests, the method of Evenhuis *et al.*[15], was evaluated but the technique manifested poor recoveries and interferences at the retention time of 4-AP. In addition, the extraction procedure produced a gelatinous emulsion which proved difficult to separate from the extraction solvent and required a subsequent alumina column cleanup procedure. After several unsuccessful attempts at modifying this procedure, a new method was developed.

Several gas chromatographic columns were tried, but overall performance was optimized with the use of 10% FFAP on 80–100-mesh Chromosorb W HP. Both OV-1 and OV-17 showed considerable tailing of the peak corresponding to deriva-

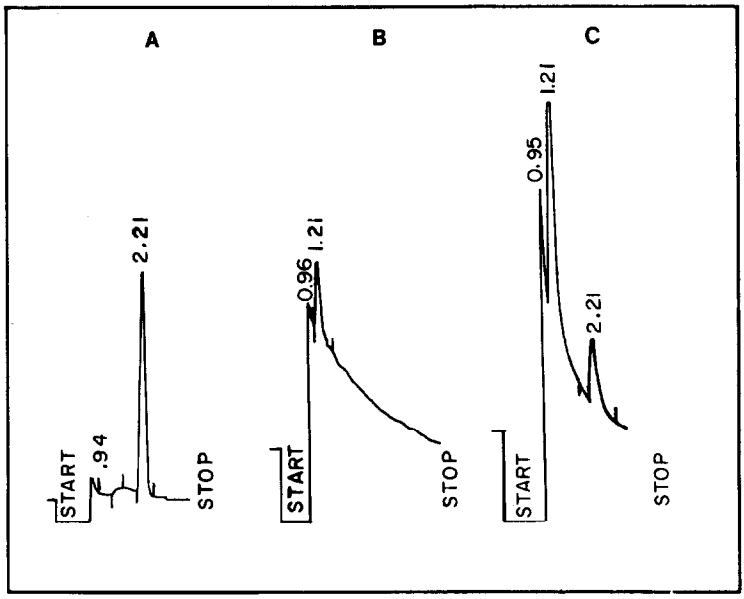

Fig. 1. Chromatograms of derivatized standard (A), control sample (B) and sample fortified with 26 ng/ml of 4-AP (C). Retention time for the 4-AP peak was 2.21 min.

tized 4-AP, especially at 1–3% liquid phase loadings. In addition, both OV-1 and OV-17 columns resulted in small shifts in retention time along with gradual deterioration.

Several chromatographic methods for determination of 4-AP in body fluids have been described and reviewed[16–18]. While these methods have proven to be accurate and reliable, we chose to develop a gas chromatographic method for analysis of 4-AP in plasma because of the selectivity and speed which the assay offered. Recoveries of fortified control plasma averaged 98% and this technique is useful for determining the amount of 4-AP in plasma following its administration in the horse and possibly would be useful for other mammalian species.

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