Journal of Chromatography, 230 (1982) 363—372
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1261

ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF 4-AMINOPYRIDINE IN SERUM

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(First received October 20th, 1981; revised manuscript received February 15th, 1982)

SUMMARY

An assay for the quantitative estimation of 4-aminopyridine in biological fluids has been developed using 2-aminopyridine as internal standard and ion-pair reversed-phase (C_{18}) high-performance liquid chromatography with detection at 263 nm. A 7.5% solution of acetonitrile in water containing tetrabutylammonium iodide and sodium heptanesulfonate buffered at pH 3.0 provided excellent separation of the analytes from each other and from an interfering peak that was occasionally observed in the outdated human sera used in these studies. Sensitivity, specificity, precision, accuracy and reproducibility all were judged sufficient for the routine use of this assay for pharmacokinetic and pharmacodynamic studies.

INTRODUCTION

The actions of 4-aminopyridine (4-AP) on the processes responsible for transmitter release at autonomic ganglia [1, 2], neuroeffector junctions [3—8], and the neuromuscular junctions in skeletal muscle [9—12] are well documented. Recent studies [13, 14] have shown that 4-AP exerts its action on evoked transmitter release by enhancing dramatically the amount of calcium ions which enter the nerve terminal at the time of depolarization, an event on which release of all neurotransmitters is dependent. This drug has found useful clinical applications in reversing the effects of non-depolarizing neuromuscular blocking agents [15] and in the treatment of myasthenia gravis [16], the Eaton—Lambert myasthenie syndrome [17], and botulism [18].

0378-4347/82/0000—0000/$02.75

Furthermore, it has been demonstrated that 4-AP antagonizes the neuromuscular blockade produced by most antibiotics [19, 20].

In view of the basic and clinical pharmacological importance of 4-AP, we have initiated studies designed to characterize pharmacokinetic and pharmacodynamic properties and the metabolic fate of this prototype drug. In this regard we have focussed our attention on the development of an adequately sensitive, specific and rigorous assay that will provide reliable quantitative estimations of this drug in biological fluids. Although gas–liquid chromatography (GLC) [21, 22] and high-performance liquid chromatography (HPLC) [23] methods for 4-AP have been reported, these methods in general do not provide the accuracy and sensitivity that we anticipate will be required for our work. More recently more sophisticated GLC [24, 25] and HPLC [26] procedures have appeared in the literature. Based on a comparison of the results reported in this paper, we submit that the ion-pair based HPLC procedure described here is the method of choice for the quantitative analysis of 4-AP in biological fluids.

EXPERIMENTAL

Chemicals and reagents

The 4-AP was purchased from Aldrich (Milwaukee, WI, U.S.A.) and was recrystallized from acetonitrile. The internal standard 2-aminopyridine (2-AP) was obtained from Aldrich (Gold Label 99+%) and was used without further purification. The acetonitrile was of chromatographic purity and was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The water used for all solutions and the mobile phase was deionized and purified by the Milli-Q-System (Millipore, Bedford, MA, U.S.A.). Analytical grade dichloromethane, methanol, trifluoroacetic acid, and tetrabutylammonium iodide were obtained from Aldrich. Sodium heptanesulfonate was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Sodium dihydrogen phosphate and phosphoric acid were Baker analyzed reagents (J.T. Baker, Phillipsburg, NJ, U.S.A.).

Stock solutions

Stock solutions of 4-AP (4.92 mg per 100 ml) and 2-AP (4.97 mg per 100 ml) were prepared in water. Storage of these solutions at 4°C did not result in detectable decomposition. All chromatographic analyses were performed by diluting stock solutions or dissolving residues of sample extracts in 10% acetonitrile.

Chromatography

The high-performance liquid chromatograph consisted of a Beckman (Fullerton, CA, U.S.A.) Model 100A solvent metering system, an Altex (Berkeley, CA, U.S.A.) Model 155 variable-wavelength detector (detection at 263 nm, 0.01 a.u.f.s.), an Altex Series 210 sample injection valve with a 50-μl loop, and a Soltec (Encino, CA, U.S.A.) Model 233 linear recorder (input 0.1 V and 1.0 V).

A prepacked Ultrasphere C_{18} reversed-phase column (250 × 4.6 mm I.D.; particle size 5 μm; Altex) was used. In order to avoid contamination of the

analytical column, a pre-column (50 × 3.2 mm I.D.) tapfilled with Vydac RP (particle size 30—44 μm; Altex) was placed between the injector and the analytical column. The mobile phase consisted of a mixture of 0.015 *M* sodium heptanesulfonate, 0.002 *M* tetrabutylammonium iodide, and 0.01 *M* phosphate buffer (pH 3.0; 0.01 *M* sodium dihydrogen phosphate + 0.01 *M* phosphoric acid) in acetonitrile—water (7.5:92.5, v/v). The mobile phase was degassed by ultrasonic vibration prior to the addition of the ion-pair reagents and then filtered through a 0.45-μm filter. The assays were performed at ambient temperature with a flow-rate of 1 ml/min, generating a pressure of about 160 bar.

Preparation of sample

To a PTFE-lined screw-cap culture tube (150 × 16 mm) were added 1.0 ml of serum and 2.485 μg of 2-AP dissolved in 0.1 ml water. The samples were made alkaline (pH 12) with 20 μl of 2 *N* sodium hydroxide solution and extracted with dichloromethane (10 ml) by gently rotating the tubes on a rotary disc for 30 min. After centrifugation for 5 min at 3000 *g*, the upper (aqueous) layer was carefully removed with the aid of a Pasteur pipet. The organic layer was pipetted into a PTFE-lined screw-cap conical centrifuge tube (134 × 17 mm) to which was added 0.1 ml of 2 *M* trifluoroacetic acid in methanol. After vortex-mixing, the solvent was evaporated to dryness under a stream of nitrogen at 40°C using an N-Evap apparatus (Organomation, Northborough, MA, U.S.A.). The residue was dissolved in 0.1 ml of 10% acetonitrile by ultrasonification for 5 min. After centrifugation (2000 *g*, 1 min) a 50-μl aliquot of the solution was subjected to the liquid chromatographic analysis.

Quantitation

The procedure was standardized by analyzing drug-free serum samples spiked with known amounts of 4-AP. Peak height ratios of 4-AP vs. the internal standard 2-AP were used to establish calibration curves.

RESULTS AND DISCUSSION

Chromatographic conditions

Since 4-AP is a strong base (pK_a of 9.2 [27]) and is water soluble, both reversed-phase and ion-pair based chromatographic procedures should provide the resolving power to develop a quantitative assay. Previous reports and our own experience revealed that reversed-phase chromatography using methanol—acetic acid [23] or acetonitrile—methanol—ammonium carbonate [26] as the mobile phase gave tailing peaks. The problem may be explained by ion-exchange or absorption reactions of ionized 4-AP with non-bonded silanols on the stationary phase which may behave as a weak ion-exchange resin. Increasing the pH of the mobile phase to suppress ionization would be likely to improve the reversed-phase chromatographic characteristics of 4-AP. Unfortunately, silica based reversed-phase columns deteriorate when exposed to solutions at pH values greater than 7.4 [28].

Ion-pair chromatography techniques have provided excellent separations of amines and quaternary ammonium compounds without column degrada-

tion [29—31]. Reversed-phase ion-pair chromatography also allows one to separate both ionized and un-ionized compounds in biological samples under the same chromatographic conditions. These properties suggested to us that a reversed-phase ion-pair chromatographic method might offer a promising approach to the quantitative analysis of 4-AP. The discussion which follows summarizes our efforts to optimize the parameters of an ion-pair based HPLC assay for 4-AP on a C_{18} reversed-phase column.

Optimization of pH

The pH was an important parameter to evaluate in the development of this assay since the pH of the mobile phase will dictate the concentration of the ionic form of an organic base such as 4-AP. The pH of the mobile phase can be safely varied from 2 to 7.4 for use with an alkyl-bonded silica column. At pH 7.4, 4-AP is essentially completely protonated. The internal standard 2-AP has a pK_a of 6.9 [27] and will be fully ionized at pH values below 5.0. Therefore, the influence of the pH on chromatographic behavior was investigated in the pH range from 2 to 6. The results are summarized in Fig. 1 and

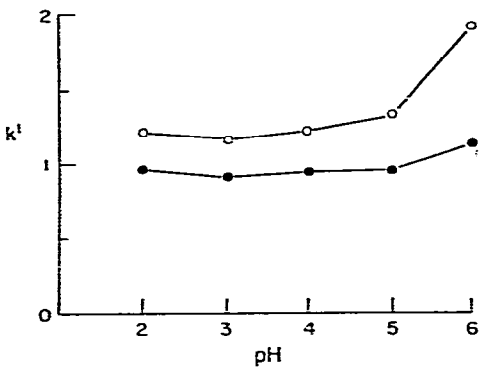

Fig. 1. Effect of pH on capacity ratio (k') of 4-AP (•——•) and 2-AP (○——○). Mobile phase (0.02 M sodium heptanesulfonate, 0.002 M tetrabutylammonium iodide, and 0.01 M phosphate buffer) in acetonitrile—water (10:90, v/v).

TABLE I

EFFECT OF pH ON CHROMATOGRAPHIC BEHAVIOR

pH	Peak asymmetry factor*		Plate count**	
	2-AP	4-AP	2-AP	4-AP
2	1.0	1.0	4800	9300
3	1.0	1.0	5300	8600
4	1.3	1.0	5600	7500
5	2.3	1.6	2900	4400
6	13.0	2.0	670	5300

*At 10% of peak height.

**$N = 5.54 \cdot \left(\frac{\text{retention time}}{W_{1/2}} \right)^2$.

Table I. At pH > 5, the peak asymmetry factor increased. Excellent resolution and column efficiency as indicated in the plate count values (Table I) were achieved at pH ≤ 4. The pH chosen for this assay was 3.0.

Hydrophobic cation

It is known that the addition of a hydrophobic cation such as tetramethylammonium has a pronounced effect on the peak shape in the separation of cationic compounds [29—32]. Presumably the cation competitively inhibits the partitioning of the sample cation in nonbonded silanols on the stationary phase. This results in a repulsion or a decreased availability of binding sites for sample cation interactions. In the present work, the addition of tetrabutylammonium iodide had a pronounced effect on the peak shape. Alterations in the concentration of tetrabutylammonium iodide also effected the retention volume with increasing concentrations causing decreases in the retention volume (Fig. 2). Similar results have been reported for the acetylcholinesterase inhibitors neostigmine and pyridostigmine [32]. The time to achieve equilibration of the column also could be regulated by changing the concentration of tetrabutylammonium iodide (Table II). The higher the concentration the shorter the time required to achieve initial equilibrium conditions. In view of these results 2 m*M* tetrabutylammonium iodide was employed in the analysis of 4-AP.

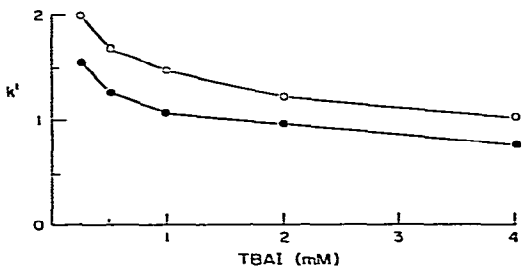

Fig. 2. Effect of tetrabutylammonium iodide (TBAI) concentration on capacity ratio (k') of 4-AP (●——●) and 2-AP (○——○). Mobile phase (0.01 *M* phosphate buffer, pH 3.0, and 0.02 *M* sodium heptanesulfonate) in acetonitrile—water (10:90, v/v).

TABLE II

EQUILIBRATION VOLUME VS. TETRABUTYLAMMONIUM IODIDE (TBAI) CONCENTRATION

Concentration TBAI (m*M*)	Column volumes to achieve equilibration
0.25	166 (690 ml)
0.5	90 (370 ml)
1.0	50 (210 ml)
2.0	30 (120 ml)
4.0	20 (85 ml)

Counter ion

The capacity ratio (k') can be varied by the choice of the counter ion. For protonated bases, alkylsulfonate (RSO_3^-) derivatives can be used. The following three sodium alkylsulfonates were examined: sodium butanesulfonate ($C_4H_9SO_3Na$), sodium pentanesulfonate ($C_5H_{11}SO_3Na$), and sodium heptanesulfonate ($C_7H_{15}SO_3Na$). Sodium butanesulfonate gave a rather low retention volume for 4-AP and 2-AP. An increase in the size of the counter ion increased the capacity ratio. Sodium heptanesulfonate gave the best peak characteristics and was selected for this assay.

The final regulation of the retention time was made by changing the concentration of the counter ion in the mobile phase. Fig. 3 shows the relationship between the counter-ion concentration and retention time. At higher concentrations ($\geqslant$ 35 mM) the k' vs. sodium heptanesulfonate concentration curve plateaued and the peak shapes deteriorated dramatically. Therefore 20 mM sodium heptanesulfonate proved to be satisfactory for the chromatographic separation of the pure analytes.

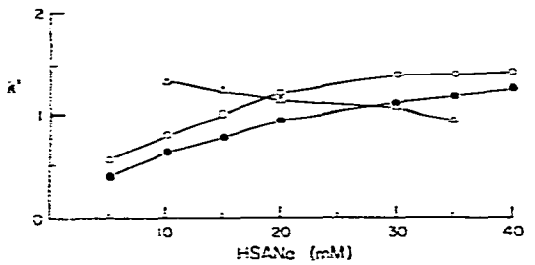

Fig. 3. Effect of sodium heptanesulfonate (HSANa) concentration on capacity ratio (k') of human serum interference (△———△). The corresponding curves for 4-AP (●———●) and 2-AP (○———○) are included. Mobile phase (0.01 M phosphate buffer, pH 3.0, and 0.002 M tetrabutylammonium iodide) in acetonitrile—water (10:90, v/v).

Organic solvent

The most commonly employed solvent combinations for reversed-phase ion-pair chromatography are water—methanol and water—acetonitrile. It is known that acetonitrile offers better column efficiencies due to its lower viscosity [28] and therefore we choose water—acetonitrile for our studies. The retention time could be regulated by changing the acetonitrile concentration. When the acetonitrile concentration exceeded 15%, a loss of resolution between 4-AP and 2-AP resulted. Moreover, 4-AP eluted near the solvent peak: 15% acetonitrile, $k' = 0.59$; 20% acetonitrile, $k' = 0.37$. For the pure analytes 5—10% acetonitrile provided satisfactory resolution although the retention volume was significantly increased at the lower concentration.

Detection

In the mobile solvent used in the assay, 4-AP and 2-AP give absorption maxima at 263 nm and 229 nm, respectively, values which are essentially identical to those published in the literature [33, 34]. Molar absorptivities are 16,900 for 4-AP and 9200 for 2-AP. In order to obtain greater sensitivity for 4-AP, the wavelength chosen for the assay was 263 nm. The lower limit

of detection for 4-AP was found to be 0.25 ng injected (signal-to-noise ratio $\geqslant 4$).

Chromatograms

Chromatography of blank serum extracts showed several peaks on the HPLC tracing other than those from the analytes. Occasionally human sera displayed a large peak (I, Fig. 4) that interfered with the analytes. The concentration of sodium heptanesulfonate in the mobile phase could be used to improve the separation of analytes from the interfering peak (Fig. 3). Use of 15 m*M* sodium heptanesulfonate provided the optimum separation. The final regulation of the retention volume was made by changing the concentration of acetonitrile. In order to optimize both resolution and elution time the amount of acetonitrile in the mobile phase was fixed at 7.5%. Fig. 4 shows chromatograms for blank sera and sera containing 98.4 ng/ml of 4-AP and 2.5 μg/ml of 2-AP. The retention times for 4-AP and 2-AP are 4.0 and 4.7 min, respectively.

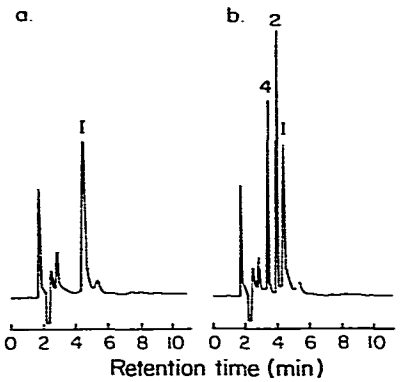

Fig. 4. HPLC tracings of extracts from (a) blank and (b) spiked human serum samples. Mobile phase (0.01 *M* phosphate buffer, pH 3.0, 0.015 *M* sodium heptanesulfonate, and 0.002 *M* tetrabutylammonium iodide) in acetonitrile—water (7.5:92.5, v/v). Peaks: (2) 2-AP, 2.5 μg/ml, (4) 4-AP, 98.4 ng/ml, (I) interference.

Recovery

The absolute analytical recoveries from human serum of 4-AP and internal standard were estimated by comparing the peak heights obtained from the injection of known quantities of the analytes with peak heights obtained from the injection of extracts of serum samples spiked with the analytes. Preliminary experiments revealed that analyses of spiked serum or aqueous samples afforded low recoveries and irreproducible results unless trifluoroacetic acid was added to the extraction solvent. These results may be explained by losses due to the volatility of the aminopyridines, especially 2-AP, during the evaporation. In attempting to overcome this problem, Uges and Bouma [26] added pentanol to prevent evaporation of aminopyridines. In our hands however, this procedure did not provide reproducible recoveries. Hengen and Hengen [35] reported that evaporation of the volatile alkaloid nicotine was prevented by the addition of hydrochloric acid to form the corresponding nonvolatile

hydrochloride salt. Since trifluoroacetic acid is volatile and easily removed by evaporation after salt formation, it was decided to add trifluoroactic acid to the extracts. The recovery of the aminopyridines was checked by adding different concentrations of trifluoroacetic acid to aqueous extract samples (196.8 ng 4-AP and 4.97 μg 2-AP per ml water). At a concentration of trifluoroacetic acid ⩽ 20 μmole recoveries improved but still were not quantitative. Addition of 200 μmole of trifluoroacetic acid to serum extracts increased extraction reproducibility and recovery to a satisfactory level (Table III).

TABLE III

ABSOLUTE ANALYTICAL RECOVERY (n = 8)

Substance	Concentration in serum (ng/ml)	x (%)	S.D. (%)	C.V. (%)
4-AP	492.0	67.81	1.55	2.29
	196.8	66.09	2.72	4.12
	98.4	67.78	1.87	2.76
	49.2	66.38	1.48	2.23
	24.6	67.47	2.21	3.28
	9.84	64.08	3.13	4.88
	4.92	64.34	3.53	5.49
	1.968	66.79	4.21	6.30
2-AP	2485.0	93.20	1.36	1.46

TABLE IV

ACCURACY

4-AP (ng/ml)	Peak height ratio Mean ± S.D. (n = 4)	C.V. (%)	Expected ratio	Relative error (%)
492.0	3.6523 ± 0.0951	2.60	3.6488	0.10
196.8	1.4499 ± 0.0272	1.88	1.4584	−0.58
98.4	0.7288 ± 0.0207	2.84	0.7283	0.07
49.2	0.3570 ± 0.0066	1.85	0.3632	1.88
24.6	0.1873 ± 0.0047	2.51	0.1807	3.65
9.84	0.0712 ± 0.0013	1.83	0.0712	0.00
4.92	0.0358 ± 0.0014	3.91	0.0347	3.17
1.968	0.0138 ± 0.0008	5.80	0.0128	7.81

Calibration curve

The calibration curve was obtained by plotting the peak height ratios of 4-AP to internal standard using drug-free pooled human sera containing 4-AP in the range of 2—492 ng/ml. A linear relationship was observed over this range ($y = 0.00742x - 0.00183$, $r = 1.0000$). The lowest quantifiable level of drug was 1 ng/ml serum (signal-to-noise ratio > 3).

Accuracy

In amounts of 2—492 ng 4-AP was added to 1.0-ml aliquots of pooled human sera and the samples were analyzed by the present method. The results presented in Table IV show that the estimated amounts of 4-AP added were in good agreement with the actual amounts.

Specificity

In order to assess the potential application of this assay to problems involving multiple-drug regimens, sera containing pancuronium, pyridostigmine, and neostigmine, each at a concentration of 1 μg/ml serum, were examined. Pancuronium could not be detected under the conditions of this analysis. Retention times for pyridostigmine and neostigmine were 3.7 and 6.2 min, respectively. Furthermore, these quaternary ammonium compounds were not extracted efficiently and did not interfere with the analysis of 4-AP and 2-AP.

Reproducibility

Within-day precision of the assay was determined by performing ten replicate analyses on aliquots of a drug-free serum sample to which 4-AP was added to give a concentration of 98.4 ng/ml. The coefficient of variation was 2.84%. Day-to-day precision was estimated for the same test material over a period of ten working days. The coefficient of variation was 2.50%. The results demonstrate excellent reproducibility.

The assay method developed allows the simple, sensitive, rapid, and selective determination of 4-AP in human serum with good accuracy and precision. The method will be used to perform pharmacokinetic and metabolic studies of 4-AP.

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