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Short communication

Optimization of the determination of 4-aminopyridine in human serum and urine by column liquid chromatography

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Abstract

Two convenient reversed-phase column liquid chromatographic procedures are described for the determination of 4-aminopyridine in human serum and urine. A 0.5-ml aliquot of serum after the addition of a 0.5-ml solution of 4-(aminomethyl)pyridine in 0.1 *M* Na_2HPO_4 as the internal standard is passed through a 1-ml BondElut C_{18} silica extraction column. The column is selectively washed to remove acidic, neutral and weakly basic compounds. The desired compounds are eluted with a 0.3-ml aliquot of 35% perchloric acid–methanol (1:100, v/v). A 10-μl aliquot of the eluate is injected onto a 150 × 4.6 mm I.D. column packed with 5-μm C_{18} silica particles that is eluted at ambient temperature with a mobile phase containing octanesulfonic acid as the ion-pairing agent. The peaks are monitored at 263 nm. A 0.25-ml aliquot of urine or 0.5 ml of serum is mixed with N-propionylprocainamide as the internal standard and subjected to benzoylation by Schotten Baumann reaction. The reaction mixture is adjusted to pH 5.5–6 and extracted with a BondElut C_{18} extraction column. An aliquot of the eluate is chromatographed at ambient temperature with a mobile phase containing tetramethylammonium perchlorate. The peaks are monitored at 278 nm.

Keywords: 4-Aminopyridine

1. Introduction

4-Aminopyridine (4-AP), a potassium channel blocking agent, is being investigated for a variety of neurological disorders. It has been suggested that the concentration of 4-AP in biological fluids should be monitored in clinical trials involving the use of 4-AP because of its high toxicity [1,2]. At present, column liquid chromatography (LC), particularly in the reversed-phase mode, is the most popular and practical technique for the determination of drugs in biological fluids. A number of LC procedures [3–7] have been described for the determination of 4-AP in serum. Uges and Bouma's procedure [3] has also been applied for the analysis of urine.

4-Aminopyridine is a polar molecule of small size. Extensive purification of the biological sample is required to separate 4-AP from endogenous compounds. The procedure of simple protein precipitation suggested by Lamiable and Millart [5] is not applicable to human serum where a detection limit of 5 ng/ml or lower is required or, even to human urine where the detection limit may be as high as 50 ng/ml. Horst et al. [6] have described a solid-phase

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extraction (SPE) for the determination of 4-AP in serum. This procedure is based on a SPE procedure described by Leslie and Bever [8] for the determination of 3,4-diaminopyridine. However, in a recent report Fossler et al. [7] used only liquid–liquid extraction for the determination of 4-AP. The previously described SPE procedure [6] gave poor and inconsistent recovery of 4-AP in our laboratory. It was the objective of this investigation to select SPE conditions for optimal recovery of 4-AP from biological samples and to understand the possible cause of our discrepant results from those of Horst et al. [6].

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Deionized water was distilled in an all-glass still. N-Propionylprocainamide and 4-AP were obtained from Sigma (St. Louis, MO, USA) and 4-(aminomethyl)pyridine was obtained from Aldrich (Milwaukee, WI, USA). Stock solutions of these compounds at a concentration of 1 mg/ml were prepared in methanol. The solutions were stored at −20°C. A serum standard of 4-AP of 800 ng/ml was prepared in human serum which was then serially diluted to make 6 standards. A urine standard of 4-AP at 8 μg/ml was prepared which was then serially diluted to obtain 6 standards.

2.1.1. Extraction

The required number of 1-ml BondElut C_{18} extraction columns (Varian, Harbor City, CA, USA) was placed on a VacElut system. The columns were washed once (one column volume) with 1 *M* HCl, twice with methanol and once with water, each time aspirating the liquid completely with suction. A 0.5-ml aliquot of the working internal standard solution prepared by mixing 50 μl stock solution of 4-(aminomethyl)pyridine with 10 ml of 0.1 *M* Na_2HPO_4, was placed in each column and then 0.5 ml of serum sample or 0.25 ml of urine sample was applied to each column. Mild suction was applied so that the liquid passed through the column at a flow-rate of approximately 1 ml/min. The columns were washed twice with water and twice with 0.4 ml aliquots of acetonitrile making sure that each column was drained completely after every wash. The tips of the columns were wiped with tissue and placed on 16 × 100 mm glass tubes containing 1.5 ml plastic cups. An aliquot of 0.3 ml of methanol containing 1 ml/100 ml of 35% perchloric acid, was applied to each column. The liquid was allowed to pass through the column bed by gravity and finally drained completely by centrifugation for 20 s at 800 *g*. A 10-μl aliquot of serum extract (or a 5-μl aliquot of urine extract) was injected onto the chromatographic system.

2.1.2. Benzoylation and extraction

Aliquots of samples of urine (0.25 ml) or serum (0.5 ml) and aliquots of 100 μl (for urine) or 50 μl of a 20 μg/ml solution of N-propionylprocainamide in 0.1 *M* HCl were placed in 12 × 75 mm disposable glass tubes. To each tube were added 0.25 ml of 4 *M* NaOH and 25 μl of a freshly prepared solution of benzoyl chloride in acetonitrile (1:1, v/v). The tubes were vortex-mixed vigorously and allowed to stand at room temperature for 15 min with occasional vortexing. Water (0.4 ml) was added to each tube and the pH of the mixture was adjusted to 5.5–6 with 4 *M* acetic acid. The tubes were vortex-mixed and centrifuged for 2 min at 1200 *g*. The supernatant was applied to a washed extraction column and the column was washed and eluted as described above.

2.2. Chromatography

A modular chromatographic system consisting of a Model LC-6A pump, a Model-10A absorbance detector, a Model Sil-9A autosampler and a Model CR501 integrator plotter (all from Shimadzu, Columbia, MD, USA) was used. A 150 × 4.6 mm I.D. Ultrasphere ODS reversed-phase column packed with 5-μm C_{18} silica particles (Beckman Instruments, San Ramon, CA, USA) protected by a 15 × 3.2 mm I.D. RP-18 guard cartridge packed with 7-μm silica particles (Applied Biosystems, San Jose, CA, USA) was used as the analytical column. Mobile phase A consisting of 2 m*M* octanesulfonic acid in 10 m*M* NaH_2PO_4–acetonitrile (90:10, v/v), pH 4.2, containing 10% H_3PO_4 was pumped at a

flow-rate of 1 ml/min. In this system the peaks were monitored at 263 nm.

Mobile phase B, consisting of 0.1% tetramethylammonium perchlorate–acetonitrile (84:16, v/v), pH 4.2, containing 10% perchloric acid was pumped at a flow-rate of 1.2 ml/min. In this system the peaks were monitored at 278 nm. Chromatography in both cases was performed at ambient temperature.

3. Results and discussion

3.1. Detection

4-AP does not show any native fluorescence and is not strongly electroactive. However, it shows a strong UV absorbance. At acidic pH, the absorption maximum is at 263 nm with a molar absorptivity of 13 900, whereas at neutral pH, the absorption maximum is at 246 nm with a molar absorpitivity of 10 900 (Sadtler UV spectrum # 20871). The pH of the mobile phase (pH 4.2) is acidic enough [4] to give an absorption maximum at 263 nm and therefore this wavelength was selected to monitor 4-AP peaks. Shinohara et al. [4] have used 263 nm at pH 3, Lamiable and Millart [5] used 260 nm at pH 7.4 and Fossler et al. [7] have used the absorbance at 260 nm, at pH 7, to monitor 4-AP. On the other hand, Uges and Bouma [3] and Horst et al. [6] used 246 nm at alkaline pH to monitor 4-AP. Monitoring of non-ionized 4-AP at 246 nm is only 78% as sensitive as monitoring of 4-AP as a cation at 263 nm.

After benzoylation, 4-AP shows an absorption maximum at 278 nm in the cationic form. There is about a 20% decrease in the peak height of benzoyl-4-AP when monitored at 263 nm compared to when it is monitored at 278 nm.

3.2. Choice of internal standard

Lamiable and Millart [5] did not use any internal standard. Uges and Bouma [3] and Horst et al. [6] used 3,4-diaminopyridine, Shinohara et al. [4] used 2-aminopyridine (2-AP) and Fossler et al. [7] have used propranolol as the internal standard for the determination of 4-AP. We tried a number of isomers and homologues of 4-AP as the internal standard for the determination of 4-AP. In the described chromatographic system, 3-aminopyridine (3-AP) and 3,4-diaminopyridine elute close to 4-AP. The separation of these compounds from 4-AP could not be satisfactorily improved by modifying the mobile phase or by changing to a C_8 column. However, there was a good separation of 2-AP from 4-AP and 2-AP behaved similarly to 4-AP during liquid–liquid extraction with dichloromethane. However, 2-AP behaved quite differently from 4-AP under the optimal conditions for SPE of 4-AP. Then 4-(aminomethyl)pyridine was selected as the internal standard for the determination of 4-AP as both of these compounds behaved similarly during SPE. 4-(Aminomethyl)pyridine has an absorption maximum at 257 nm at acidic pH with a molar absorpitivity of only 3870 (Sadtler UV spectrum # 6135). As a result, a relatively large amount of 4-(aminomethyl)pyridine was used as the internal standard.

Despite our extensive efforts, a suitable pyridine which behaved similarly to 4-AP during benzoylation could not be found. 4-AP is unique in that it exclusively forms a monobenzoyl derivative under Schotten Baumann conditions (Beilstein **22**, 433), while other substituted pyridine amines including 2-AP, 3-AP and 4-(aminomethyl)pyridine form mixtures of mono- and di-benzoyl derivatives resulting in poor precision and possible interferences from the late-eluting peaks of dibenzoyl derivatives from the previous injections. 4-Amino-3-methylpyridine, which is expected to form only a monobenzoyl derivative and which was used by Watson [9] as the internal standard for the gas chromatographic determination of 4-AP is no longer available. Finally, we selected N-propionylprocainamide, which does not undergo benzoylation, as the internal standard. However, it is a suitable compound as an internal standard from every other aspect.

3.3. Extraction

Liquid–liquid extraction remains the most popular technique for the determination of 4-AP. Uges and Bouma [3] and Shinohara et al. [4] have used dichloromethane and recently Fossler et al. [7] used ethyl acetate for the extraction of 4-AP from serum samples. In all these procedures, the extraction is

only a single step procedure as the organic extract obtained at alkaline pH is evaporated and the residue reconstituted in a small volume for chromatography. Initially, we applied the technique of Shinohara et al. [4] for the extraction of serum and urine. There were many extraneous peaks and late-eluting peaks from previous injections. We modified Shinohara's procedure by drying the organic extract with anhydrous sodium sulfate and by back-extracting the basic compounds from 7 ml of the organic extract with 0.4 ml of 0.1 *M* phosphoric acid, by mixing on a rotary mixer for 10 min. The desired compounds were efficiently extracted into phosphoric acid and the resulting chromatogram was cleaner than that obtained after evaporation of the organic extract. However, urine extracts were still quite dirty and 4-AP could not be quantitated.

3.3.1. Derivatization

We considered derivatization of 4-AP to improve its separation from endogenous compounds present in biological fluids. It was thought that derivatization of the amino group of 4-AP will separate it from other primary and secondary amines because of the presence of a second tertiary amino group of the pyridine ring and because the increase in the size of 4-AP as a result of derivatization will separate 4-AP from other polar tertiary amines which do not undergo derivatization. Initially, preparation of 2,4-dinitrophenyl derivatives was attempted. However, there was a considerable reduction in the sensitivity of 4-AP determination after derivatization and liquid–liquid extraction. Benzoylation proved easy and efficient. Liquid–liquid extraction procedure remained the same except for the addition of benzoyl chloride and additional sodium hydroxide prior to extraction. The procedure was validated for linearity and reproducibility [10] and successfully applied for the analysis of clinical samples [11].

3.3.2. Solid-phase extraction

The procedure of Horst et al. [6] gave poor recovery of 4-AP with the use of BondElut C_{18} extraction columns. Extraction of basic drugs with the use of reversed-phase silica extraction columns is a complex process and requires optimization of different steps. To optimize the pH of the matrix, a serum standard of 1 μg/ml was mixed with an equal volume of 0.1 *M* phosphate buffers of pH 5.5, 6.5, 7, 7.5, 8.5, and carbonate buffer of 9.5 and 10.5 and extracted with the described SPE procedure. The best recovery of 4-AP (82–94%) was obtained at pH 8.5. The retention of 4-AP on an extraction column was poor when the pH of the matrix was below 7 and there were extensive losses of 4-AP during acetonitrile washes when the pH of the matrix was between 7–8. Horst et al. [6] used only the physiological pH (7.4) of the sample and did not use any additional buffer for SPE of 4-AP.

To understand the discrepancy of our observation of the recovery of 4-AP by SPE from that reported by Horst et al. [6], 1 ml (100 mg) C_{18} extraction columns marketed by 3 additional vendors were evaluated. All of these columns also gave best results when the pH of the matrix was adjusted to ca 8.5. However, columns marketed by Baker (used by Horst et al. [6]) and by Alltech (Deerfield, IL, USA) also gave good recoveries at the physiological pH. Horst et al. [6] evaporated the extract to ca. 20 μl with a stream of nitrogen for adequate sensitivity. However, in the present procedure aliquots of the extract are chromatographed directly to avoid the possible loss of 4-AP during evaporation.

Fig. 1A shows the chromatogram of a drug-free serum sample. The chromatogram is quite clean. Fig.

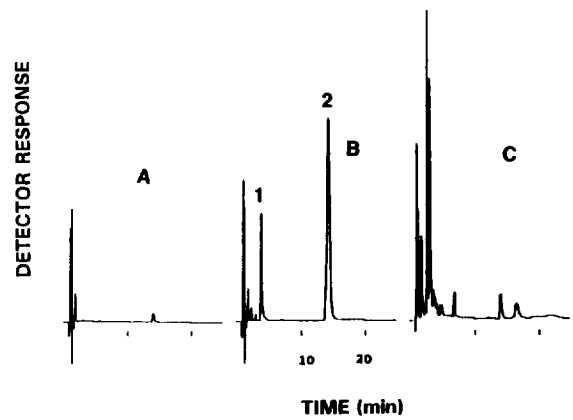

Fig. 1. Chromatograms of (A) a 10-μl injection of an extract of drug-free serum; (B) a 10-μl injection of an extract of serum from a patient after infusion of 4-AP; (C) a 5-μl injection of an extract of drug-free urine. Peaks: 1=4-aminopyridine and 2=4-(aminomethyl)pyridine (internal standard). Detector sensitivity, 0.01 AUFS. Integrator attenuation, 2; chart speed, 2 mm/min. Mobile phase A was used in all cases.

1C shows the chromatogram of a drug-free urine sample. This chromatogram shows many extraneous peaks even though the detection sensitivity is only 25% of that of plasma extract (0.25 ml of urine sample compared to 0.5 ml of serum sample and 5 μl of urine extract injected compared to a 10-μl injection of serum extract). There was no significant reduction in the extraneous peaks when the extraction column was washed for the third time with another aliquot (0.4 ml) of acetonitrile.

3.3.3. Solid-phase extraction of benzoyl derivative

A very different pH of the matrix after derivatization is required for SPE of the derivatized 4-AP. At pH > 6.5, N-benzoyl-4-AP is extensively lost during SPE and for good recovery a pH of < 6 is required. The peak height of benzoylated 4-AP monitored at 278 nm, when an aqueous 4-AP standard was subjected to benzoylation and SPE, was similar to the peak height of 4-AP monitored at 263 nm when the same standard was directly extracted by SPE. The recovery of benzoylated 4-AP from serum standards by SPE is about 10% less than that of urine standards of the same concentration. There is a voluminous protein precipitate when the benzoylation reaction mixture of a serum sample is adjusted to pH 5.5–6 for SPE. It is likely that the reduction in the recovery of 4-AP from serum sample is due to protein precipitation. Fig. 2A shows a chromatogram of drug free serum and Fig. 2C shows a chromatogram of a drug free urine sample. Here, both the chromatograms are clean.

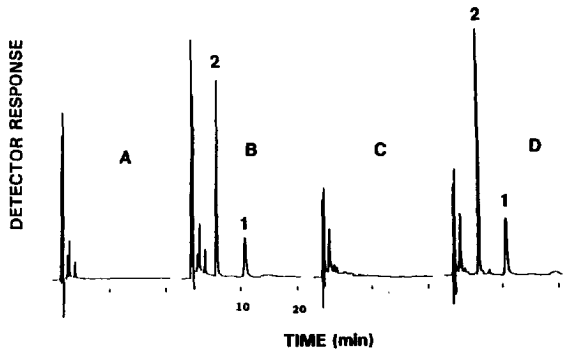

Fig. 2. Chromatograms of (A) a 10-μl injection of an extract of drug-free serum; (B) a 10-μl injection of an extract of serum from a patient after infusion of 4-AP; (C) a 5-μl injection of an extract of drug-free urine; (D) a 5-μl injection of an extract of urine collected 1 h after the start of 4-AP infusion. Peaks: 1=N-benzoyl-4-aminopyridine and 2=N-propionylprocainamide (internal standard). Detector sensitivity, 0.01 AUFS. Integrator attenuation, 3, chart speed, 2 mm/min. Mobile phase B was used in all cases.

3.4. Chromatography

Uges and Bouma [3] and Horst et al. [6] used C_{18} silica columns with a mobile phase of pH > 8. At this pH 4-AP is very firmly bound to the column and a mobile-phase of about 95% organic modifier is required. To prolong the life of a silica column in an alkaline environment, Horst et al. [6] saturated the mobile phase with silica. Lamiable and Millart [5] used a C_{18} silica column with a mobile phase of pH 7.4 requiring 60% acetonitrile. Fossler et al. [7] used a cyano column with a mobile phase of pH 7 containing 1-decanesulfonic acid as the ion-pairing agent requiring 50% acetonitrile. On the other hand, Shinohara et al. [4] used a C_{18} silica column with a mobile phase of pH 3 containing 1-heptanesulfonic acid as the ion-pairing agent requiring only 7.5% acetonitrile. This mobile phase also included 2 m*M* tetrabutylammonium iodide as an amine modifier to improve the peak shape.

It was the object of the present study to select the chromatographic conditions requiring the least amount of chemicals for economic and environmental reasons. The described mobile phase A uses 1-octanesulfonic acid as the ion-pair reagent at a concentration of only 2 m*M* as compared to 15 m*M* concentration of the ion pair reagent used by Shinohara et al. [4], 10% acetonitrile and no amine modifier. As seeen in Fig. 1B, the peaks of 4-AP and the internal standard are quite sharp and are well separated from each other and from the solvent peaks. Endogenous urine peaks could not be adequately separated from the desired peaks by increasing the amount of 1-octanesulfonic acid up to 5 m*M* and using a mixture of acetonitrile and methanol as the organic modifier.

N-Benzoyl-4-AP has adequate retention on reversed-phase columns and does not require a large ion-pair reagent. Tetramethylammonium perchlorate, used in the mobile phase B, acts as an amine modifier and a mild ion pair reagent and does not require any other buffer. As seen in Fig. 2B and 2D,

the peaks of 4-AP and the internal standards are quite sharp and well separated from the solvent peaks.

3.5. Method validation

3.5.1. Unchanged drug

The relation between the ratios of peak areas of 4-AP/internal standard was linear for the range tested for serum standards (25–800 ng/ml) and the curve passes through the origin ($y = -0.005 + 0.002x$, $r = 1$). Analysis of serum spiked with a high concentration (400 ng/ml) of 4-AP showed a within-batch C.V. of 1.9% (mean = 412 ng/ml, $n = 6$) and of serum spiked with a low concentration (50 ng/ml) of 4-AP showed a within-batch C.V. of 4.7% (mean = 46 ng/ml, $n = 6$). The percentage bias from the spiked concentration of +3% for the high concentration and −8% for the low concentration shows satisfactory accuracy of the procedure. A between-batch C.V. of 3.9% (mean = 419 ng/ml, $n = 8$) for the high control and a C.V. of 6.6% (mean = 47 ng/ml, $n = 8$) for the low control was observed.

3.5.2. Derivatized drug

The relation between the ratios of peak areas of 4-AP/internal standard for the urine standards (0.25–8 μg/ml) was linear and the curve passed through the origin ($y = -0.029 + 0.115x$, $r = 1$). The limit of quantitation of 20 ng/ml can be lowered to 5 ng/ml by injecting 20 μl of the extract. Within-batch C.V. of low-urine control spiked with 0.15 μg/ml of 4-AP was 2.4% (mean = 0.15 μg/ml, $n = 8$) and a high-urine control spiked with 8 μg/ml of 4-AP showed a C.V. of 3.9%. Corresponding between-batch C.V.'s were 6.4 and 5.3%. Fig. 2D shows a chromatogram of an extract of a urine sample collected from a patient 1 h after the start of 4-AP infusion. The concentration of 4-AP is 0.7 μg/ml.

The benzoylation technique also gave a linear relationship between serum 4-AP concentrations (25–800 ng/ml) and the ratios of peak areas of 4-AP/internal standard and the curve passed through the origin ($y = -0.001 + 0.002x$, $r=1$). Reproducibility data of serum controls spiked with 50 and 400 ng/ml is similar to that of urine controls. Good extraction recovery and high detector response allow the quantitation of 4-AP to levels as low as 5 ng/ml when only 10 μl aliquots of the extract are injected. The limit of quantitation can be lowered to 3 ng/ml by injecting 20 μl of the extract. There is no distortion of peaks when up to 30 μl of the extract are injected. Fig. 1B and Fig. 2B show the chromatograms of serum extracts obtained by the two procedures of a blood sample collected from a patient at the end of a continuous infusion of 4-AP over a period of 2 h. The concentration of 4-AP in Fig. 1A is 152 ng/ml and in Fig. 2B the concentration of 4-AP is 157 ng/ml.

3.6. Selectivity

Neutral and acidic compounds are removed during acetonitrile washes in both of the SPE procedures. In the benzoylation procedure monofunctional primary and secondary amines are also expected to be removed due to their conversion to neutral compounds but may be co-extracted in the non-derivatization procedure. Tertiary amines are expected to be co-extracted in both of the procedures and their separation from the desired peaks depends upon the chromatographic system. Most of the commonly prescribed drugs, e.g. benzodiazepines and antidepressants have strong retention in both the systems and stay on the column. However, small molecules, e.g. 3-AP or 2,4-diaminopyridine, elute close to 4-AP in the non-derivatization procedure. 3-AP and 3,4-diaminopyridine are converted to dibenzoyl derivatives and stay on the column. Thus the benzoylation procedure offers much better selectivity than the non-derivatization procedure. However, in most situations a direct extraction procedure is adequate for the analysis of serum samples as other pyridines are not administered with clinical trials using 4-AP.

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