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Short Communication

Determination of 4-aminopyridine in serum by solid-phase extraction and high-performance liquid chromatography

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ABSTRACT

An assay for the determination of 4-aminopyridine in serum has been developed using 3,4-diaminopyridine as internal standard and reversed-phase high-performance liquid chromatography with detection at 244 nm. A mobile phase of acetonitrile–methanol–ethanol–1% ammonium carbonate (75:10:10:5) provided excellent separation of both compounds. Samples were extracted on solid-phase columns. The linearity, precision, recovery and the limit of detection were all sufficient for the routine use of this assay in clinical studies of patients treated with 4-aminopyridine.

INTRODUCTION

4-Aminopyridine (4-AP), a potassium channel blocker, is able to restore a nerve conduction block in demyelinated nerve fibres by prolonging the duration of the action potential [1]. In a few small clinical trials in patients with multiple sclerosis (MS), transient improvements in visual, oculomotor and motor functions have been demonstrated after intravenous or oral administration of 4-AP [2–4].

About two years ago a randomized double-blind placebo-controlled cross-over study was started at our institution to evaluate the effect of 4-AP on the clinical signs in MS. Seventy patients with MS were included. 4-AP was administered both intravenously and orally (dosage ranged from 0.07 to 0.59 mg per kg body weight). The clinical results of this study will be published.

In order to correlate clinical changes induced by 4-AP with serum blood levels of 4-AP, it was necessary to develop a sensitive method for the determination of 4-AP concentration in blood. The high-performance liquid chromatographic (HPLC) determination of 4-AP and 3,4-diaminopyridine (3,4-DAP) with the precipitation of serum proteins, as described by Lamiable and Millart [5] had a limit of detection of 50 ng 4-AP/ml.

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This method was not appropriate because the serum blood levels of 4-AP in our study were expected to be lower. Extraction methods using dichloromethane followed by reversed-phase HPLC assay have been described [6,7]. However, because of a bad reproducibility due to the type of HPLC columns used, the method of Uges and Bouma [6] did not produce satisfactory results for the analysis of 4-AP in our laboratory. Shinohara *et al.* [7] claimed a satisfactory reproducibility for their ion-pair HPLC method, but the separation between 4-AP and 3,4-DAP is not stated. A gas chromatographic method has been described [8]. The ion-pair HPLC analysis of 3,4-DAP following solid-phase extraction has been described by Leslie and Bever [9]. The disadvantage of the latter method is the coelution of 4-AP and 3,4-DAP, so that an internal standard other than an aminopyridine has to be used.

In this study the development of a method for the HPLC analysis of 4-AP after solid-phase extraction is described. With this method 4-AP concentration in serum could be measured with satisfactory precision and a limit of quantitation of 3 ng/ml.

EXPERIMENTAL

Materials

4-AP was obtained from Bufa-Chemie (Castricum, Netherlands) and 3,4-DAP from Sigma (Flow-Amstelstad, Zwanenburg, Netherlands). Acetonitrile, methanol, ethanol and ammonium carbonate, all of analytical quality, were purchased from Merck (Amsterdam, Netherlands), and phosphate-buffered salt USP XXI (PBS) was produced by our own pharmacy production unit.

Instrumentation and chromatographic conditions

A Model M-6000 A solvent-delivery system, a Waters Intelligent Sample Processor (WISP) and a Model 481 variable-wavelength detector (all from Waters, Etten-Leur, Netherlands) were used. The HPLC analytical column was a μBondapak C_{18} 300 × 3.9 mm I.D. (10 μm particle size) with a Guard-Pak μBondapak C_{18} pre-column 4 × 6 mm I.D. (10 μm particle size) (Waters). The solid-phase extraction column was a 1 ml Bakerbond Spe C_{18} in a column processor Spe-21 (Baker, Deventer, Netherlands). The detector was connected to a Shimadzu Chromatopac C-R3A integrator (Chrompack, Middelburg, Netherlands).

The flow-rate of the mobile phase was 1.5 ml/min. The effluent from the column was monitored at a wavelength of 244 nm.

Mobile phase and extraction solvent

The mobile phase was acetonitrile–methanol–ethanol–1% ammonium carbonate (75:10:10:5, v/v). It was saturated with silica gel, kept overnight, and filtered and degassed by vacuum before use. The solid-phase extraction solvent was acetonitrile–methanol–ethanol–1% ammonium carbonate (25:30:30:30, v/v). The chromatographic system and column were cleaned daily by purging with water and methanol, respectively.

Preparation of standards and calculations

Stock solutions of 4-AP and the internal standard 3,4-DAP were prepared in water. Further dilution steps were made in PBS. The solutions were stored at 4°C. The final dilutions for the 4-AP standards were prepared with "new-born bovine" serum. The internal standard method using peak-height calculations of the integrator was used to obtain the resulting serum concentrations.

Sample preparation and injection

The solid-phase extraction columns were conditioned by purging with 2 ml of methanol, followed by 2 ml of water using the Spe-21 column processor at a vacuum of 40–60 kPa. Aliquots of 500 μl of standard or sample were applied to the conditioned column, followed by 100 μl of internal standard solution (1.6 mg/l 3,4-DAP). The column was purged with 2 ml of water and then twice with 250 μl of acetonitrile; the washing solutions were discarded. The components, which were retained on the column, were eluted with three portions of 250 μl of the extraction solvent. The extract was collected in 10-ml glass tubes and concentrated to *ca.* 20 μl by evaporation under a stream of air at 20°C. The residue was mixed with *ca.* 100 μl of mobile phase, and 30 μl were injected into the HPLC system.

RESULTS

Fig. 1 shows two examples of chromatograms obtained from patients' sera. Patient I received a placebo and patient II received 4-AP. The retention times of 4-AP and 3,4-DAP were 4 and 5 min, respectively.

No interference was observed from several drugs commonly used by MS patients, such as muscle relaxants (baclofen and tizanidine), NSAIDs (ibuprofen, indomethacin), antidepressants (amitriptyline/nortriptyline, mianserine, doxepine), benzodiazepines (lorazepam, temazepam, diazepam, flurazepam, flunitrazepam) and parasympaticolytics (oxybutidine).

During the assay of over 300 serum samples we used three analytical columns and four batches of SPE columns, all with the same results.

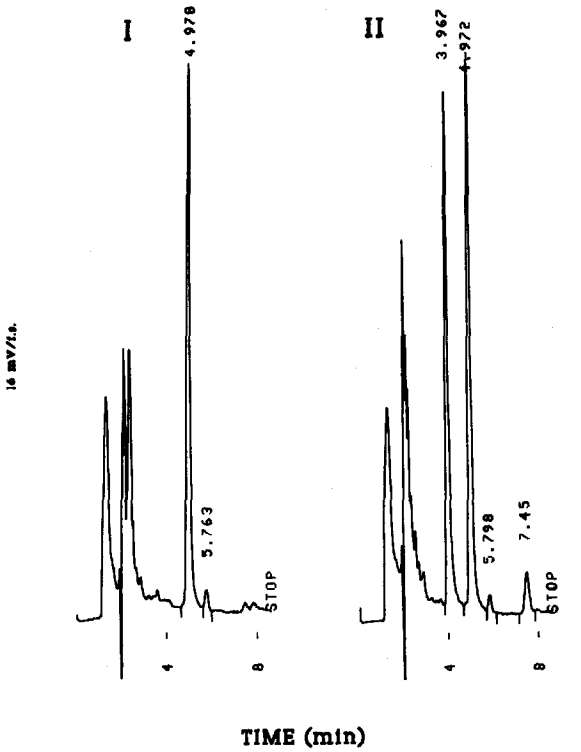

Fig. 1. Chromatograms obtained from (I) serum of patient receiving a placebo without 4-AP, (II) serum of a patient after oral administration of 3 × 10 mg of 4-AP. Sampling time 1.45 h after dose. Calculated concentration 99 ng/ml 4-AP. Retention times: 4-AP, 4 min; 3,4-DAP, 5 min. Peaks at 5.7 and 7.5 min are serum peaks. Concentration of 3,4-DAP is *ca.* 40 ng/ml.

TABLE I

DAY-TO-DAY PRECISION DATA FOR 4-AP DETERMINATION

Concentration of 4-AP (mean) (ng/ml)	*n*	Coefficient of variation (%)
11.1	5	8.2
41.0	6	2.7
85.1	6	2.7

Extraction efficiency

The recovery of 4-AP and 3,4-DAP from the extraction column was tested by adding known amounts of the compounds to drug-free human serum. The samples were extracted as previously described, with the difference that the internal standard was added after the extraction. The recovery was determined by comparing peak heights with those obtained from the injection of equivalent concentrations of pure drug in mobile phase. The extraction efficiency of 4-AP was 89% and that of 3,4-DAP was 92% ($n = 6$).

Precision

The day-to-day precision was estimated with the same test material of three different samples, which were stored at −20°C, over six working days. The precision data are presented in Table I.

Linearity

The linearity was tested by constructing standard curves with spiked 4-AP serum samples. The calibration samples were prepared daily by diluting appropriate amounts of the 4-AP solution with 0.5 ml of bovine serum. The standard curves were linear over the range of interest, 10–125 ng 4-AP/ml. The correlation coefficient of several curves was 0.9994 ($n = 6$).

Limit of quantification

The limit of quantitation of this method was 3 ng/ml. This was equal to the concentration where the signal was three times the relative deviation of the noise. The noise was the response from a serum injection without 4-AP measured at the retention time of 4-AP.

DISCUSSION

With our method we were able to determine very low 4-AP serum concentrations in our clinical study. The method has proved to be suitable and fast during the assay of over 300 patient sera.

As shown in Fig. 1, 4-AP and 3,4-DAP were very well separated so the method can also be applied for the assay of 3,4-DAP. This is an advantage over the method of Leslie and Bever [9], who were not able to measure the two substances at the same time. Furthermore, 3,4-DAP is a better internal standard, because of its related molecular structure, than N-methylclonazepam, which was used by Leslie and Bever. Compared with the methods of Uges and Bouma [6] and Shinohara *et al.* [7] the variation of the extraction efficiency and the reproducibility of our method were better. The detection limits were about equal.

CONCLUSION

The combination of solid-phase extraction with reversed-phase HPLC, which separates 4-AP and 3,4-DAP, offers the possibility of determining 4-AP in serum with sufficient precision and an adequate limit of quantitation.

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