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Letter to the Editor

High-performance liquid chromatographic determination of 3,4-diaminopyridine in human plasma

Sir,

4-Aminopyridine (4-AP) has been used in clinical practice for the treatment of human neuromuscular diseases [1—4]. We have previously developed a method for determining 4-AP and 3,4-diaminopyridine (3,4-DAP) in rat cerebrospinal fluid and serum [5], but this method is not sensitive enough for pharmacokinetic studies in humans. High-performance liquid chromatographic (HPLC) methods for the determination of 4-AP in body fluids have been reported [6]. This paper describes a sensitive assay for the determination of 3,4-DAP in human plasma which is useful for its pharmacokinetic study in humans.

Methanol, acetonitrile and dichloromethane, HPLC grade, and potassium dihydrogen phosphate and potassium carbonate, analytical reagent grade, were purchased from E. Merck (Darmstadt, F.R.G.). 4-AP and 3,4-DAP were from Aldrich-Europe (Beerse, Belgium). Water was deionized and then double-distilled.

Stock solutions of 3,4-DAP and 4-AP (internal standard) were prepared in methanol at the concentration of 1 mg/ml and were stored at 0—5°C. These solutions were prepared every week.

A liquid chromatograph (Varian Model 5000) equipped with a variable-wavelength detector (Varichrom, Varian) was used in a reversed-phase system with a Micropak C_{18} column as the stationary phase (300 × 4 mm I.D., particle size 10 μm; MCH 10, Varian). The mobile phase was made up of acetonitrile—phosphate buffer (0.05 *M*, pH 7.4) + tetramethylammonium chloride (0.02 *M*) (23:77). The volume of sample injected was 20 μl (Valco valve). The effluent was monitored at 288 nm with a sensitivity of 0.02 a.u.f.s. The mobile phase flow-rate was 1.3 ml/min and the chart-speed 0.25 cm/min.

For the sample preparation, about 300 mg of potassium carbonate and 50 μl of the internal standard (solution of 4-AP, 10 mg/l in water) were added to 500 μl of plasma. The mixture was stirred (10 sec on a Vortex mixer), extracted twice with 5 ml of dichloromethane (1 min on Vortex) and centrifuged (2600 *g* for 1 min). The organic phase was transferred to another tube and re-extracted with 50 μl of 0.1 *M* hydrochloric acid (1 min on Vortex).

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After centrifugation (2600 *g* for 1 min), the supernatant was transferred into a cone-shaped tube and 20 μl of the aqueous phase were injected onto the column.

Calibration curves were constructed by spiking blank serum samples with an aqueous solution of 3,4-DAP giving plasma concentrations ranging from 6.25 to 400 μg/l. The ratios between the peak heights of 3,4-DAP and the internal standard versus the concentrations of 3,4-DAP were used to construct the calibration curves.

Typical chromatograms are shown in Fig. 1. The retention times of 3,4-DAP and 4-AP were 228 and 288 sec, respectively.

The recovery of 3,4-DAP was 75.6 ± 6.9% (mean ± S.D., n = 5) and 78.8 ± 5.8% (mean ± S.D., n = 5) at concentrations of 50 μg/l and 400 μg/l, respectively. For 4-AP the recovery was 86.7 ± 2.4% (mean ± S.D., n = 5) at a concentration of 500 μg/l.

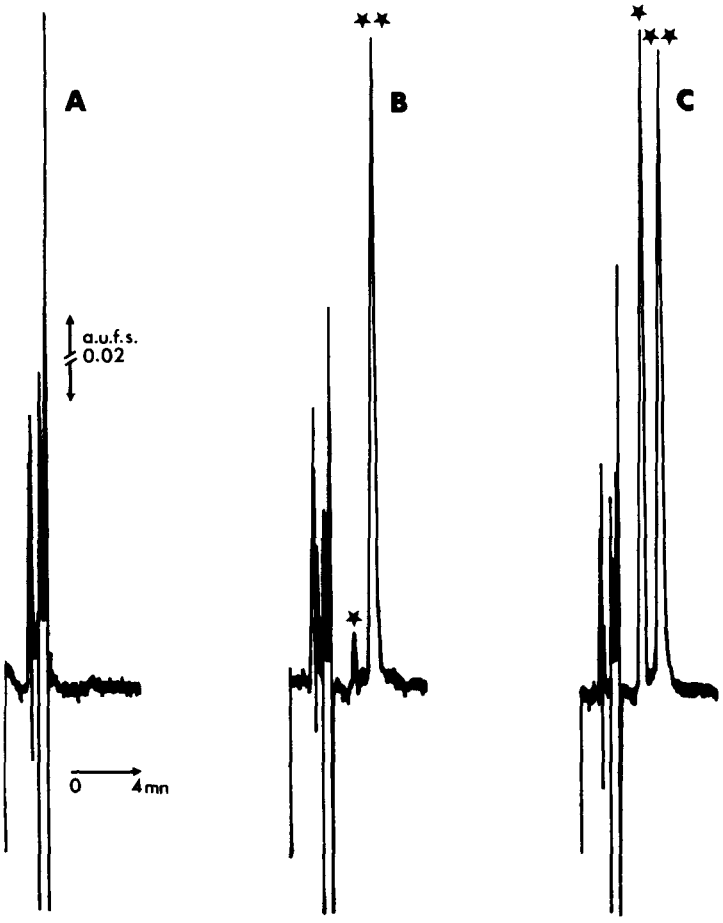

Fig. 1. (A) Chromatogram obtained from analysis of a blank human plasma sample. (B) Chromatogram obtained from analysis of a spiked human plasma sample containing 12.5 μg/l of 3,4-DAP (⋆) and 400 μg/l of 4-AP (⋆⋆) (internal standard). (C) Chromatogram obtained from analysis of a spiked human plasma sample containing 200 μg/l of 3,4-DAP (⋆) and 400 μg/l of 4-AP (⋆⋆) (internal standard).

The within-run precision was evaluated by analysing plasma samples (n = 5) spiked with known amounts of 3,4-DAP, and was found to be 10.8% and 6.8% at concentrations of 50 μg/l and 400 μg/l, respectively.

A linear relationship was observed between the peak height ratio of 3,4-DAP/4-AP (Y) and the amount of 3,4-DAP added to the plasma (X, μg/l): $Y = 0.0054X - 0.0018$; $r = 0.9983$; $n = 16$; concentration range 6.25—400 μg/l.

No interfering peaks with the same retention times as 3,4-DAP and 4-AP were present in blank plasma. The limit of detection in plasma allowing a signal-to-noise ratio of 2 was 3.4 μg/l.

The reported procedure was capable of quantitating 3,4-DAP in plasma to a concentration as low as 3 μg/l with a 0.5-ml plasma sample, and this was useful for its kinetic study in humans.

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