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Note

High-performance liquid chromatographic determination of 4-aminopyridine and 3,4-diaminopyridine in rat cerebrospinal fluid and serum

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As far as we know, only 4-aminopyridine (4-AP) has been used in clinical practice for treatment of human neuromuscular diseases [1—4], but its usefulness has been limited by its central nervous system stimulant effect [5]. 3,4-Diaminopyridine (3,4-DAP) has been shown to be six to ten times more potent than 4-AP in increasing evolked transmitter release at the neuromuscular junction in vitro and two times less convulsant and toxic than 4-AP after acute intravenous injection in mice.

The existing high-performance liquid chromatographic method was developed for determining 4-AP in stomach contents of horses and was not applicable for biological fluids [6]. This paper describes a rapid, sensitive and selective assay developed to compare the ability of 4-AP and 3,4-DAP to cross the blood—brain barrier.

EXPERIMENTAL

Materials

4-AP and 3,4-DAP were purchased from Aldrich-Europe (Beerse, Belgium). Acetonitrile and methanol were of analytical grade. Potassium dihydrogen phosphate buffer (0.05 *M*), containing trimethylammonium chloride (0.02 *M*), was prepared in freshly glass-bidistilled water and adjusted to pH 7.4. Stocks solutions of 4-AP and 3,4-DAP were prepared at the concentrations of 100 μg/ml and 150 μg/ml, respectively, in methanol. Standard solutions of 4-AP and 3,4-DAP were prepared in methanol at the concentrations of 10 μg/ml and 15 μg/ml, respectively.

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Sample preparation

To 100 μl of serum were added 200 μl of methanol. The mixture was stirred for 30 sec on a Vortex Genie Mixer (Scientific Industries, Bohemia, NY, U.S.A.), centrifuged for 5 min at 2600 *g* and a 10μl portion of the supernatant was injected into the chromatograph. The cerebrospinal fluid (CSF) samples were shaken, centrifuged at 2600 *g* for 5 min and a 10-μl portion of the supernatant was injected directly into the chromatograph.

Calibration

4-Aminopyridine. Serum calibration curves were constructed by adding 0.25, 0.5, 1, and 2 μg of 4-AP to 100-μl serum samples via the 200 μl of methanol used for the deproteinization (2, 5, 10, 20 mg/l 4-AP). CSF calibration curves were constructed by dilution of the standard solution in water (0.5, 1, 3, and 5 mg/l 4-AP).

3,4-Diaminopyridine. Serum calibration curves were constructed in the same way as for 4-AP, but the final concentrations were 3.75, 7.5, 15, and 30 mg of 3,4-DAP per l of serum. For the CSF samples, the final dilutions were 0.25, 0.75, 1.5, and 3 mg of 3,4-DAP per l of water.

The peak heights were plotted against the concentration of the standards to give the calibration curves. Equations of the computed regression lines and correlation coefficients were calculated.

Chromatography

A liquid chromatograph (Varian Model 5000 liquid chromatograph) equipped with a variable-wavelength detector (Varichrom, Varian) was used in a reversed-phase system with MicroPak C_{18} as the stationary phase (300 × 4 mm I.D.; particle size 10 μm) (MCH 10, Varian) and acetonitrile–phosphate buffer (0.05 *M*) + tetramethylammonium chloride (0.02 *M*) (60:40) as the mobile phase. The volume of sample injected was 10 μl (Valco Valve). The effluent was monitored at 260 nm for 4-AP and 290 nm for 3,4-DAP with a sensitivity of 0.02 or 0.01 absorbance unit full scale (a.u.f.s.). The mobile phase flow-rate was 2 ml/min and the chart speed 0.25 cm/min.

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 1. The retention times of 3,4-DAP and 4-AP were 175 sec and 216 sec, respectively.

Extraction efficiency

Known amounts of each component were added to serum free from drug. All samples were extracted as previously described. The results were compared with those obtained from the injection of equivalent concentrations of pure drugs in water. The results for extraction efficiency are summarised in Table I.

Precision

The within-run precision was established by spiking serum and CSF with 3,4-

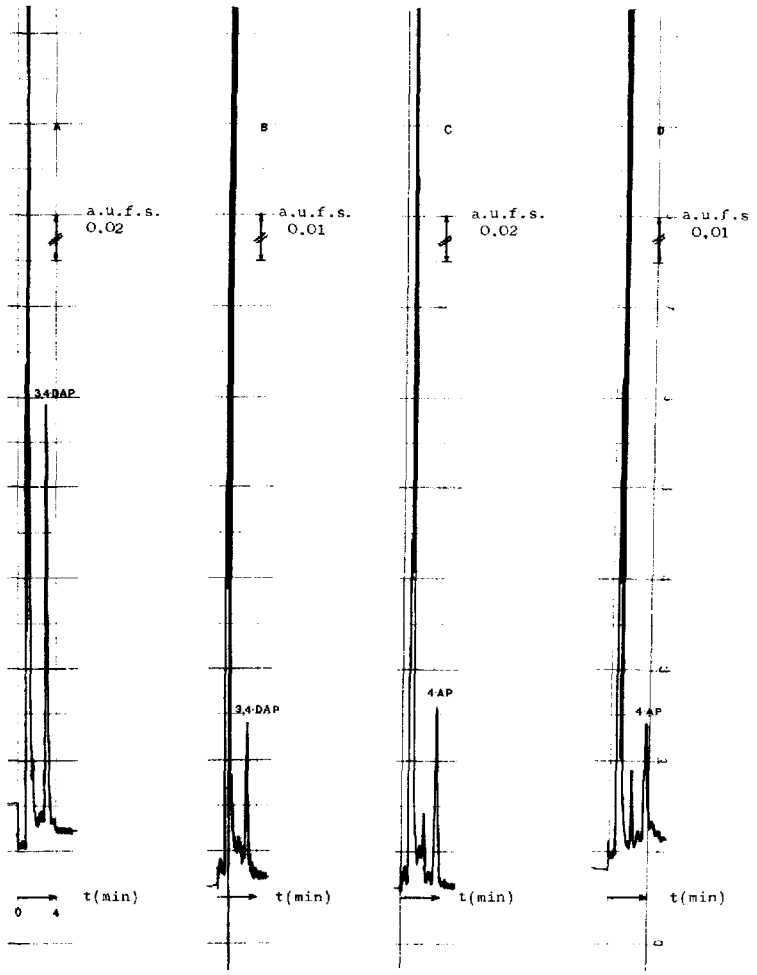

Fig. 1. Chromatograms obtained from rat serum and CSF samples after intravenous injection of 4-AP (7 mg/kg) or 3,4-DAP (16 mg/kg). (A) serum sample containing 20 mg/l 3,4-DPA, (B) CSF sample containing 1.3 mg/l 3,4-DAP, (C) serum sample containing 6 mg/l 4-AP and (D) CSF sample containing 1.6 mg/l 4-AP.

TABLE I

EXTRACTION EFFICIENCY OF 3,4-DAP AND 4-AP IN SERUM

Compound	Concentration (mg/l)	*n*	Mean ± S.D. (%)
3,4-DAP	15	5	99.8 ± 3
4-AP	5	5	97.9 ± 2

DAP and 4-AP in a concentration corresponding, approximately, to the middle range to be encountered after a single intravenous dose of 16 mg/kg 3,4-DAP or 7 mg/kg 4-AP. The results are summarised in Table II. The higher levels of 3-4 DAP and 4-AP in serum explain the better precision for serum than for

TABLE II

PRECISION OF THE METHOD (WITHIN-RUN)

Compound analyzed	Biological fluid	Concentration (mg/l)	n	C.V. (%)
3,4-DAP	CSF	1.5	8	5.07
	serum	15	10	2.31
4-AP	CSF	1	9	5.46
	serum	5	10	3.05

CSF. The use of an internal standard (3,4-DAP for the determination of 4-AP and 4-AP for the determination of 3,4-DAP) was not found to improve the precision of the assay, and has, therefore, been omitted.

Linearity

A linear relationship was observed between the peak height (Y, mm) and the amount of 3,4-DAP added to serum (X, mg/l) ($Y = 6.892\ X - 2.913$; $r = 0.999$; concentration range 3.75—30 mg/l; a.u.f.s. = 0.02) and 3,4-DAP added to water ($Y = 38.623\ X + 1.132$; $r = 0.995$; concentration range 0.25—3 mg/l; a.u.f.s. = 0.01). The same was observed with amount of 4-AP added to serum ($Y = 9.655\ X + 2.719$; $r = 0.998$; concentration range 2—20 mg/l; a.u.f.s. = 0.02) and to water ($Y = 25.263\ X + 2.829$; $r = 0.998$; concentration range 0.5—5 mg/l, a.u.f.s. = 0.01).

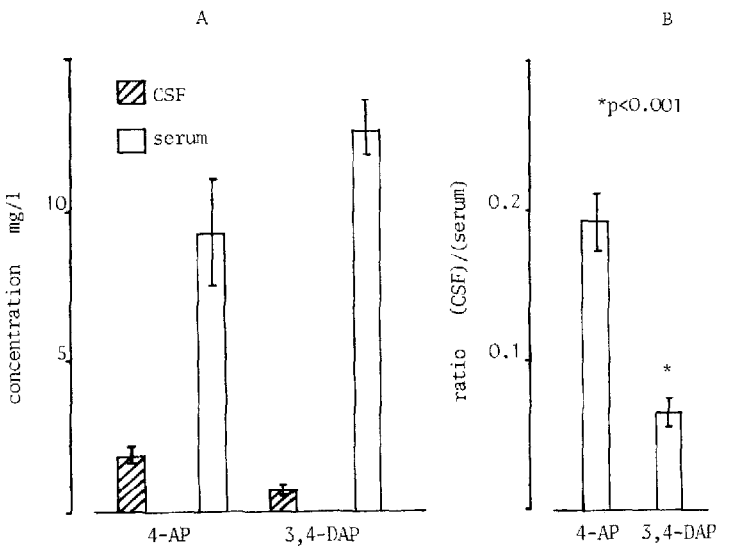

Fig. 2. (A) Mean concentrations in the CSF and serum for 4-AP (7 mg/kg, $n = 7$) and 3,4-DAP (16 mg/kg, $n = 8$) 5 min after intravenous injection. (B) Mean ratios of the concentrations (CSF/serum) with significant difference between 4-AP and 3,4-DAP.

Detection limit

No interfering peaks were present in pre-dose plasma or CSF with the same retention time as 3,4-DAP or 4-AP. The lower limits of detection in serum measured at a detector sensitivity of 0.02 a.u.f.s. and allowing a signal-to-noise ratio of two are 100 μg/l for 3,4-DAP and 50 μg/l for 4-AP.

Concentration of the drugs in rat CSF and serum

The concentrations of the drugs were measured in the CSF and in the serum of anesthetized rats (urethane) 5 min after intravenous injection of 4-AP (7 mg/kg) or 3,4-DAP (16 mg/kg). The mean concentration of 3,4-DAP in the CSF was lower than that of 4-AP. The ratios of the concentrations found in the CSF to those found in the serum were significantly higher with 4-AP than with 3,4-DAP (Fig. 2) [7].

CONCLUSION

In conclusion, 3,4-DAP does not cross the blood–brain barrier as easily as 4-AP and this can account for its lower central nervous system stimulant action and toxicity in vivo.

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