Journal of Chromatography, 421 (1987) 392–395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3833

Note

High-performance liquid chromatographic determination of 2,4-diaminopyridine in plasma and urine

P.T.M. BIESSELS* and S. AGOSTON

Department of Pharmacology/Clinical Pharmacology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen (The Netherlands)

and

A.S. HORN

Department of Pharmacy, University of Groningen, Groningen (The Netherlands)

(First received March 9th, 1987; revised manuscript received June 17th, 1987)

The use of 4-aminopyridine (4-AP) as a reversal agent for non-depolarizing muscle relaxants [1] or in neuromuscular disorders [2] is limited by its central stimulating side-effects, which can cause restlessness, excitement and in higher doses convulsions [3]. One of our aims in the search for more selectively acting derivatives of 4-AP was to find compounds that had no or hardly any central stimulating effects but which have the same antagonistic potency as 4-AP against neuromuscular blocking agents. So far two compounds, 2,4-diaminopyridine (2,4-DAP) and LF-14 [3-(dimethylamino)carbonylamino-4-aminopyridine] have shown promising results [4]. In a preliminary toxicological study 2,4-DAP proved to be less toxic than LF-14 [5]. For further pharmacokinetic studies on 2,4-DAP we needed to develop an assay method for this compound.

Several methods for the determination of 4-AP in body fluids have been described [6–8]. We have investigated them for the determination of 2,4-DAP and have found that one method [9], with some modifications, is suitable for determination of 2,4-DAP in body fluids, using 4-AP as an internal standard.

EXPERIMENTAL

Materials

2,4-DAP was synthesized and a recrystallized from toluene to at least 99% purity. 4-AP was purchased from Aldrich (Brussels, Belgium) and recrystallized

0378-4347/87/$03.50

from ethanol–diethyl ether. All other reagents were of analytical grade and were used without further purification. Dichloromethane, acetonitrile, trifluoroacetic acid and tetrabutylammonium iodide were obtained from Merck (Darmstadt, F.R.G.), and sodium heptane sulphonate was from Janssen (Beerse, Belgium).

Fresh bidistilled water was used throughout. Stock solutions of 2,4-DAP (15 mg per 100 ml) and 4-AP (25 mg per 100 ml) were prepared in water. Storage of these solutions at 4 °C did not result in detectable decomposition.

Chromatography

We used a Model 6000A solvent-delivery system from Waters Assoc. (Milford, MA, U.S.A.) equipped with a variable-wavelength PU 4020 UV detector from Pye Unicam (Cambridge, U.K.) at 272 nm (sensitivity 0.01 a.u.f.s.) and a standard dual-line compact recorder. A prepacked Versapack C_{18} reversed-phase column was used (30 cm × 4.1 mm I.D., particle size 5 μm) (Alltech, Nazareth, Belgium).

The mobile phase consisted of a mixture of 0.012 *M* sodium heptane sulphonate, 0.002 *M* tetrabutylammonium iodide and 0.01 *M* sodium dihydrogenphosphate buffer in acetonitrile–water (10:90, v/v). The mobile phase was degassed ultrasonically and filtered through a 0.2-μm filter. The assays were performed at ambient temperature, with a flow-rate of 1.2 ml/min.

Sample preparation

Plasma. To 0.5 ml of plasma in a glass centrifuge tube were added 500 mg of potassium carbonate. This was vortex-mixed for 3 s, and 40 μl of 4-AP solution (2.5 μg/ml) were added. Then 7.5 ml of dichloromethane were added, and the plasma was extracted by rotating the tubes on a rotary disc for 30 min. After centrifugation for 5 min (2600 *g*), the organic layer was decanted, 10 μl of TFA were added, and the samples were evaporated under a stream of air on a water-bath at 37 °C. The residue was dissolved in 10% acetonitrile by whirl-mixing for 10 s, and 50 μl were injected on to the column.

Urine (alternative method). To 1.0 ml of urine were added 1.5 g of potassium carbonate. This was mixed on a vortex mixer for 10 s, and 2.5 ml of dichloromethane were added. After rotation for 30 min (60 rpm) and centrifugation for 5 min (2600 *g*), 50 μl of the organic layer were injected on to the column. For this alternative method a flow-rate of 2.0 ml/min was chosen.

RESULTS

Typical chromatograms are shown in Figs. 1 and 2. The retention times of 2,4-DAP and 4-AP were 5.7 and 7.6 min, respectively. For urine extracts the retention time of 2,4-DAP was 3.0 min. The higher flow-rate for urine detection was chosen because of the large peaks that we found at retention times of 15–20 min, which made the usual method very time-consuming.

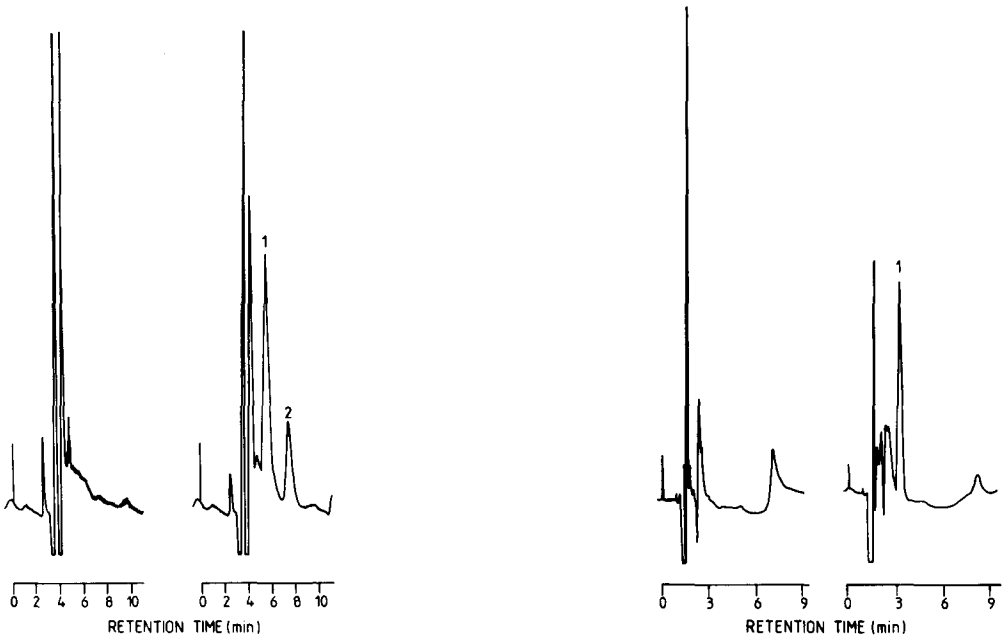

Fig. 1. Chromatogram of cat plasma 45 min after intravenous injection of 750 μg/kg 2,4-DAP (concentration, 104 μg/l) (right) and of blank plasma of the same cat (left). Flow-rate, 1.2 ml/min. Peaks: 1 = internal standard, 4-AP; 2 = 2,4-DAP.

Fig. 2. Chromatogram of cat urine taken 4 h after intravenous injection of 750 μg/kg 2,4-DAP (concentration, 1.3 mg/l) (right) and of blank urine of the same cat (left). Flow-rate, 2.0 ml/min. Peak 1 = 2,4-DAP.

Extraction efficiency

Known amounts of the two compounds were added to drug-free plasma. All the samples were extracted as previously described. The results were compared with those obtained from known amounts of the compounds in water directly injected on to the column. The results are summarized in Table I.

TABLE I

ANALYTICAL RECOVERY

Substance	Concentration (ng/ml)		Recovery (%)	C.V. (%)	Concentration found (ng/ml)
	Plasma	Urine			
4-AP	100		83.2	3.3	101 ± 2.4
2,4-DAP	50		76.3	5.1	49 ± 2.8
	200		74.2	4.6	197 ± 4.1
	800		74.8	3.9	805 ± 10.7
		500	97.6	6.2	507 ± 9.6
		2000	92.3	3.7	2023 ± 19.8
		8000	89.7	3.1	7935 ± 73.5

Precision

The within-day precision of the assay was determined by performing ten replicate analyses on aliquots spiked with 200 ng of 2,4-DAP and 100 ng of 4-AP. The coefficient of variation (C.V.) was 4.7% for 2,4-DAP and 3.1% for 4-AP, and 3.9% for the alternative method. Day-to-day precision was estimated for the same test material over five working days. The C.V. was 5.5 and 4.7% for the alternative method. The results demonstrate a good reproducibility.

Linearity

The calibration curve was obtained by plotting the peak-height ratios of 2,4-DAP to internal standard using drug-free plasma containing 2,4-DAP in the range 25–800 ng/ml. A linear relationship was observed over this range ($y=0.003+0.036x$, $r=0.9995$). The lowest quantifiable level of drug was 5 ng/ml (signal-to-noise ratio greater than 3). For the alternative method, urine was spiked with known amounts of 2,4-DAP (0.5–8 μg/ml). A linear relationship was observed over this range ($y=19+8.675x$, $r=0.998$).

CONCLUSION

Of the two methods we have described one is suitable for the determination of 2,4-DAP in the urine. This is an easy and rapid method, but not sensitive enough to determine low concentrations of 2,4-DAP (less than 250 ng/ml). The other method is more time-consuming but also more sensitive. Both methods will be used for further pharmacokinetic investigations. They also proved to be useful for the determination of 2,4-DAP in body fluids of the cat and in lyophilised calf serum, a biofluid that we use for routine analyses and for obtaining calibration curves.

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