Pergamon

0731-7085(95)01295-0

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 6, pp. 791–794, 1995
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0731–7085/95 $9.50 + 0.00

Short Communication

Determination of 3,4-diaminopyridine in human plasma by high-performance liquid chromatography

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Keywords: *3,4-Diaminopyridine; HPLC; 2,6-diaminopyridine.*

Introduction

3,4-Diaminopyridine (3,4-DAP) is an effective drug in improving neuromuscular transmission, with limited CNS toxicity, compared to its analogue, 4 aminopyridine (4-AP). 3,4-DAP is used in the treatment of neuromuscular diseases, such as Lambert–Eaton Syndrome [1]. A number of HPLC methods for the quantitation of 3,4-DAP and the related 4-AP have been reported. The method of 3,4-DAP quantification in the supernatant after precipitation of serum proteins described by Lamiable and Millart [2] lacks the sensitivity (limit of detection 100 ng ml^{-1}) required for the measurement of the plasma drug concentrations following therapeutic dosing. Extraction of the drug by organic solvents as described by Uges and Bouma [3] and Shinohara *et al.* [4] also lack in precision and accuracy, due to the volatile nature of the drug. The two other HPLC methods described by Leslie and Bever [5], and van der Horst *et al.* [6] use solid-phase extraction, but the so-called internal standard is added after the drug is extracted. Here we report a sensitive and reproducible method for the measurement of 3,4-DAP in plasma which utilizes solid-phase extraction of both the drug and the internal standard simultaneously, followed by reversed-phase ion pair isocratic high-performance liquid chromatography.

Materials and Methods

Apparatus

The chromatographic system employed was an automated Kontron system consisting of a data system (model 450), a dual piston (model 420 pump), an autosampler (model 460) and an ultraviolet detector (model 432). The column was a C_{18} μBondapack (10 μM particle size, 150 mm × 3.9 mm i.d.; Waters Millipore Corporation, USA).

Chemicals

3,4-DAP, 2,6 diaminopyridine (2,6-DAP) [internal standard] were purchased from the Sigma Chemical Company, (St Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from FSA Laboratory Supplies (Loughborough, UK). Disodium hydrogen phosphate was obtained from BDH (Poole, UK) and heptane sulphonic acid (HPLC grade) was purchased from Fisons (Loughborough, UK). Water was distilled and deionized using a Nanopure system (Barnstead, Fisons, UK).

High-performance liquid chromatography

All experiments were carried out at ambient room temperature (approximately 20°C). The column was equilibrated with the mobile phase for at least 30 min prior to analysis of samples. The mobile phase was disodium hydrogen

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phosphate (pH 4.0; 50 mM) containing heptane sulphonic acid (10 mM)–acetonitrile (90:10 v/v) pumped at a flow rate of 1.5 ml min^{-1}, pressure 73 bar (1095 psi). 3,4 Diaminopyridine and 2,6 diaminopyridine were detected by UV absorbance at 229 nm (AUFS 0.001).

Sample preparation

Stock solutions of 3,4-DAP and 2,6-DAP were prepared in water. Further dilution steps were made in plasma obtained from healthy subjects. The standards were prepared by adding appropriate volumes 10–15% of the final plasma volume (1 ml), so that the integrity of the samples were preserved. The quality control samples were also prepared in the same way, using a separately weighed stock solution. The final concentration of 3,4-DAP in plasma standards were 5, 10, 20, 25, 50, 100, 250 and 500 ng ml^{-1}. Two quality controls at 32 and 130 ng ml^{-1} were prepared, and after aliquoting, 1 ml samples were stored at −20°C until analysis. A 100 μg ml^{-1} solution of 2,6-DAP was prepared and 100 μl was added to each sample, vortex mixed for 10 s, and extracted, using Bond Elut C_{18} (3 ml) cartridges (Varian, CA, USA). The cartridges were washed and preconditioned prior to extraction step, by passing through methanol (2 ml) followed by water (2 ml) under vacuum (20 kPa), using a Vac Elut manifold (Anachem International, UK). The 3,4-DAP and 2,6-DAP were then desorbed with 100 μl of disodium hydrogen phosphate (pH 4.0; 50 mM) containing heptane sulphonic acid (60 mM)–acetonitrile (40:60, v/v), followed by water (500 μl). An aliquot (80 μl) was injected into the HPLC system.

The within-day and between-day precision was established by assaying four different concentrations of 3,4-DAP (25, 100, 250 and 500 ng ml^{-1}) in duplicate at six different times.

The assay was evaluated in one patient who received an oral dose of 3,4-DAP (10 mg). Blood (10 ml) samples were collected at 0 (pre-dose), 30, 80, 120 and 190 min post dose and were later centrifuged at 2500*g* for 10 min to separate the plasma. Concentrations of 3,4-DAP in plasma were evaluated by determining the ratio of peak area for the compound to that of the internal standard, then comparing with the standard curve obtained after regression analysis of the calibration samples.

Results and Discussion

The mean recovery for 3,4-DAP was more than 80% (relative standard deviation <10%) at the four different concentrations of the drug studied, compared to the direct injection of the standard dissolved in water (Table 1). Figure 1 represents typical elution profiles for a blank plasma and a sample containing 3,4-DAP obtained from a patient with myasthenia gravis, 30 min following an oral dose (10 mg) of 3,4-DAP. The retention times for 3,4-DAP and 2,6-DAP were 2.3 and 3.6 min, respectively. The standard curve for 3,4-DAP was linear over the concentration range (5–500 ng ml^{-1}) studied. The correlation coefficients were greater than 0.99 for each regression line and intercepts were all close to zero (Table 2). The relative standard deviation for 3,4-DAP ranged 2.5–6.5% for within-day and 0.0–12.0% for between-day (Table 3). The limit of quantification for 3,4-DAP was 5 ng ml^{-1} (signal/noise ratio = 3). Figure 2 represents the concentrations of 3,4-DAP in plasma over a 4-h period from a patient who had received an oral dose (10 mg) of the drug.

Table 1
Recovery of 3,4 diaminopyridine and internal standard (n = 6)

	3,4-DAP				
% Extracted	25	100	250 (ng ml^{-1})	500	IS 17 (ng ml^{-1})
1	83.4	86.4	82.1	79.5	63.4
2	86.4	87.9	81.7	80.3	63.0
3	94.4	88.5	80.7	80.0	60.5
4	90.4	87.4	82.1	79.5	61.1
5	89.6	87.1	82.8	80.2	62.8
6	88.7	88.0	81.8	80.3	60.8
Mean % recovery	88.8	87.6	81.9	80.0	61.9
RSD (%)	3.2	0.8	0.9	0.5	2.1

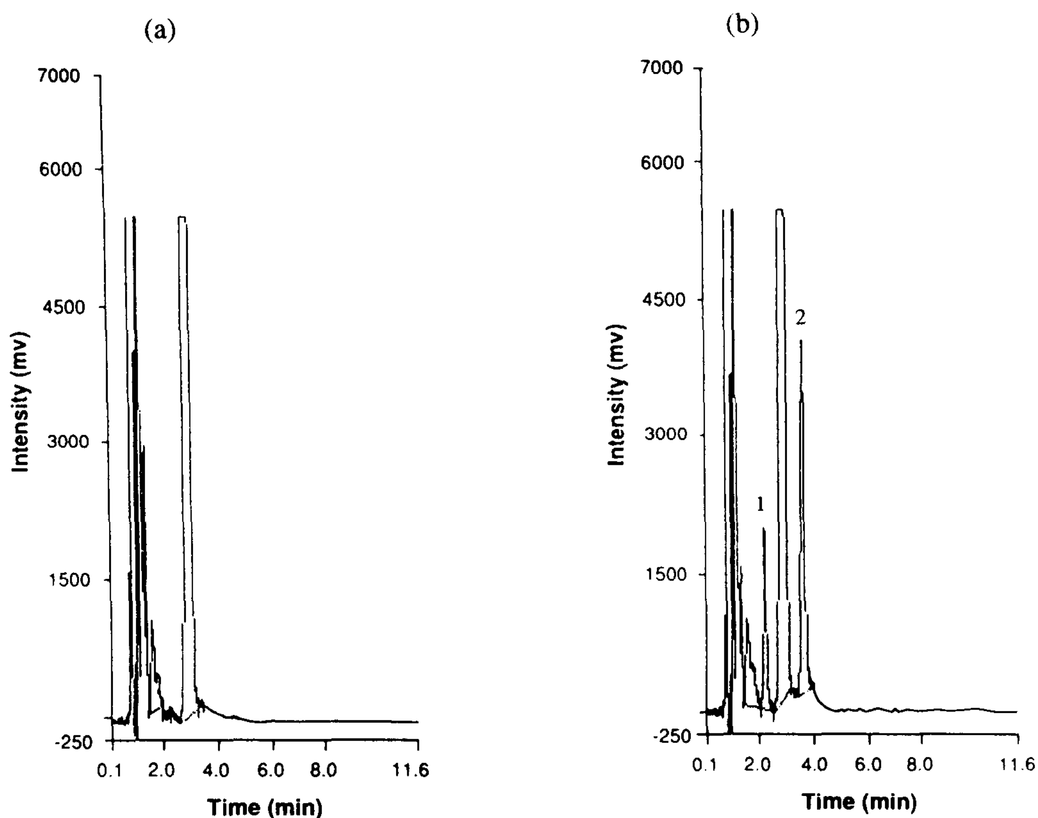

Figure 1
Elution pattern for 3,4-DAP and 2,6-DAP. (a) blank plasma and (b) plasma from a patient 30 min after receiving an oral dose (10 mg) of DAP. 1 = 3,4-DAP, corresponding to 51 ng ml^{-1}, and 2 = 2,6-DAP. Injection volume 80 μl. (AUFS 0.001).

Table 2
Between-day standard curve statistics for 3,4-diaminopyridine

	Slope	y-intercept	x-intercept	Correlation coefficient
1	0.0142	0.0089	−0.6242	0.9925
2	0.0133	0.0128	−0.9644	0.9940
3	0.0129	0.0368	−0.2854	0.9924
4	0.0152	0.0068	−0.4503	0.9960
5	0.0156	0.0065	−0.4182	0.9978
Mean	0.0142			0.9945
RSD (%)	8.45	0.0143	0.5485	0.23

Table 3
Precision data for 3,4 diaminopyridine

Within-day (n = 6) conc. (mean ± SD) (ng ml^{-1})	RSD (%)	Between-day (n = 6) conc. (mean ± SD) (ng ml^{-1})	RSD (%)
476.0 ± 16.7	3.5	446.7 ± 12.1	2.7
280.0 ± 7.0	2.5	243.0 ± 19.8	8.1
140.0 ± 9.0	6.3	130.0 ± 0.00	0.0
27.9 ± 1.8	6.5	29.9 ± 3.6	12.0

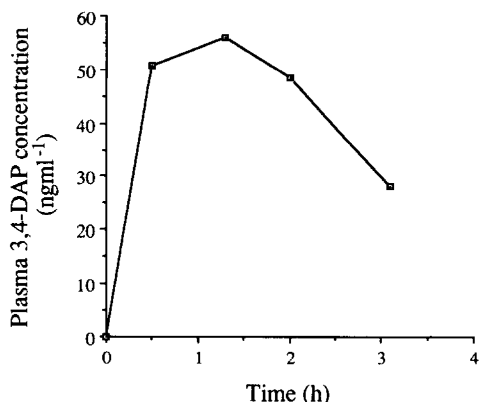

Figure 2
Plasma 3,4-DAP concentrations following oral administration of 3,4-DAP (10 mg) in a patient with myasthenia gravis.

Conclusions

The objective of this work was to develop a separation method that was both sensitive and simple and which was applicable for the analysis of a large number of samples in a pharmacokinetic study. When compared to other methods, the advantages of this method lie in its assay sensitivity, reproducibility and its reliability. This method is currently being used to determine 3,4-DAP pharmacokinetics in patients treated with the drug.

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[Received for review 28 September 1994; revised manuscript received 10 November 1994]