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Determination of A 3,4-diaminopyridine in plasma by liquid chromatography with electrochemical detection using solid-phase extraction

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Abstract

In order to quantify a small amount of a drug, 3,4-diaminopyridine (3,4-DAP), in animal plasma samples, an analytical method was developed. It involved an extraction of 3,4-DAP and phenylephrine, used as internal standard (IS), from plasma with solid-phase extraction (SPE) on C18 cartridges. This analytical method is a hyphenated technique based on high-performance liquid chromatography with electrochemical detection (HPLC–EC) whose purpose is to obtain first a sensitive method and second a satisfying separation between 3,4-DAP and phenylephrine. The analytical method is accurate, specific, and linear between 10 and 500 μg of 3,4-DAP per litre. The recovery of 3,4-DAP is estimated at 70.8% with a 95% confidence interval of (66.0–75.6%). Intermediate precision was evaluated on three quality control samples; the intra-day precision was estimated at 13.5, 9.1, 7.8% and the inter-day precision at 17.9, 8.4, 9.3%. The limit of quantification of the method was evaluated at 10 $\mu g\,l^{-1}$. First toxicokinetic parameters determined on dogs plasma samples after one 3,4-DAP oral administration of 1 $mg\,kg^{-1}$ were: $C_{max} = 395.7\ \mu g\,l^{-1}$; $T_{max} = 15$ min; $t_{1/2} = 113.6$ min; Clearance/$F = 16.8\ ml\,kg^{-1}\,min^{-1}$ and Vd/$F = 2.7\ l\,kg^{-1}$.

Keyword: 3,4-Diaminopyridine

1. Introduction

Lambert-Eaton myasthenia syndrome is a rare disease whose clinical expressions are myasthenic features in the form of variable muscle weakness and the fatigability. This pathology is due to disorders of neuromuscular junctions resulting in blocking acetylcholine release. 3,4-Diaminopyridine (3,4-DAP) is usually prescribed to treat this disorder: it liberates acetylcholine, and it is more efficient and less toxic than other treatments [1–6]. The Pharmacist Establishment of Paris Hospitals (EPHP) controls and distributes the 3,4-DAP raw material to hospital pharmacies which manufacture the 3,4-DAP capsules. Recently, the EPHP was asked by an association of patients ("Association Française contre les Myopathies"), to supply a medicinal treatment to patients affected by Lambert-Eaton myasthenic syndrome.

In order to determine in vivo toxicokinetic profiles of 3,4-DAP as part of toxicological studies on two animals species (dog and rat), the quantification of the active substance in plasma samples was necessary. Since the amounts of 3,4-DAP, administered in animals by gavage, are relatively low ($<1.0\ mg\,kg^{-1}$ 3,4-DAP) particularly in chronic toxicity, the toxicokinetic studies require an outstanding analytical method in terms of sensitivity and specificity.

In the analytical field, very few techniques have been described that allow the quantification of 3,4-DAP in biological fluids. The major aim of these previous techniques involved hyphenated methods combining high-performance liquid chromatography and spectrometry in UV [7–9]. The amounts of 3,4-DAP were also determined after several steps leading to purification and concentration of the studied samples. Endogenous substances that represent potential interfering compounds were removed by solid-phase extraction (SPE) using silica base with C18 coating cartridges.

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However, in these previous studies, the existing analytical methods did not allow sufficient sensitivity for accurate quantification of the active substance during the elimination phase of the drug, despite of good extraction recoveries of 3,4-DAP in plasma samples.

Therefore, this work describes an analytical approach leading to a significant lower limit of quantification of 3,4-DAP in plasma, based on the techniques present in our laboratory.

It is based on high-performance liquid chromatography coupled with electrochemical detection (HPLC–EC). The choice of such detection mode was supported by preliminary studies on the oxidation mechanisms of the active substance by hydrogenperoxide. However, after extraction, some residues of biological endogenous substances were remaining in sample which would lead to passive the glassy carbon electrode. Therefore, two steps of purification of the studied samples, consisting of a solid-phase extraction and a suppression of residual impurities during chromatography were included in the analytical methodology.

2. Experimental

2.1. Analytical standard and reagents

3,4-Diaminopyridine salt was purchased from SERATEC (Courville sur Eure, France). Phenylephrine hydrochloride, used as internal standard (IS) was purchased from Sigma–Aldrich (Steinheim, Germany). Methanol (VWR International, Fontenay sous bois, France), Ethanol (Merck, Darmstadt, Germany), Acetonitrile (Sigma–Aldrich, Seelze, Germany), Acetone (Merck Eurolab, Briaire le canal, France), Ammonium and sodium acetate (Merck, Darmstadt, Germany). All solvents were of HPLC grade.

2.2. Apparatus and chromatographic condition

2.2.1. Solid-phase extraction

The SPE procedure was performed on an automatic system (ASPEC XL4, GILSON; Villiers-le-Bel, France) piloted by 735 Sampler Software.

The extraction procedure was performed on SPE cartridges, C18 (500 mg, 3 ml), purchased from Varian® (Les Ulis, France).

2.2.2. Chromatographic system and detection

2.2.2.1. Chromatographic system. The chromatographic system was composed of a HPLC apparatus from ThermoSeparationProduct (TSP) (Les Ulis, France) which includes helium degasser (SCM400), a quadratic pump (SpectraSystem P1000 XR) and an auto sampler (AS 3000). This system was piloted by the Chromquest® software.

Chromatographic analysis of plasma samples were performed on two columns: during the first step of the chromatographic separation, the 3,4-DAP and its internal standard were retained on the cyano column while the endogenous substances of the plasma samples were eluted to the waste owing to an automatic three-port valve. In the second time, after 1.4 min, the commutation valve switched automatically to a second cyano column where the compounds were separated. The first column was a Discovery®Cyano 100 μm × 4.6 μm × 5 μm purchased from Supelco (Bellafonte, USA). The second one was a Zorbax SB-CN 250 μm × 4.6 μm × 5 μm from Interchim (Montluçons, France).

For the isocratic mobile phase, a binary mixture 75/25 (v/v) acetonitrile/ammonium acetate (6 mM), sodium acetate (50 mM) adjusted to pH 6.0 with acetic acid, was selected. Flow rate was set at 1.2 ml min^{-1}.

2.2.2.2. Electrochemical detection. The amperometric detector was an EC2000 purchased from TSP (Les Ulis, France). The electrochemical detection was carried out in the oxidation mode at +1.0 V on a carbon glass working electrode versus a reference electrode of Ag/AgCl.

2.3. Preparation of standard solutions and calibration samples

A solution was prepared by dissolving an accurately weighed amount of 3,4-DAP in water to give a concentration of approximately 1.0 g l^{-1}. This solution was then successively diluted in water to give a final concentration of 2 mg l^{-1} of 3,4-DAP. It was used to spike the drug free plasma to cover the calibration range of 1–500 μg l^{-1} and to prepare quality control of 20, 80 and 160 μg l^{-1}. These spiked plasma samples were stored at −20 °C.

2.4. Extraction method

Two hundred and fifty microliters of plasma, 250 μl of internal standard solution (125 μg l^{-1}) and 1.5 ml of phosphate buffer pH 7.2 were added in a 10 ml tube and mixed. SPE cartridges were conditioned successively with 3 ml of methanol and 2 ml of water. Then 9 ml of the sample tube content were loaded on the SPE cartridge. The SPE cartridge was washed with 3 ml of water. Then 3,4-DAP and phenylephrine were twice eluted by 1 ml of acetonitrile/ethanol/ammonium carbonate 1% (w/v) in the following ratio 30/60/30 (v/v/v) in a 6 ml tube. The eluent was evaporated to dryness at 55 °C under a gentle stream of nitrogen. To accelerate the evaporation, 2 ml of acetone were added to the eluent leading to an azeotrope mixture. The residue was dissolved in 200 μl of water and transferred into a micro insert (250 μl). Fifty microliters were injected in the chromatographic system.

2.5. Quantification

The quality control solutions were used to determinate the intra- and inter-days variation of the tests ($n = 6$). The analytical method validation was performed by estimating

its specificity, sensibility (the limit of detection and the limit of quantification), linearity and accuracy in order to define a dynamic range in which theses qualities are respected. This was performed by following recommendations of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [10–13] and the Internationnal Conference of Harmonisation (ICH) [14,15].

2.6. Recovery

Two hundred and fifty microliter of various stock solutions of 3,4-DAP (1, 5, 10, 25, 200, 300, 400 and 500 $\mu g\,l^{-1}$) were mixed with 250 μl of plasma and 1.5 ml of phosphate buffer (pH 7.2). They were extracted following the described extraction procedure. Two hundred and fifty microliter of phenylephrine solution (125 $\mu g\,l^{-1}$) was added before drying samples.

A second series of samples was prepared simultaneously by the extraction of 250 μl of blank plasma mixed with 250 μl of water and 1.5 ml of phosphate buffer (pH 7.2). 3,4-DAP and phenylephrine solutions were added to the extraction residue at the concentration noted above before drying samples. Then 200 μl of water were added, and 50 μl were injected in the chromatographic system.

The analytical recovery was calculated by comparing the slope of the first calibration curve to the second one. The value was presented in their confidence limits at 95% level established on several tests ($n = 6$).

2.7. Preclinical study

The analytical method was applied to toxic kinetic dog samples. A female beagle dog (Marshall farm, New York, USA), 10 months old and weighting 7.9 kg, was given 1 mg of 3,4-diaminopyridine kg^{-1} once by oral gavage. Blood sampling for toxic kinetic evaluation was performed on the animal at different times: before dosing, 5, 15, 30 and 45 min, 1 h, 1 h 30 min, 2 h, 2 h 30 min, 3 h, 3 h 30 min, 4, 6, 12 and 24 h. Two hundred and fifty microliter of plasma samples were analysed with the analytical method previously described.

3. Results and discussion

3.1. SPE performances: recovery

Because of the instability of 3,4-DAP and of phenylephrine at high pH values, samples were prepared in phosphate buffer at pH 7.2. As for the pK_a values of 3,4-DAP [16] (0.49 and 9.2) and of phenylephrine (10.1) [17], the two amino compounds are protonated under this condition and theirfore their retention on the hydrophobic C18 stationary phase could not be explained by the only partitioning mechanism. Thus, their was shown by small recovery extraction when samples were prepared at pH 3.

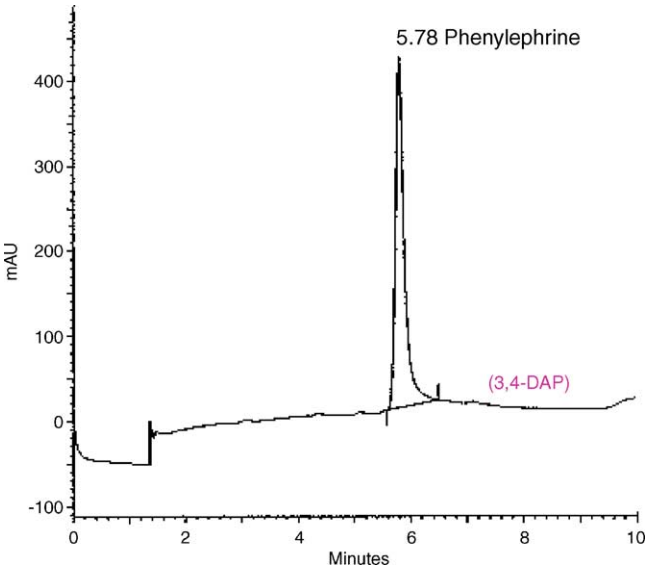

Fig. 1. Blank serum spiked with phenylephrine (125 $\mu g\,l^{-1}$).

The extraction recovery for 3,4-DAP was calculated by the slopes ratio between the two calibration curves: the calibration curve obtained from the extracted plasma samples and the one obtained from the samples to which the 3,4-DAP and the internal standard had been added after extraction. The recovery of 3,4-DAP was evaluated at 70.8% with the 95% interval of confidence of 66.0–75.6% and the recovery of IS was evaluated at 90.0% with the 95% interval of confidence of 80.1–100.0%.

3.2. HPLC system performance

Fig. 1 presents a blank serum spiked with IS. As shown in Figs. 2 and 3, a good resolution was observed between 3,4-DAP and its internal standard (Rs = 4.0 ± 0.2),

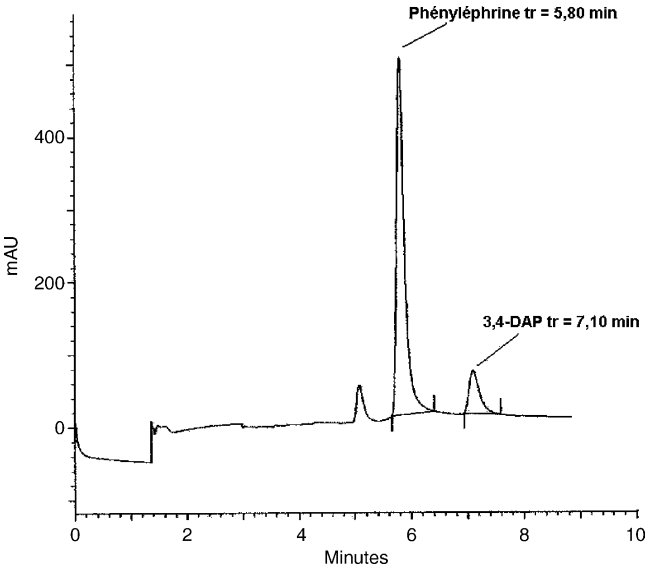

Fig. 2. Chromatogram of the analysis of a standard containing 25 $\mu g\,l^{-1}$ of 3,4-DAP and 125 $\mu g\,l^{-1}$ of phenylephrine.

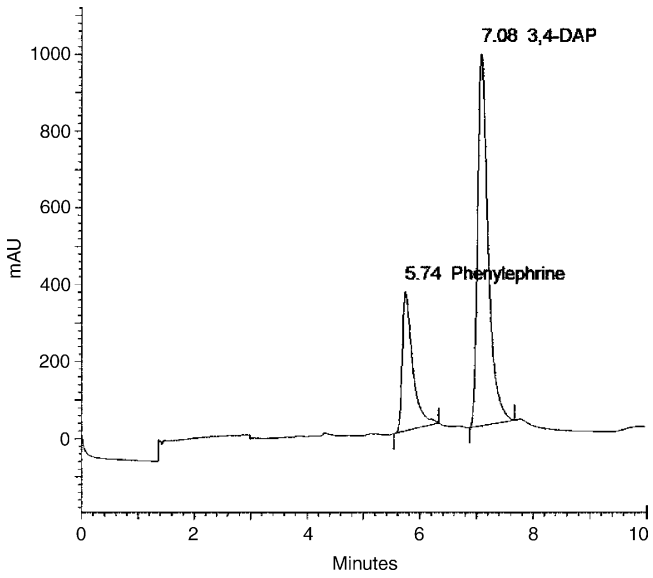

Fig. 3. Chromatogram of dog plasma sample containing 333.3 $\mu g\,l^{-1}$ after oral administration of 0.3 $mg\,kg^{-1}$ of 3,4-DAP.

phenylephrine without interferences due to endogenous plasma compounds. This chromatographic approach allowed satisfactory elimination of residual impurities from plasma and constituted a necessary step for the carbon glass working electrode protection. Capacity factor of 3,4-DAP and phenylephrine was found to be respectively around 0.66 and 1.03. This assembly system did not increase the pressure drop nor alter the efficiency of the two chromatographic peaks. The mobile phase had been optimised for the separation of the studied compounds.

3.3. Electrochemical detection performances

Preliminary voltametric studies have been performed for the determination of the oxidation potential of 3,4-DAP. The data analysis showed that oxidation of 3,4-DAP began at +0.8 V and the detector response was enhanced as the working electrode potential was increased from +0.8 to +1.0 V. With additional applied potential from +1.0 to +1.3 V, no significant increase in 3,4-DAP peak area occurred. Consequently, the operating oxidation voltage was set at +1.0 V in order to reduce damage caused on the working electrode and to maintain electrolysis recovery of 3,4-DAP. Moreover, the EC detection performance was influenced by the ionic strength of the mobile phase. The best condition to ensure the most sensitive detection was a concentration of salt of about 50–100 mM. Due to the chromatographic effect of sodium and ammonium ions on 3,4-DAP and phenylephrine elution, the ratio between the two salts was adjusted to obtain a satisfying resolution.

3.3.1. Limits of detection and quantification

The limit of detection of the method was 5 $\mu g\,l^{-1}$ of 3,4-DAP using 250 μl of plasma. For this minimum detectable concentration, a signal-to-noise ratio of approximately 3:1 was observed. The limit of quantification of the method was evaluated by establishing a linear relation between variation coefficient (%) and the inverse of 3,4-DAP concentration (Fig. 4). Then the limit of quantification was the concentration of 5 $\mu g\,l^{-1}$ with 15% of coefficient of variation.

3.3.2. Linearity

The method linearity was verified by using statistical tests. They showed that the relation between detector response and concentration expressed as square root was linear between 1 and 500 $\mu g\,l^{-1}$.

3.3.3. Precision and accuracy

The precision (expressed by the coefficient of variation of replicate analyses) was estimated on three quality control levels and accuracy (expressed by the ratio between

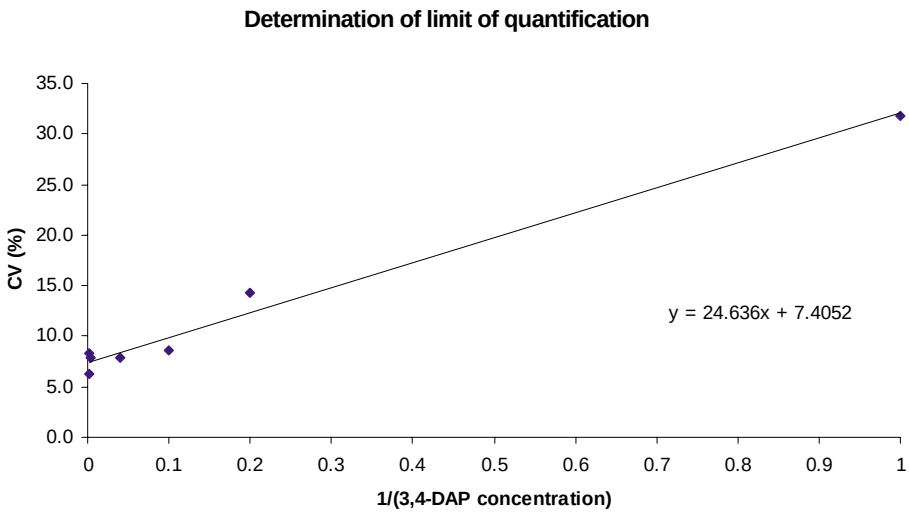

Fig. 4. Linear relation between 3,4-DAP concentration and variation coefficients ($n = 6$).

Table 1
Accuracy profile of 3,4-DAP determination in plasma

3,4-DAP concentration, $n = 6$ (ng ml^{-1})	Mean	CV (%)	Accuracy ($IC_{95\%}$)
1	2.5	30.1	[1.9; 3.1]
5	3.8	13.4	[3.4; 4.2]
10	11.1	7.9	[10.3; 11.7]
25	22.2	7.8	[20.8; 23.6]
200	182.8	14.9	[161.1; 204.6]
300	301.7	8.6	[280.9; 322.5]
400	421.7	6.7	[399.2; 444.1]
500	499.0	8.3	[465.7; 532.3]
Calibration curve	$y = 193.42x - 3.3311$		
Correlation coefficient (r)	0.999		

measured and theoretical concentration) of the method was evaluated between 1 and 500 μg l^{-1} of 3,4-DAP.

The intra-day precision was determined by analysing six quality controls in 1 day. The inter-day precision was evaluated on six quality controls analysed on different days. The precision was estimated at 13.5, 9.1, 7.8% and 17.9, 8.4, 9.3% for, respectively, intra- and inter-day precisions.

Table 1 presents the accuracy profile of 3,4-DAP by this analytical method. Each calibration point was represented as a percentage of measured concentration on theoretical concentration. This profile showed that the first points (1–5 μg l^{-1}) could not be taken into account in the experimental field because they could not be accurately quantified. The analytical method was specific, since no endogenous compound interfered with 3,4-DAP and phenylephrine.

The analytical validation evaluated by previous criteria showed that quantification of 3,4-DAP in plasma could be accurately determined with acceptable precision inside the concentration range of 10–500 μg l^{-1}.

3.4. Pre-clinical study

Little information on 3,4-DAP pharmacokinetic parameters exists in literature for either human beings or animals.

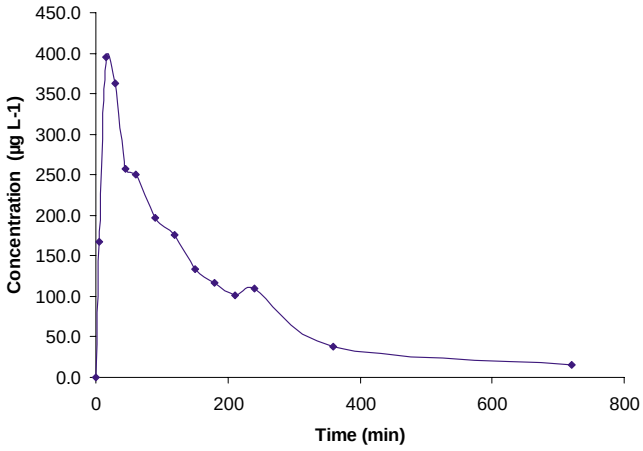

Fig. 5. Plasma-concentration–time course of 3,4-DAP following a single oral administration dose of 1 mg kg^{-1} to a female dog.

One publication gave inaccurate pharmacokinetic parameters for human being [18]. The analytical method was applied on dog plasma samples. Fig. 5 shows a plasma-concentration profile of 3,4-DAP after oral administration of 1 mg kg^{-1}. The excellent precision of the method and its large dynamic range allowed the determination of high and low concentrations, without re-analyse, and with accurate determination of pharmacokinetic parameters: $C_{max} = 395.7$ μg l^{-1}; $T_{max} = 15$ min; $t_{1/2} = 113.6$ min; clearance/$F = 16.8$ ml kg^{-1} min^{-1} and Vd/$F = 2.7$ l kg^{-1}.

4. Conclusion

The principal difficulty of this development is to give accurate and sensitive results with a sensitive detection from techniques used in our laboratory. Electrochemical detection after HPLC separation and SPE extraction allowed the detection of small amounts of 3,4-DAP where UV detection method have failed. But this kind of hyphenated method requires special attention, such as the elimination of all endogenous oxidable compounds, to ensure method quality. It also provides a specific separation of 3,4-DAP and its internal standard from endogenous plasma components owing to SPE extraction and the use of a rotary valve permitting the elimination of residual impurities. The analytical method was sufficiently specific and sensitive to determine 3,4-DAP concentrations on a large dynamic range with accuracy and precision. Its application to the analysis of animal plasma samples leads to the accurate determination of 3,4-DAP concentrations and toxicokinetic parameters for a preclinical study on dogs.

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