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Note

Analysis of 3,4-diaminopyridine in human serum by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection

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Potassium channel blockers such as 4-aminopyridine (4-AP) have been proposed for the symptomatic treatment of multiple sclerosis based on advances in the understanding of action potential propagation along myelinated axons [1,2] Although some improvements were observed with 4-AP, concerns about its toxicity have led to studies of the related but less toxic 3,4-diaminopyridine (3,4-DAP, Fig 1) [3,4] In order to correlate any observed toxic symptoms with the serum concentration of the drug during a clinical trial of orally administered 3,4-DAP to patients with multiple sclerosis an assay for this compound in serum was required. The high-performance liquid chromatographic (HPLC) quantification of 3,4-DAP in the supernatant after precipitation of serum proteins described by Lamiable and Millart [5] had a reported sensitivity of 100 ng/ml, which was insufficiently sensitive for our needs Extraction methods using organic solvents followed by HPLC are reported to have sufficient sensitivity for the related compounds 4-AP [6,7] and 2,4-DAP [8] but did not produce satisfactory results for the analysis of 3,4-DAP in our laboratory. A gas chromatographic method has been described [9]. We thus have

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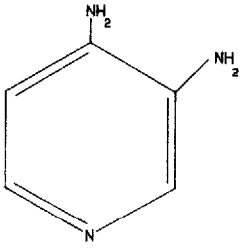

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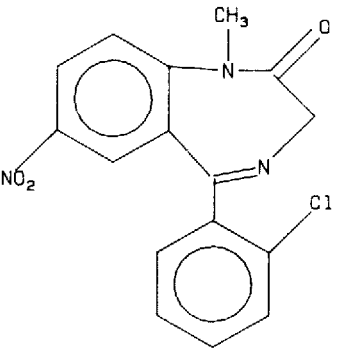

(II)

Fig 1 Chemical structures of 3,4-diaminopyridine (I) and N-methylclonazepam, the internal standard (II)

developed a method in which the 3,4-DAP is extracted onto a solid-phase column sample, eluted off the column and quantified by HPLC. Using this procedure 3,4-DAP concentrations in serum as low as 5 ng/ml can be measured with satisfactory precision.

EXPERIMENTAL

Materials

The 3,4-DAP was purchased from Regis (Morton Grove, IL, U S.A) A stock aqueous solution of 3,4-DAP was prepared by dissolving 10.0 mg in 250 ml purified water to yield a concentration of 40 μg/ml; 1 0 ml of this solution was diluted to 25 ml with drug-free human serum This 800 ng/ml solution was serially diluted with the drug-free serum to obtain standards in the appropriate concentration range. Serum standards and patient samples were stored at $-20°$C Trimethylammonium chloride was obtained from Aldrich (Milwaukee, WI, U.S A.) and potassium dihydrogenphosphate from J T Baker (Phillipsburg, NJ, U.S A) HPLC-grade acetonitrile and methanol were also obtained from J T Baker Water was purified using a Millipore Milli-Q system (Millipore, Bedford, MA, U S.A.). A stock solution (100 μg/ml) of N-methylclonazepam (Fig. 1) (Hoffmann-La Roche, Nutley, NJ, U.S.A.) was prepared by dissolving 10 0 mg of the compound in 100 ml of acetonitrile A 1-ml volume of this stock solution was diluted to 200 ml with water to yield a working internal standard solution with a concentration of 500 ng/ml

Sample preparation

C_{18} Bond Elut 1-ml solid-phase extraction columns (Analytichem International, Harbor City, CA, U.S.A) were conditioned by passing 2 ml of methanol through them, followed by 2 ml of water using a vacuum of 40–60 kPa in a Vac Elut manifold (Analytichem International) A 500-μl volume of serum standard or sample was added to the conditioned column and washed with 2 ml of water and then by two 250-μl portions of acetonitrile; the washes were discarded The 3,4-DAP, which was retained on the column, was eluted with 100 μl of a solution containing 100 mg trimethylammonium chloride dissolved in a mixture of 80 ml acetonitrile and 20 ml water, followed by 100 μl of water The eluates were collected in 1.5-ml polypropylene tubes (VWR Scientific, San Francisco, CA, U S A.). A 100-μl volume of the working N-methylclonazepam solution was added and the solution thoroughly mixed Of this solution 100 μl were injected into the equilibrated HPLC system.

Chromatography

The chromatographic system used consisted typically of a Waters (Milford, MA, U.S.A) M6000 solvent delivery system set at 1 0 ml/min. The mobile phase was prepared by mixing 600 ml of acetonitrile with 330 ml of water and 70 ml of a buffer (3 5 g of potassium dihydrogenphosphate and 1 g of trimethylammonium chloride per liter, adjusted to pH 7 4 with 10 *M* sodium hydroxide), filtered through a 0 45-μm filter (47-mm Nylon-66, Rainin, Woburn, MA, U S A) and degassed by ultrasonication The analytical column was from Phenomenex (Rancho Palo Verdes, CA, U S.A.) packed with C_{18} μBondapak (Waters), particle size 10 μm (30 cm $\times$ 3 9 mm I.D), fitted with a Waters guard column (84550) packed with Pellicular ODS packing, 30 μm particle size (Whatman, Clifton, NJ, U.S.A.). Injections were made either with a Perkin-Elmer (Norwalk, CT, U.S.A.) ISS-100 autoinjector or a 7125 manual injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 100-μl loop. The eluent was monitored with a Perkin-Elmer LC235 ultraviolet detector set at 290 nm and connected to either a C-R3A (Shimadzu, Columbia, MD, U S.A) or a 3390A integrator (Hewlett-Packard, Avondale, PA, U.S A.) in the peak-height mode at an attenuation of 2.

RESULTS

Typical chromatograms of drug-free serum and serum from a patient who had been administered 3,4-DAP are shown in Fig 2. The 3,4-DAP and N-methylclonazepam elute at 10 and 5 min, respectively

Extraction efficiency

By comparing the peak heights of aqueous 3,4-DAP solutions and 3,4-DAP in serum processed through the extraction procedure with aqueous solutions injected directly into the chromatographic system it was ascertained that re-

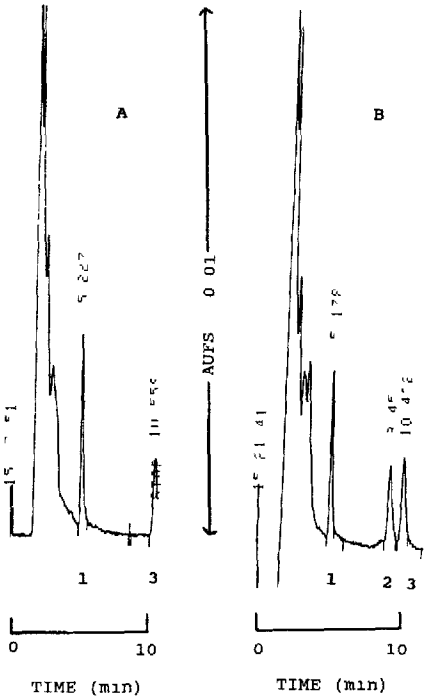

Fig 2 Chromatograms from an extract of a patient sample (A) before administration of DAP and (B) after oral administration of DAP The peak corresponds to a concentration of 49 6 ng/ml Peaks 1 = internal standard, 2 = DAP, 3 = serum peak

covery of the drug from the columns was virtually 100%. However, because 500 μl of serum are used and, in effect, reconstituted into less than a 300-μl volume, the 3,4-DAP is concentrated in the process

Detection limit

Using the procedure described a concentration of 5 ng/ml could be quantified in serum with an intra-assay precision (coefficient of variation, C.V.) of 13%. At higher concentrations the C.V. was less than 10%. At a signal-to-noise ratio of 3, the minimum amount of 3,4-DAP detectable on the column was 0.5 ng.

Selectivity

Drug-free normal sera and sera from patients with multiple sclerosis before DAP administration showed no interfering peaks at the retention times of the drug or internal standard when processed as described above. The possibility of the in vitro degradation or in vivo metabolism of 3,4-DAP to 3-AP or 4-AP was considered. 3-AP was eluted at a much shorter retention time than the parent drug while 4-AP eluted at essentially the same retention time (Table

TABLE I

RETENTION TIMES OF DRUGS

Compound	Retention time (min)	Compound	Retention time (min)
3,4-Diaminopyridine	10 0	Desipramine	>30
N-Methylclonazepam	5 0	Diazepam	6 0
2-Aminopyridine	3 4	Ditrepan	3 7
3-Aminopyridine	3 4	Doxepin	>30
2,3-Diaminopyridine	3 2	Ibuprofen	<3
2,6-Diaminopyridine	3 2	Imipramine	>30
4-Aminopyridine	10 1	Nortriptyline	>30
Acetaminophen	<4	Oxazepam	3 8
Amitriptyline	>30	Protriptyline	>30
Baclofen	3 2	Salicylic acid	<3
		Trimipramine	>30

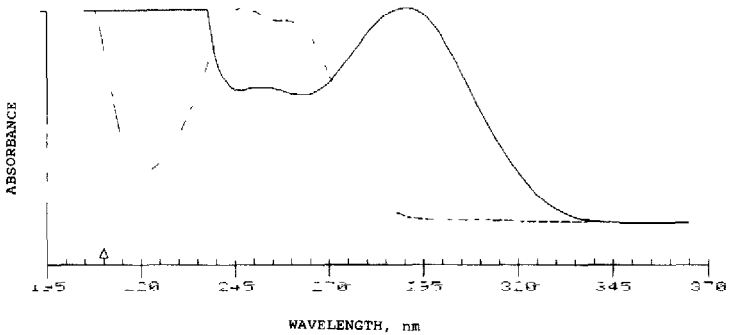

Fig 3 Ultraviolet absorption spectra of 3,4-DAP and 4-AP obtained from chromatographic peaks after injection of 20 μl of 40 μg/ml solutions of the pure compounds The absorbance maximum for 3,4-DAP (solid line) is 0 0658 at 290 nm and for 4-AP (broken line) it is 0 1052 at 247 nm

I). However, the ultraviolet absorption spectra of 3,4-DAP and 4-AP are very much different as shown in Fig. 3, with 3,4-DAP and 4-AP having absorption maxima at 290 and 247 nm, respectively That 4-AP poses little potential for interference in the quantification of 3,4-DAP is supported by the data in Fig. 4, which shows that at comparable concentrations the peak height of 4-AP at 290 nm is negligible compared with that of 3,4-DAP. Therefore, the absorption spectra of the peaks observed for 3,4-DAP were routinely monitored utilizing the diode array capabilities of the LC235 detector to generate an ultraviolet spectrum of the peak compound. There was no evidence of any formation of 4-AP in patient samples. 4-AP and 3,4-DAP could only be satisfactorily separated by lowering the acetonitrile concentration in the mobile phase to 5%

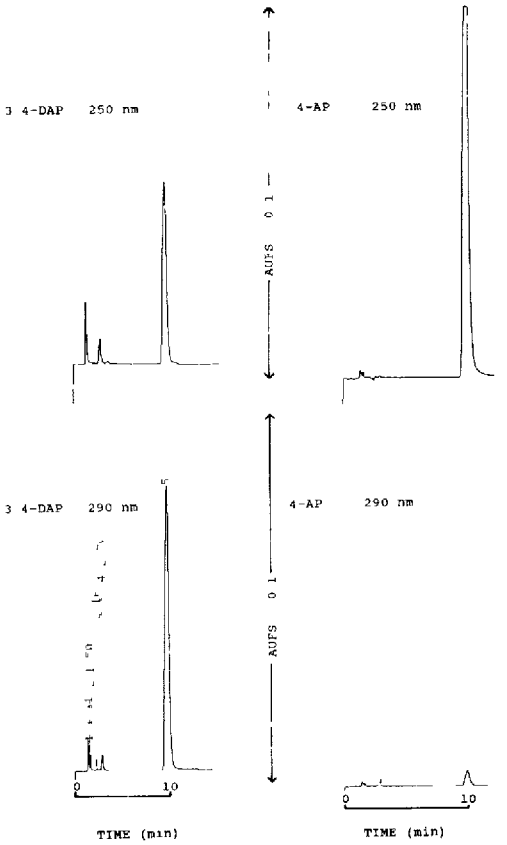

Fig 4 Chromatogram of 4-AP monitored simultaneously at 250 and 290 nm after injection of 20 μl of a 40 μg/ml solution of the pure compound A similar chromatogram is shown for 3,4-DAP

(v/v), but late-eluting serum component peaks precluded using this mobile phase in the routine assay of 3,4-DAP

An endogenous compound eluted shortly after the 3,4-DAP and the degree of its separation from the drug varied with the previous use of the column, but the peaks could be resolved by slight modification to the proportion of the buffer component in the mobile phase. The retention time of the 3,4-DAP was relatively insensitive to the concentration of the acetonitrile in the mobile phase but was strongly dependent on the concentration of the trimethylammonium chloride The mobile phase composition described above was suitable for a new column, but a higher proportion of the amine–phosphate was necessary for an older column.

A number of compounds, including other aminopyridines, common analgesic compounds and muscle relaxants, were injected into the chromatographic system. The compounds and their retention times are listed in Table I; none

of them, except for 4-AP discussed above, interfered with 3,4-DAP or the internal standard.

Linearity

When sera spiked with 3,4-DAP at concentrations of 5, 10, 25, 50, 75, 100 and 200 ng/ml were processed the ratio of drug peak to that of internal standard versus concentration was linear The equation of the line is $y=0.00835x+0.0227$, with $r=0.998$. The mean response factor (ratio divided by concentration) for the seven standards was 0 00912 with a C.V. of 8.1%.

Precision and accuracy

The intra-run precision of the assay was ascertained by processing a series of spiked sera through the assay procedure in the concentration range 5–100 ng/ml. The results are summarized in Table II and show that acceptable precision and accuracy are obtained in this concentration range.

TABLE II

INTRA-ASSAY PRECISION AND ACCURACY

Concentration added (ng/ml)	*n*	Concentration found (mean ± S D) (ng/ml)	C V	Percentage deviation from mean
5 0	5	5 25 ± 0 70	13 2	+5 0
10 0	5	10 1 ± 0 59	5 9	+1 0
25 0	3	25 4 ± 0 96	3 8	+1 6
50 0	3	50 1 ± 0 98	2 0	+0 2
75 0	6	73 5 ± 6 25	8 5	−2 0
100	3	104 ± 6 35	6 1	+0 4

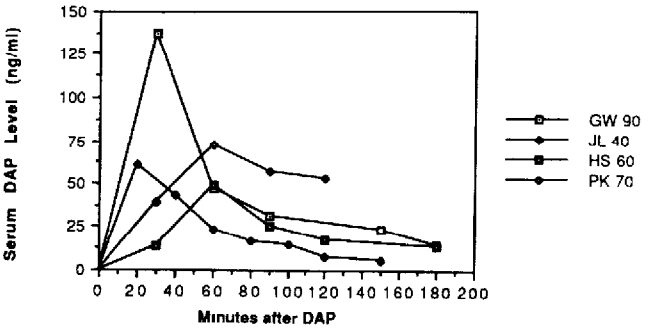

Fig 5 Serum 3,4-DAP concentrations in four patients with multiple sclerosis (male, age range 38–61 years) after oral administration of the drug The numbers after the initials are the doses (in mg) administered

Application

Oral doses of 3,4-DAP were administered to patients with multiple sclerosis and their sera assayed for the drug. A set of standards was processed with each batch of patient samples and control samples were placed after each batch of eight samples The concentration of 3,4-DAP in patient samples was routinely calculated using the mean response factor obtained for the standards. The control samples were generally well within 10% of the nominal value. Typical results for the patient samples are shown in Fig. 5.

DISCUSSION

The method described here is simple, reliable and fast enough to be used for the routine monitoring of therapeutic concentrations of 3,4-DAP in patients and sensitive enough for serum samples obtained in pharmacokinetic studies. Initial results show that 3,4-DAP has a half-life of about 2 h in patients with multiple sclerosis and that there is a high degree of inter-patient variability in the serum level concentrations for a given dose of drug.

The method is more sensitive (down to 5 ng/ml) for 3,4-DAP than the previously described method involving protein precipitation which had a sensitivity of 100 ng/ml [5]. The solid-phase extraction is more convenient than solvent extraction which has been used in sensitive methods for the assay of 4-AP [6,7] and 2,4-DAP [8] and obviates the potential loss of the volatile aminopyridines which has to be minimized by the addition of trifluoroacetic acid or pentanol in the solvent evaporation stages

A difficulty in developing the assay was in obtaining an internal standard that would have behave in a similar manner to 3,4-DAP on the extraction column and yet have acceptable ultraviolet absorption and chromatographic retention times. Tricyclic antidepressants and aminopyridines related to 3,4-DAP were suitable for the extraction process but had unsuitable chromatographic retention times, either coeluting with peaks from endogenous compounds or eluting too late (Table I). Since the recovery of 3,4-DAP from the extraction column is 100% a source of variability was deduced to be due to the differences in the small volumes in the final elution steps from the extraction columns. For this reason the N-methylclonazepam, which was found to elute at a suitable retention time in the chromatography, was added to the final eluted sample.

Although the method was specifically developed for the assay of 3,4-DAP in serum, preliminary results indicate that with minor modification it can also be used for the assay of 3,4-DAP in cerebrospinal fluid

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