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Note

A simple and sensitive method for the determination of perhexiline in plasma using gas—liquid chromatography with nitrogen—phosphorus detection

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Perhexiline is a calcium agonist used in the treatment of angina pectoris. The use of this drug is limited to patients who do not respond to conventional drug therapy and who are considered unsuitable for surgery. The reason for the poor general acceptance of perhexiline is the incidence of serious side effects that have been reported to occur during treatment with the drug [1].

Although it has been suggested that the kinetics of the drug are non-linear, very little is known of the disposition of perhexiline [2]. To a large extent this is due to poor analytical methods available for the measurement of perhexiline. Available methods have used laborious extraction techniques and then measurement by gas—liquid chromatography (GLC) with either flame ionization or electron-capture detection [3—5]. Detection by electron capture requires derivatisation with trifluoroacetic anhydride or heptafluorobutyric anhydride. Similarly, a high-performance liquid chromatographic (HPLC) method has recently been developed which also involves an extraction procedure and then derivatisation (with Dns chloride) to form a fluorescent product [2]. While this method has good sensitivity, the chromatography time is long.

The GLC method described here is precise, reproducible and requires only a protein precipitation step followed by a back extraction into solvent. No solvent evaporation or derivatisation procedure is required and the use of nitrogen—phosphorus detection provides good specificity and sensitivity.

EXPERIMENTAL

Analyses were performed on a Hewlett-Packard 5730A gas chromatograph fitted with a nitrogen—phosphorus detector. The following chromatographic conditions were employed: glass column (1.6 m × 2 mm I.D.) containing 3%

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OV-101 on Gas-Chrom Q, 100—120 mesh (Applied Sciences Labs., State College, PA, U.S.A.), column temperature 218°C, injector port and detector temperatures 250°C and nitrogen carrier gas flow-rate 20 ml/min.

Reagents and standards

A pure sample of perhexiline maleate was supplied by the William S. Merrell Company (Sydney, Australia) and benzhexol, the internal standard, was extracted from a tablet preparation (Artane, Cyanamid Australia, Sydney, Australia). Silyl-8 was supplied by Pierce (Rockford, IL, U.S.A.).

A standard solution of perhexiline was prepared by dissolving perhexiline maleate (0.14188 g) in 100 ml of ethanol giving a concentration of 1000 mg/l perhexiline. A 10-ml aliquot of this was further diluted to 100 ml with 1.0 *N* hydrochloric acid giving a standard solution containing 100 mg/l perhexiline. Working standard solutions containing 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/l perhexiline were prepared daily by making dilutions from the 100 mg/l stock solution with distilled water. Plasma standard solutions containing 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/l perhexiline were prepared by taking 0.1-ml aliquots of the working standards and adding 0.9 ml of drug-free plasma.

The internal standard was prepared by extracting a tablet containing benzhexol hydrochloride (5 mg) with 30 ml of methanol. The extraction was aided by breaking up the tablet with a spatula while shaking with methanol. The insoluble tablet matrix was removed by filtration through a Whatman No. 1 filter. The filtrate was made up to 100 ml with methanol. A 1.0-ml aliquot of this solution was then diluted to 50 ml with methanol to give a final concentration of approximately 1.0 mg/l.

Extraction

To a 5-ml disposable plastic tube fitted with a cap was added 1.0 ml of plasma (patient sample or standard) followed by 1.0 ml of methanol containing the internal standard, and then 0.5 ml of 5 *N* hydrochloric acid. The mixture was vortex mixed for 3 min and the samples allowed to settle for 5 min before centrifuging at 1500 *g* for 15 min. The supernatant solutions were decanted into conical centrifuge tubes followed by the addition of 0.5 ml 8 *N* sodium hydroxide. The tubes were allowed to cool and then 0.1 ml of 1,2-dichloroethane—hexane (8:2) was added. The contents of the centrifuge tubes were vortex mixed for 1 min and then centrifuged at 1500 *g* for 2 min to partition the aqueous and organic phases. The vortex mixing and centrifugation steps were repeated and finally 3.0 μl of the lower organic phase taken for injection into the gas chromatograph.

RESULTS AND DISCUSSION

Protein precipitation with reagents such as acetonitrile, methanol and aqueous solutions of trichloroacetic acid or mineral acids is frequently used in the preparation of plasma samples for analysis by HPLC [6]. The procedure is simple and rapid but nevertheless provides a matrix of suitable purity for analysis by this technique. However, injection of the plasma supernatant into a gas—liquid chromatograph after a protein precipitation step produces problems.

Firstly, endogenous material from plasma may interfere by giving an excessively large solvent front and pyrolysis products of plasma material could give a noisy baseline and extraneous peaks. Secondly, the small volume capacity of GLC (usually 10 μl) prevents the injection of larger volumes to improve poor sensitivity resulting from sample dilution by the protein precipitating agent. These two problems can be solved by extraction into a smaller volume of organic solvent after the protein precipitation procedure. The organic phase is then cleaner and has a higher concentration of analyte, thus making it suitable for analysis by GLC. Protein precipitation is quick and involves relatively small amounts of sample and reagent in comparison with other published methods. Although the use of a preliminary protein precipitation step in a GLC method is unusual, extremely clean chromatograms can be obtained. Fig. 1A is the chromatogram of a blank plasma sample showing it to be free of endogenous peaks which may interfere with perhexiline or the internal standard.

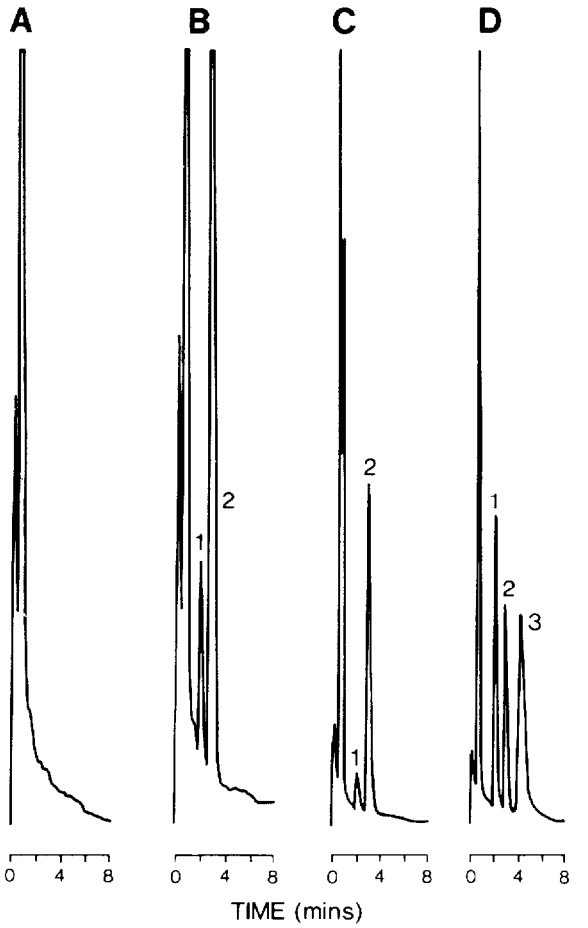

Fig. 1. GLC tracings of extracted plasma samples containing perhexiline and the internal standard, benzhexol. A, plasma blank; B, plasma perhexiline standard at 0.1 mg/l; C, plasma perhexiline standard at 0.1 mg/l but recorded at a 5-times reduced sensitivity of B; D, patient sample. Peaks: 1 = perhexiline, 2 = the internal standard, benzhexol, and 3 = an extraneous substance in the plasma.

Initially, a double extraction technique was investigated in order to obtain a clean and more concentrated sample for analysis. Various organic solvents were evaluated in extracting perhexiline from an alkaline sample of plasma and it was found that the following solvents extracted perhexiline in order of increasing recovery, diethyl ether < hexane < toluene < ethyl acetate. However, the quantitative back extraction of perhexiline from these organic solvents into a smaller volume of acid solution was not possible because the salts of perhexiline are very soluble in organic solvents. Of the following acids, hydrochloric, perchloric, nitric, sulphuric and phosphoric, only phosphoric acid gave a salt that would back extract into an aqueous phase to any significant extent. Attempted isolation of the acid extract by freezing, decanting of the organic phase then alkalinisation and extraction into chloroform also gave low recoveries and was not reproducible.

An alternative approach was to recover the perhexiline after extraction from plasma with an organic solvent. Although this technique is successful, as shown by Cooper and Turnell [3], the time involved in processing large numbers of samples was considered unacceptable.

In the method described in this paper, methanol and hydrochloric acid are used to precipitate protein. Protein precipitation using only concentrated acid gave very low recoveries (10%), presumably because the perhexiline binds to the protein pellet. Methanol is essential for high recoveries and the ratio of reagents has been optimised to give the best results. The following acids, 10% trichloroacetic, 10% perchloric and 5 *N* nitric were also tried in conjunction with methanol as precipitating agents and all were inferior to 5 *N* hydrochloric acid and methanol.

In the extraction procedure, dichloromethane and chloroform were tested but both are unsuitable because they evaporate easily during vortex mixing in the final extraction step. The less volatile 1,2-dichloroethane works well and the specificity of the extraction is improved by adding hexane (20%).

All the chromatopgraphic data were recorded on a dual-pen recorder with the pens set at 1 and 5 mV for full scale deflection. The attenuation of the GLC was set at 128 and with this arrangement the peak heights for perhexiline could be measured over the concentration range of 0.02—1.00 mg/l. Figs. 1B and C are chromatograms obtained for a 0.10 mg/l standard where B has 5 times the sensitivity of C. Fig. 1D is the chromatogram of a typical patient sample having a perhexiline concentration of 1.08 mg/l. The retention times of perhexiline and benzhexol (internal standard) are 2 min 24 sec and 3 min 30 sec, respectively.

The calibration curve is linear over the range 0.02—1.00 mg/l when peak height ratios for the standards are plotted against concentration. Although peak symmetry and linearity deteriorate with time, this can be corrected by injection of Silyl-8 and all new columns should be silanised with the reagent during the conditioning period.

Blank plasma was spiked with perhexiline to give solutions containing 0.05 and 0.50 mg/l. Each test solution was analysed in replicate on a between-day and within-day assessment. For the between-day analysis at a concentration of 0.05 mg/l (n = 8), the mean was 0.0484 ± 0.0026 S.D. with a coefficient of variation of 5.4%. At 0.50 mg/l (n =8), the mean was 0.4673 ± 0.0282 S.D. with a coefficient of variation of 6.0%. For the within-day analysis at the 0.05

mg/l concentration (n =10), the mean was 0.0463 ± 0.0013 S.D. with a coefficient of variation of 2.8%. Similarly, within-day analysis at the 0.50 mg/l sample gave a mean of 0.4861 ± 0.0072 S.D. with a coefficient of variation of 1.5%.

The recovery of perhexiline was determined by comparing (in triplicate) the peak heights for perhexiline obtained from plasma standards containing 0.1, 0.5 and 1.00 mg/l with standards made up in dichloromethane obtaining 1.0, 5.0 and 10.0 mg/l. Using this procedure the recovery of perhexiline was shown to be essentially quantitative, with the mean recovery over the 0.1 to 1.00 mg/l range being 94%. The recovery for the internal standard was determined in the same way (in triplicate) and was 103%.

Drugs that are used to supplement perhexiline therapy do not interfere with this assay procedure. These are beta-adrenoreceptor antagonists, metoprolol, pindalol, propranolol, labetalol, alprenolol, timolol, practolol and antenolol, the antiarrhythmic drugs mexiletine, procainamide, disopyramide and quinidine; and other drugs used in the treatment of angina, sorbide nitrate, nitroglycerine and verapamil.

Drugs that are extracted and interfere are the tricyclic antidepressants, amitriptyline, nortriptyline and doxepin. These are not used in the treatment of angina but may be encountered occasionally.

REFERENCES

1 M. Chevais, Rev. Med., 22 (1981) 335.
2 J.D. Horowitz, P.M. Morris, O.H. Drummer, A.J. Goble and W.J. Louis, J. Pharm. Sci., 70 (1981) 320.
3 J.D.H. Cooper and D.C. Turnell, Ann. Clin. Biochem., 17 (1980) 155.
4 G.J. Wright, G.A. Leeson, A.V. Zeiger and J.F. Lang, Postgrad. Med. J., April Suppl. (1973) 8.
5 E. Singlas, M.A. Ganjet and P. Simon, Eur. J. Clin. Pharmacol., 14 (1978) 195.
6 P.J. Meffin and J.O. Miners, in J.W. Bridges and L.F. Chasseaud (Editors), Progress in Drug Metabolism, Vol. 4, Wiley Interscience, New York, 1980, pp. 261—307.