

Method for the analysis of perhexiline and its hydroxy metabolite in plasma using high-performance liquid chromatography with precolumn derivatization

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Abstract

A high-performance liquid chromatographic method for the analysis of perhexiline and its monohydroxy metabolite in plasma has been developed. After a simple extraction procedure, the analytes are derivatized over a 30-min period with *trans*-4-nitrocinnamoyl chloride. The derivatized products are monitored at 340 nm following separation on a 5- μ m phenyl reversed-phase column under isocratic conditions. The limits of detection for perhexiline and its hydroxy metabolite are 0.03 and 0.02 mg/l, respectively. The between-day and within-day assay coefficients of variation for perhexiline and its hydroxy metabolite at concentrations of 0.2 and 1.0 mg/l were less than 10%. The method has proved robust and suitable for the routine monitoring of perhexiline and hydroxyperhexiline. © 1997 Elsevier Science B.V.

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1. Introduction

Perhexiline (2-(2,2-dicyclohexyl)ethylpiperidine) is a drug used in the treatment of angina pectoris. Although this drug has been available since 1972, it has mostly been used in cases which do not respond to conventional therapy. This is a result of the relatively high incidence of potentially serious side effects, such as hepatotoxicity and peripheral neuropathy, which have been reported with its use [1–3]. The incidence of side effects has been shown to be plasma concentration-dependent [4,5]. However, achieving effective concentrations with a minimum of side effects has always been difficult. This is due largely to the fact that perhexiline exhibits non-linear kinetics and there is a genetic polymorphism in the metabolism of the drug whereby 5–10% of

Caucasian populations are known to be poor metabolisers [6,7].

Metabolism of perhexiline is via the enzyme cytochrome P4502D6, producing the monohydroxy compound (2-[2-cyclohexyl-2-(4-hydroxycyclohexyl)ethyl]piperidine) as the major metabolite [7,8]. Incidence of side effects such as neuropathy are more common in patients with a reduced ability to metabolise the drug. As a result, the routine determination of drug oxidation phenotype has been advocated [8] in order to predict which patients might be at risk from perhexiline toxicity. However, measuring the appearance of the hydroxy metabolite from the beginning of treatment may also give an indication of a patient's ability to metabolise perhexiline. This would give clinicians the ability to better predict patients more at risk of developing toxicity.

Dose adjustments could be made earlier in treatment, thus minimising the chance of side effects. To investigate the usefulness of the monohydroxy metabolite of perhexiline as an early indicator of a patient's ability to metabolise perhexiline, a method for the routine measurement of perhexiline and its metabolite has been developed.

Several methods for the quantitation of perhexiline have been published that utilise gas–liquid chromatography (GLC) [9,10] or high-performance liquid chromatography (HPLC) [11,12], but only one of these is able to quantitate the hydroxy metabolite of perhexiline [12]. This method was not considered to be useful for the routine therapeutic monitoring of perhexiline and its metabolite because of the cumbersome extraction procedures involved and the use of a HPLC system using solvent gradient control. The method described here utilises a simple extraction procedure, a rapid derivatisation step and an isocratic HPLC system.

2. Experimental

2.1. Reagents and standards

A pure sample of perhexiline maleate was provided by Fawns and McAllen (Sydney, Australia) and a pure sample of the hydroxy metabolite of perhexiline was provided by Marion Merrell Dow (Cincinnati, OH, USA). Di-*n*-hexylamine (internal standard), di-isopropylethylamine and *trans*-4-nitrocinnamoyl chloride (derivatising agent) were purchased from Sigma-Aldrich (Sydney, Australia) and all other reagents used were supplied by Ajax Chemicals (Sydney, Australia) and were of analytical-reagent grade. The plasma used as the assay blank and for the preparation of standards was Ciba-Corning QCS 1,2 (unassayed), provided by Australia Diagnostics (Sydney, Australia).

A standard solution of perhexiline (1000 mg/l) was prepared by dissolving the maleate salt (0.1418 g) in 100 ml of methanol. Similarly, a standard solution of the metabolite (1000 mg/l) was prepared by dissolving 0.100 g of the free base in 100 ml of methanol. A combined standard containing 100 mg/l of perhexiline and 200 mg/l of the metabolite was

prepared by diluting 10 ml of the perhexiline bulk standard and 20 ml of the metabolite bulk standard to 100 ml with methanol. Combined working standards of the analytes were prepared by diluting 20.0, 10.0, 5.0 and 1.0 ml of the combined standards to 100 ml with methanol–water (1:1, v/v). This gives solutions containing 20.0, 10.0, 5.0 and 1.0 mg/l of perhexiline, respectively, and 40.0, 20.0, 10.0 and 2.0 mg/l of its hydroxy metabolite, respectively. Plasma standards were prepared by diluting 0.1 ml of each working standard with 0.9 ml of blank plasma (described above), giving a calibration range of 0.10 to 2.00 mg/l for perhexiline and 0.20 to 4.00 mg/l for the metabolite.

The internal standard solution (100 mg/l) was prepared by dissolving 0.100 g of di-*n*-hexylamine in 100 ml of 0.1 M hydrochloric acid. This was further diluted by taking 10 ml of the bulk solution and diluting it to 100 ml with distilled water. A working solution of the internal standard (2.5 mg/l) was prepared by diluting 2.5 ml of the 100 mg/l solution to 100 ml with distilled water.

A solution of the derivatising reagent (*trans*-4-nitrocinnamoyl chloride) was prepared by adding 0.1 g of the reagent to 10 ml of anhydrous acetonitrile (dried using anhydrous magnesium sulphate). This solution is stable for at least one week.

2.2. Chromatography system

The analysis was performed using a Waters Model 481 UV detector, purchased from Waters Australia (Sydney, Australia), a Waters autosampler (WISP), a Spherisorb phenyl reversed-phase column (250×4.6 mm I.D., 5 µm particle size) purchased from Alltech Associates (Sydney, Australia) and a GBC Model LC 1110 pump, purchased from GBC Scientific Equipment (Dandenong, Australia). The mobile phase consisted of 46% acetonitrile, 53.5% distilled water and 0.5% glacial acetic acid. The mobile phase was filtered through a 0.45-µm nylon filter (Alltech) prior to use. The flow-rate used was 2.0 ml/min and the eluent was monitored at 340 nm with the detector set at 0.05 AUFS. All chromatography was performed at room temperature (20°C) and the sample volume injected was 0.13 ml.

2.3. Extraction

To a 15-ml culture tube fitted with a PTFE-lined screw cap was added 0.4 ml of a patient's sample or standard, 0.4 ml of 1 M diammonium hydrogen phosphate, 0.05 ml of internal standard working solution (2.5 mg/l), 0.1 ml of 1000 mg/l diisopropylethylamine and 5 ml of isopentane–dichloromethane (3:2, v/v). The tube contents were mixed vigorously for 90 s using a vortex-mixer and then were centrifuged at 1500 g for 5 min. After centrifugation, the lower aqueous layer in the culture tube was frozen in a dry ice acetone bath. The upper organic layer was decanted into an appropriately labelled conical tube. The organic solvent in the conical tube was removed by evaporation under reduced pressure with no application of heat. To the residue was added 0.1 ml of an acetonitrile solution of the derivatising reagent (*trans*-4-nitrocinnamoyl chloride). The mixture was vortex-mixed for 15 s and then allowed to stand at room temperature for 30 min. After the 30 min reaction period, 0.1 ml of 0.025 M sodium carbonate solution was added, the mixture was vortex-mixed for 15 s and then allowed to stand for a further 5 min. Finally, 0.1 ml of 0.05 M ammonium acetate–acetonitrile (1:1, v/v) was added, followed by vortex-mixing for 30 s. The conical tube was centrifuged at 1500 g for 5 min to settle the solids that formed during the addition of sodium carbonate and ammonium acetate. As much as possible of the clear supernatant liquid was transferred to a glass WISP insert tube, with care being taken not to transfer any solid material. A 0.13-ml aliquot was taken for injection on to the column.

3. Results and discussion

3.1. Chromatography

Representative chromatography of perhexiline and its monohydroxy metabolite are illustrated in Fig. 1. Under the chromatography conditions employed, retention times for the analytes are: monohydroxy metabolite, 7.2 min; perhexiline, 18.4 min and the internal standard, 10.2 min. In the chromatogram (A)

of the plasma control, a peak appeared at approximately 9 min. This peak appears in the plasma of all patients' samples, but to a relatively negligible extent. Moreover, the internal standard is sufficiently resolved from this endogenous peak so that the latter does not cause interference. The monohydroxy metabolite similarly elutes near endogenous substances but not to an extent that causes interference. With new HPLC columns, the monohydroxy metabolite peak appears to be split. This is due to the partial resolution of the *cis*- and *trans*-isomers of the monohydroxy metabolite and should not be confused with other compounds causing interference.

The monohydroxy metabolite standard provided was a mixture of the *cis*- and *trans*-isomers, thus, absolute measurement of the relative amounts of each was not possible. For our purposes, it was not necessary to be able to determine the relative amounts of each isomer, but rather, the total amount of monohydroxylated perhexiline as an indicator of a patient's ability to metabolise perhexiline. The loss of partial resolution of the two isomers with column deterioration does not affect the quantitation of the hydroxy metabolite.

3.2. Derivatising reagent

An acyl chloride (*trans*-4-nitrocinnamoyl chloride) was chosen as the derivatising reagent because of the speed with which it reacts with perhexiline and its metabolite. This reagent reacts completely with these secondary amines within 30 min at room temperature. A benzoyl chloride (3,5-dinitrobenzoyl chloride) and a sulphonyl chloride (8-quinoline sulphonyl chloride) were also investigated, but the rate of reaction was much slower for both, requiring the application of heat and a longer reaction time to achieve complete derivatisation. The UV absorbance maximum for *trans*-4-nitrocinnamoyl chloride is at 340 nm.

The derivatisation procedure described here requires that 0.025 M sodium carbonate be added to the reaction mixture. This is necessary to quench the reaction. After this, an aliquot of 1:1 (v/v) acetonitrile–0.05 M ammonium acetate must be added. If this final reagent is not added, additional large peaks appear in the chromatographs, making it

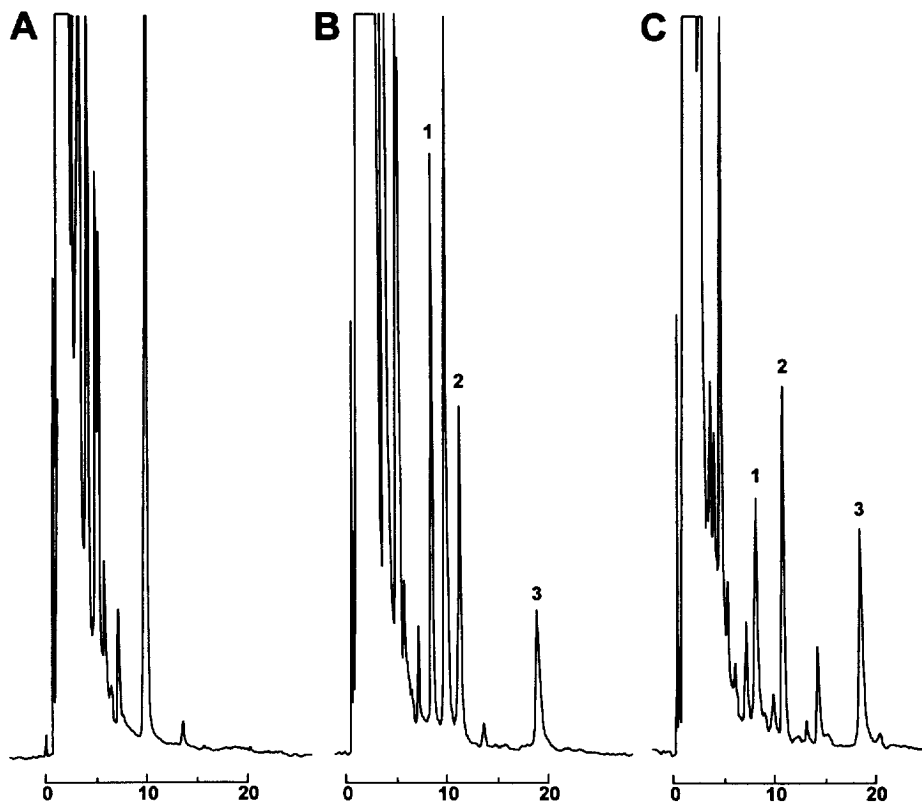


Fig. 1. (A) Plasma blank, (B) plasma standards (0.50 mg/l perhexiline and 1.0 mg/l hydroxy metabolite), (C) patient's sample containing 0.74 mg/l perhexiline and 0.35 mg/l hydroxy metabolite. In all chromatographs, peak 1 is the hydroxy metabolite, peak 2 is the internal standard (di-*n*-hexylamine) and peak 3 is perhexiline.

impossible to accurately quantitate the analytes. Presumably, the ammonium ion present reacts with remaining derivatising reagent, forming an amide. Under the chromatography conditions employed, the amide elutes with the solvent front, while the derivatising reagent has a broad peak at about 10 min.

The reaction mixture, once transferred to WISP vials with glass inserts and PTFE-lined screw caps, is stable for at least 12 h.

3.3. Extraction

The use of diisopropylethylamine in the extraction procedure serves a two-fold purpose. Firstly, as an amine, it will bind to active sites on glassware, preventing the analytes from being adsorbed and reducing recovery. Secondly, this tertiary amine is extracted with the major analytes and is part of the

residue after the organic solvent is removed. When the derivatising reagent is added, this compound then acts as a catalyst for the reaction by scavenging the hydrogen chloride generated. Recoveries run with and without diisopropylethylamine show that the recovery of the metabolite and the internal standard are unaffected, however, the recovery of perhexiline is improved 20% with this tertiary amine present. Recovery in this context refers to the sum of extraction efficiency and derivatisation efficiency.

3.4. Recoveries

The recoveries for perhexiline and the metabolite were calculated at each point of the calibration curve (0.1, 0.5, 1.0 and 2.0 mg/l for perhexiline and 0.2, 1.0, 2.0 and 4.0 mg/l for the metabolite). The mean percentage recovery (\pm the standard deviation) for

each analyte over these concentration ranges were: Perhexiline, $83.8 \pm 3.7\%$ ($n=4$) and hydroxy metabolite, $80.3 \pm 6.0\%$ ($n=4$). The recovery of the internal standard was calculated to be $79.3 \pm 4.38\%$ ($n=4$).

3.5. Accuracy and precision

Within-day accuracy and precision were determined by analysing the same sample on the same day ($n=10$). Two spiked samples were prepared containing 0.2 and 1.0 mg/l of each analyte. Between-day precision was similarly calculated by analysing standards containing these concentrations of each analyte on seven different days. The mean concentration (\pm the standard deviation) and the C.V. for each analyte at each concentration are given in Table 1. The data indicate that the method is accurate and precise for both analytes.

3.6. Linearity and limit of detection

The calibration curves for perhexiline and its metabolite are linear over the range defined in the method. The following linear equations are typical for a data set: perhexiline, $y=0.636x+0.039$, $r=0.997$; perhexiline metabolite, $y=1.311x-0.078$, $r=0.999$. The calibration range for the metabolite is twice that for the parent compound because our experience has shown that metabolite concentrations in the range of 3.0 to 4.0 mg/l may be observed.

The sensitivity, defined here as the concentration where the ratio of peak height of analyte to back-

ground is 10:1, provides a limit of detection of 0.03 mg/l for perhexiline and 0.02 mg/l for the monohydroxy metabolite.

3.7. Interference

The problem of interference from other compounds, either endogenous compounds or other therapeutic drugs, was considered. There are very few compounds (endogenous or therapeutic agents) that are likely to have a significant absorbance at 340 nm. The most likely compounds (co-administered drugs) to cause interference are primary or secondary amines that are able to be extracted and derivatised in the procedure described. These amines can be drugs such as mexiletine, which is a primary amine, or the de-alkylated products of drugs like amiodarone. The retention times of primary and secondary amines tested for interference are shown in Table 2. The retention time data indicate that dealkyldisopyramide and the tricyclic antidepressants could be sources of interference for the hydroxy metabolite of perhexiline. With the introduction of newer and more effective antiarrhythmic agents, disopyramide is seldom used. As a result, interference by its desalkyl metabolite is unlikely.

It is possible that any of the three antidepressants tested could be present, because treatment for depression is not uncommon in patients with severe heart disease. The therapeutic plasma concentrations for these drugs is quite low (0.05–0.250 mg/l) and, as a result, they are not likely to cause measurable interference.

Table 1
Accuracy and precision data

Analytes	Concentration (mg/l)	Concentration (mean \pm SD) (mg/l)	Coefficient of variation (%)
<i>A. Within-day accuracy and precision (n=10)</i>			
Perhexiline	0.2	0.180 \pm 0.004	2.2
	1.0	0.908 \pm 0.021	2.3
Metabolite	0.2	0.210 \pm 0.007	3.3
	1.0	0.975 \pm 0.045	4.6
<i>B. Between day accuracy and precision (n=7)</i>			
Perhexiline	0.2	0.208 \pm 0.007	3.4
	1.0	0.990 \pm 0.073	7.4
Metabolite	0.2	0.210 \pm 0.014	6.7
	1.0	0.997 \pm 0.073	7.3

Table 2
Drugs tested for interference

Drug tested	Retention time (min)
Desethylamiodarone	20.0
Flecainide	6.0
Desipramine	8.6
Nortriptyline	8.4
Protriptyline	8.8
Dealkyldisopyramide	8.0
Sotalol	4.8
Mexiletine	4.0
Perhexiline metabolite	7.2
Perhexiline	18.4
Dihexylamine (internal standard)	10.2

4. Conclusions

The method described here for the analysis of perhexiline and its major metabolite, monohydroxy perhexiline, is as sensitive, and easier to perform when compared to other HPLC or GLC methods used to analyse perhexiline alone. In this laboratory, the assay has proved to be robust and reliable.

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