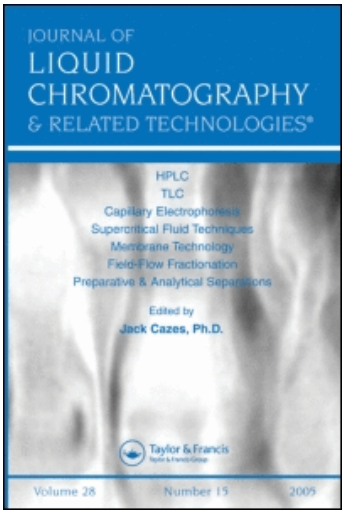

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APPLICATION OF AN IMPROVED HPLC PERHEXILINE ASSAY TO HUMAN PLASMA SPECIMENS

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ABSTRACT

The analysis of perhexiline in plasma offers an important contribution to the management of patients prescribed such therapy for refractory angina pectoris. Perhexiline has properties and complexities of non-linear kinetics and is subject to genetically predetermined metabolic variants in hydroxylation. The present communication describes a refinement of a previous high performance liquid chromatographic fluorescence method and the application of this method in the therapeutic monitoring of 100 patients at steady-state. The method described is sensitive, accurate and precise, with intra-assay CV's of 2.1%, 1.4% and 3.7% at concentrations of 150, 750 and 1500 μg/L, and between-assay CV's of 5.7%, 4.7% and 5.8% at 50, 1000 and 3000 μg/L, respectively. The review of patient specimens received in our therapeutic drug monitoring laboratory, suggested that approximately 6% appear to belong to the "poor-hydroxylation" metabolic sub-population, with a further 16% attaining steady-state plasma perhexiline concentrations above the "therapeutic range" of 150 to 600 μg/L following standard dosage schedules of 100 to 200 mg/day.

INTRODUCTION

Perhexiline [Pexid®, 2-(2,2-dicyclohexylethyl)piperidine maleate)] is an older drug [1] that has returned to favour in recent years in many centres as the agent of choice in the prophylactic treatment of angina pectoris refractory to conventional therapy including nitrates, beta-blockers or calcium antagonists in patients for whom coronary revascularization is contra-indicated [2, 3]. Early clinical experiences with perhexiline included an unacceptable frequency of toxicity, particularly neuropathy and hepato-toxicity, which is now better understood as being, (a) particularly associated with the pharmacogenetic subgroup of the population, ranging from <1% in Japanese, to approximately 5 to 10% of Caucasian and up to 31% of Chinese populations [4] who are considered "poor hydroxylators" [5], and (b) up to 50% of patients where saturation of metabolic pathways results in elevated plasma perhexiline concentrations of 720 to 2680 μg/L [6]. The "poor hydroxylator" sub-group can attain very high plasma perhexiline concentrations from relatively low doses and typically have a very long "apparent" (given its non-linear kinetics [7, 8]) elimination half-life, possibly even of the order of months (compared with 2 to 6 days in "normal" hydroxylators).

The current upper limit for a "therapeutic range" for perhexiline in human plasma is somewhat controversial. Whilst a range of 150 to 600 μg/L has been proposed and applied in several centers [6, 9], concentrations of up to 1200 μg/L have been considered in some institutions [10] to be associated with a low incidence of reversible toxic side effects [11]. In this institution patients are routinely maintained in the lower range without apparent compromise of the anti-anginal efficacy.

Treatment with perhexiline is associated with minimal effects on resting heart rate or blood pressure, however, exercise induced tachycardia may be depressed [12, 13]. Its use in this institution has

been both as a short-term adjunct to anti-anginal therapy, as well as long-term management of patients for whom coronary bypass grafting is not indicated.

Previous liquid chromatographic methods for perhexiline analysis [7] have proven only partially adequate, particularly from the perspective of the chromatographic separation obtained and the time taken for sample preparation. The plasma sample preparation is complex as parent perhexiline is neither UV-absorbant, or fluorescent, it requires both derivatization with dansyl chloride, in addition to repeated organic solvent extraction and back extraction steps.

This communication reports a refinement of the above method aimed at reducing both the protracted sample preparation time as well as further optimizing the HPLC separation resulting in markedly reduced solvent consumption. This has been achieved without compromizing analytical accuracy or sensitivity. Further, we have reviewed a cross-section of patients included in our perhexiline-treated population to identify the frequency of those who would appear to be "poor metabolizers" on the basis of the very low doses required to maintain plasma perhexiline concentrations within the "therapeutic range", as well as those patients who appear to have excessive concentrations from apparently standard dosage schedules. This represents the collated experience of our therapeutic drug monitoring (TDM) laboratory which currently performs approximately 1000 patient perhexiline plasma analyses per annum.

MATERIALS & METHODS

Stock Solutions

A 100 mg/L perhexiline-base stock solution was prepared by dissolving perhexiline maleate (Merrell Dow Pharmaceuticals

Australia Pty. Ltd.) in 0.8% dimethyl sulphoxide and an appropriate volume of 0.1 mol/L HCl. This solution was diluted to give concentrations of 10 mg/L and 1.0 mg/L in 0.1 mol/L HCl. Hexadiline HCl (the internal standard, also from Merrell Dow) was prepared in a similar manner to give 14 and 1.4 mg/L. These solutions were stored at -20°C between assays for up to 8 weeks.

The derivatization reagent, 0.005 mol/L dansyl chloride (Sigma Chemical Company, USA, part D2625), was prepared in acetone (approximately 5 ml) prior to use in each analytical run and held in a light proof container. Sodium bicarbonate solution (0.1 mol/L, Ajax Chemicals, Univar grade) was adjusted to pH=10 with NaOH. Trizma base (tris-hydroxymethyl-aminomethane, Sigma Chemical Company, USA) buffer (2 mol/L) was adjusted to a pH of 8.75 with concentrated HCl solution.

Plasma Extraction

Each assay was calibrated by adding appropriate volumes of the perhexiline stock solutions to a series of 0.5 ml perhexiline-free heparinized plasma aliquots to give final concentrations of 0, 50, 250, 500, 1000, 2000, 3000 µg/L. Quality control was assessed in each run by analysing aliquots from 3 separate plasma pools which had been spiked with perhexiline to give concentrations of 150, 750 and 1500 µg/L. Patient blood samples collected for TDM purposes were drawn into lithium heparin blood collection tubes at a "trough" time with respect to the previous dose and the plasma fraction separated by centrifugation at 1000xg for 10 min. Specimens were stored at -20°C prior to analysis.

The 0.5 ml calibration standards, controls and samples were all spiked with; 200 µl of 1.4 mg/L of the internal standard, 50 µl of Trizma buffer (2.0 mol/L, pH = 8.75) and 4 ml of n-hexane (BDH/Merck, Hypersolv grade) in 15 ml disposable borosilicate glass screw-capped

tubes. This mixture was shaken on a horizontal mixer for 15 min at 100 oscillations per min, followed by centrifugation at 2500 rpm for 10 min. The organic layer was transferred to a 5 ml disposable borosilicate glass tube and evaporated to dryness at 60°C under a gentle nitrogen stream. When dry, 100 µl of sodium bicarbonate solution and 100 µl of derivatizing reagent were added and vortex mixed. Tubes were immediately capped and placed in a water bath at 37°C for 20 min. Following this incubation, 1.5 ml of n-hexane was added and vortex mixed. Following centrifugation for 3 min at 2500 rpm at 10°C, phases were separated by snap freezing the aqueous layer in a dry-ice/ethanol bath, and the organic layer decanted into a second 5 ml tube. Following a further drying step at 60°C under nitrogen, the residue was dissolved in 100 µl of the mobile phase solution, described below, and 25 µl injected for chromatographic separation.

Chromatography

The mobile phase consisted of a mixture of methanol (BDH/Merck Hipersolv grade) and water (glass distilled) in a ratio of 86:14 (v:v). This mixture was filtered (0.2 µm, Millipore, part number GVWP-04700) under vacuum before use, degassed continuously with helium and pumped (Millipore/Waters, model 510) at 0.5 ml/min via an autosampler (Millipore/Waters WISP, model 710B) through a 3 µm column (Velosep 10 cm x 3.2 mm, Brownlee Laboratories, part BLV18-103) maintained at 45°C. Compounds separated by this system were quantified by fluorescence detection (Perkin Elmer, model LS-1) at excitation and emission wavelengths of 366 and 470 nm, respectively. This detector was operated using a "fixed scale" attenuation of 7.5 units. The output to a dual-pen chart recorder was plotted at both 2 and 20 mV at a chart speed of 0.25 cm/min. The retention time of the 2 hexadiline and perhexiline derivatives were 15.9, 16.8 and 19.5 min, respectively, with baseline separation.

Statistical Considerations

The method was validated within a single run by assaying 6 replicates at 150, 750 and 1500 μg/L. Between-run performance was assessed by considering the run-to-run (n=10) reproducibility of 3 of the calibration standards, ie., at 50, 1000 and 3000 μg/L. The performance of each assay run was considered both by the coefficient of variation (CV%) of the concentration-corrected peak height ratio for each calibration standard (ie., the apparent slope of the calibration curve indicated by each calibration standard), as well as by the concentration derived from the calibration curve for each of the 3 quality control samples. Runs were accepted when the calibration standard values were within a ±10% range of the spiked concentration, and each of the controls within ±2 SD of their respective target concentrations.

Patient Data

A retrospective review was undertaken of individual patients from whom "trough" blood specimens had been received for perhexiline analysis and where data were available (as supplied on the laboratory's drug assay request form) for the dosage, dosage frequency, duration of therapy (at the current dosage level) and the time of blood sample collection with respect to the previous dose. Internal laboratory records were also used to establish/confirm the duration of therapy at a particular dosage. The aim was to record 100 different patients who were considered to be at "steady state" with respect to their plasma perhexiline concentration. These data were recorded along with measured plasma perhexiline concentrations as a means of establishing a dosage versus plasma concentration profile, as well as estimating the frequency of patients who might be classified into the "poor hydroxylator" sub-group, ie., those who appeared to have "therapeutic" steady-state plasma perhexiline concentrations but were being maintained on apparently very low dosages (mean of less than 50 mg/day).

TABLE 1.

Precision and Accuracy Data of the Method Described in the Text.

Spiked Perhexiline Concentration	n	Measured Concentration (mean ± SD)	Accuracy (%)	C.V. (%)
With-in run:				
150 µg/L	6	163 ± 4	+8.6	2.1
750 µg/L	6	780 ± 11	+4.0	1.4
1500 µg/L	6	1468 ± 55	- 2.1	3.7
Between-run:				
50 µg/L	10	46.6 ± 2.7	- 6.8	5.7
1000 µg/L	10	1054 ± 50.4	+5.4	4.7
3000 µg/L	10	3025 ± 175	+0.8	5.8

RESULTS & DISCUSSION

Table 1 shows the accuracy and precision of the method described and suggests that acceptable data were observed both within and between analytical runs for TDM purposes. Within-run CV's ranged from 1.4 to 3.7%, and between-run ranged from 4.7 to 5.8%. Figure 1 shows a range of chromatograms for a perhexiline-free extract, a perhexiline-spiked calibration standard extract, and a patient perhexiline plasma extract. The double peak for the internal standard, hexadiline, has been observed in this and other laboratories [7] and the first of these peaks routinely used as the internal standard for quantitation purposes. The chromatographic run-time of approximately 20 min has been accommodated in this laboratory by overnight running of the HPLC system. The very stable baseline characteristics of the particular fluorescence detector employed offers a considerable advantage for this purpose. In more than 2.5 yr operation of the current, or previous closely related methods no chromatographic interferences have been noted.

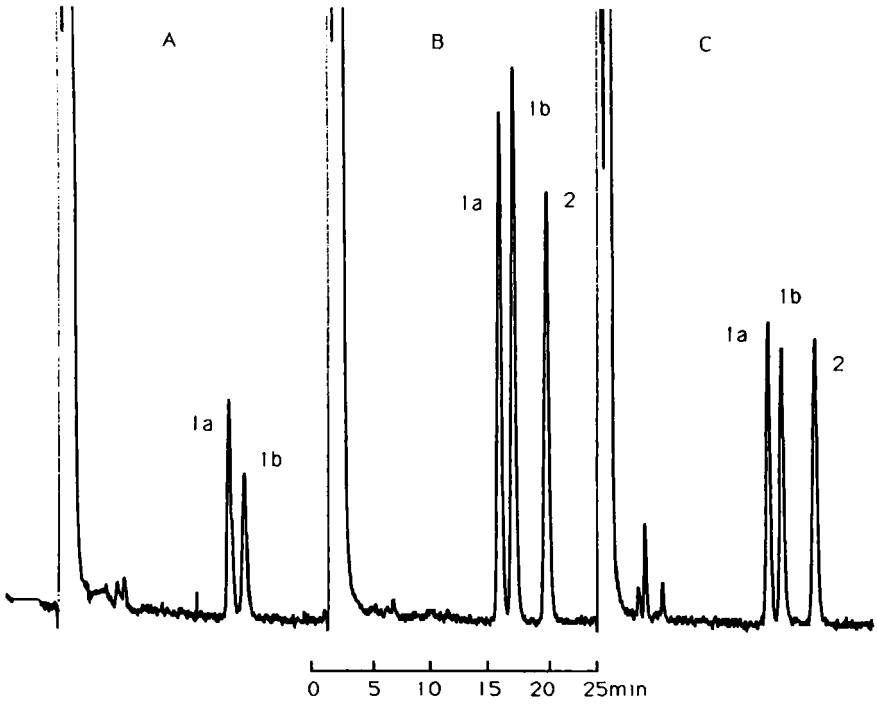

FIGURE 1. Shows representative chromatograms using the method described. Peaks 1a and 1b are the dual peaks typically observed for hexadiline (internal standard) and peak 2 is perhexiline. The panels represented are; A = perhexiline-free plasma extract, B = perhexiline-spiked calibration standard, and C = extract from a perhexiline-treated patient.

Although not shown in Figure 1, the mono- and di-hydroxy-metabolites of perhexiline have been considered in the method described and have retention times of less than 5 min. Hence these were well separated from the parent drug and internal standard peaks. Further exploration and quantification of these peaks may allow a means of identifying the "poor hydroxylator" sub-group of patients, even in the early stages of perhexiline therapy. The use of a single test-dose study, prior to commencing chronic therapy, could potentially be

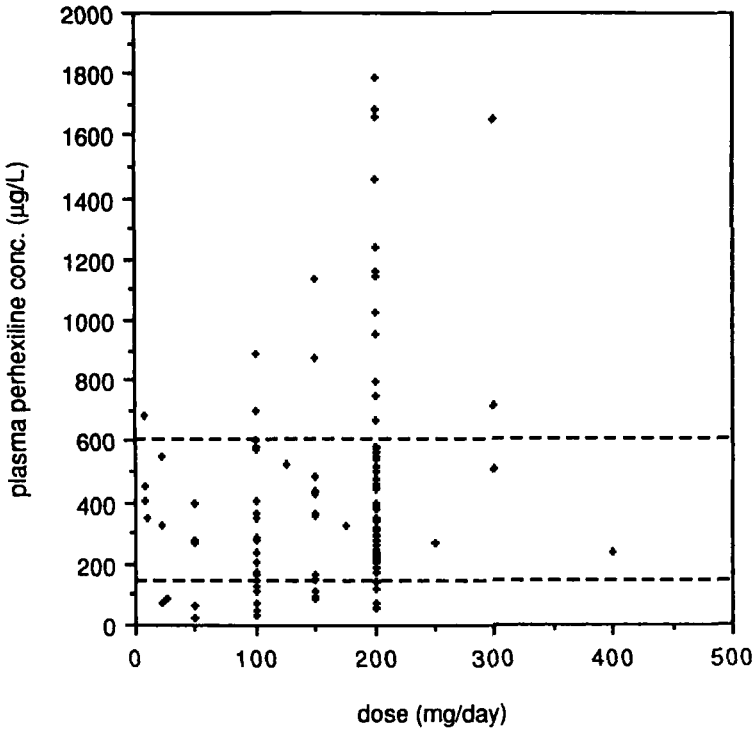

FIGURE 2. Shows the distribution of steady-state plasma perhexiline concentrations (µg/L) with doses prescribed (mg/day) in 100 patients as monitored in this laboratory. The dashed lines define the "therapeutic range" routinely employed (150-600µg/L).

applied if limits were established to define a perhexiline metabolite profile which was specific for each genotypic sub-class.

Figures 2 and 3 present the data recovered from the 100 patients, as described above. Figure 2 shows the distribution of dosages routinely used, showing that the majority (82%) of patients were prescribed 100 to 200 mg/day. Of the 48 patients prescribed 200 mg/day, 27% appeared to have plasma perhexiline concentrations greater than the recommended therapeutic range shown (150 - 600 µg/L), compared

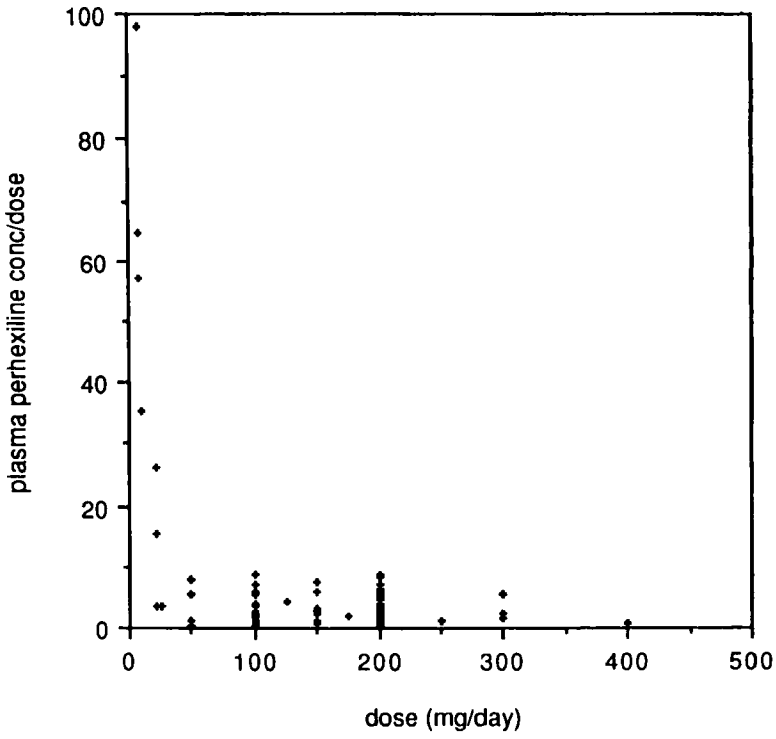

FIGURE 3. Shows the distribution of the ratio of plasma perhexiline concentrations to the dose, plotted against the dose (mg/day) for each of the 100 patients presented in figure 2. The 6 patients with "exaggerated" ratios at the low dosages were considered to be "poor hydroxylators".

with only 2 of the 20 patients prescribed 100 mg/day, suggesting that these patients were either being over-dosed, or were being prescribed dosages greater than would appear necessary based on the therapeutic range recommended so as to achieve a therapeutic outcome [6].

An interesting subgroup in Figure 2 were those 8 patients maintained on <50 mg/day, 6 of whom attained plasma perhexiline concentrations within, or in 1 case just above, the therapeutic range of

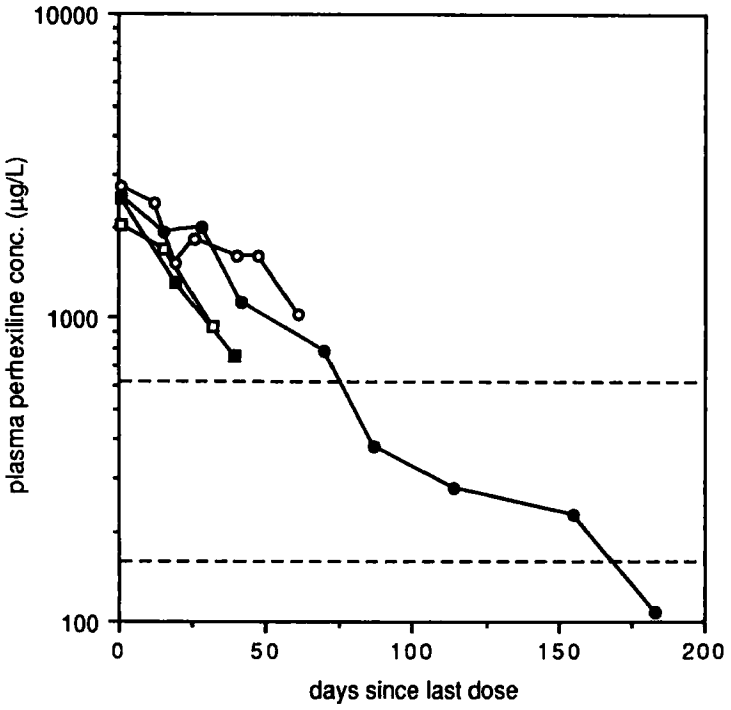

FIGURE 4. Shows the elimination curve for 4 patients, on a logarithmic scale, following very high concentrations of perhexiline from standard dosages at the commencement of therapy. Further doses were with-held in the above period.

150 to 600 µg/L. This group are more clearly identified in Figure 3 which shows the ratio of the plasma perhexiline concentration to the dose for each patient plotted against the dose. Six patients at doses of <50 mg/day stand out from the remainder of the sample as having exaggerated plasma perhexiline concentrations for the given dose. It is these patients who appear to be the "poor hydroxylators". Interestingly, this fraction (6 out of 100 patients) compares well with a debrisoquine hydroxylation study [14], also performed in the heterogeneous Australian population, where 6% were identified as poor metabolizers

of that drug. Four of the poor metabolizers in our study have been monitored over a long period by this laboratory and data were also available for their initial treatment phase where exaggerated plasma perhexiline concentrations were first observed following initial dose(s). These data are presented in Figure 4, which shows the decline in plasma perhexiline concentrations (on a logarithmic scale) over time with perhexiline therapy withheld. The one patient monitored for the longest period, maintained plasma perhexiline concentrations above or within the therapeutic range for up to approximately 170 days despite no further doses having been administered. This equated to an "apparent" plasma elimination half-life of 40 days. This patient is currently maintained with therapeutic plasma perhexiline concentrations of 400-500 μg/L on a single 50 mg dose/week.

In addition to the "poor hydroxylator" sub-group, there is also evidence in this review that 16% of patients attained plasma perhexiline concentrations greater than the "therapeutic range" despite standard dosages (100 to 200 mg/day). These patients probably represent those in whom the non-linear kinetics of perhexiline was most evident as previously described [15]. This increase in concentration has been previously associated with an increased incidence of toxicity in such patients [6,16], including neuropathy [17].

The above differences in dosage requirements strongly support the need for routine therapeutic drug monitoring of perhexiline in clinical practice, indeed without such analytical support it could be argued that the use of this drug should be restricted as there is no prospective clinical indication to suggest which phenotypic sub-group a particular patient may belong, or those patients likely to experience excessive perhexiline concentrations with standard dosages [15]. Phenotyping patients prior to commencing perhexiline therapy using a test dose of another less toxic drug which utilises this same metabolic pathway, such as debrisoquine [14] or dextromethorphan [17], would offer only a partial solution by identifying the smaller risk group of poor hydroxylators. The remainder of those at risk from excessive

concentrations, possibly as a result of perhexiline's non-linear kinetics, should be identified by therapeutic monitoring of plasma perhexiline concentrations in the early treatment phase where clinical or biochemical toxicity is not a significant risk [6].

In summary, the analytical method presented provides a sensitive and reliable HPLC-fluorescent method which has improved upon the previously described methods. The longer run time in the present method has not proved to be a detraction as the preparation of batches of up to 50 tubes is undertaken during the course of half a working day and the chromatography automatically performed over the ensuing evening. The patients sampled suggested that 6% of this population were likely to be "poor hydroxylators" of perhexiline, and a further 16% attained excessive plasma perhexiline concentrations despite standard dosages. Their role of the therapeutic drug monitoring laboratory is therefore most important in the on-going care of patients prescribed perhexiline.

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