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Determination of perhexiline and its metabolite hydroxyperhexiline in human plasma by liquid chromatography/tandem mass spectrometry

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ABSTRACT

Perhexiline is a drug that is used for treatment of moderate to severe angina pectoris that has not responded to other treatment. It has a low therapeutic index, and saturable metabolism that is also subject to genetic polymorphism (CYP2D6). Concentration monitoring of the parent drug and its major metabolite is considered necessary to optimise efficacy and reduce the risk of hepatotoxicity and neuropathy. A rapid, simple and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) assay was developed for the determination of perhexiline and its metabolite *cis*-hydroxyperhexiline in human plasma. After proteins were precipitated with acetonitrile, perhexiline, the major metabolite *cis*-hydroxyperhexiline and nordoxepin as the internal standard were resolved on a phenyl-hexyl column using gradient elution of 0.05% formic acid and methanol. The three compounds were detected using electrospray ionisation in the positive mode. Standard curves were linear over the concentration range 10–2000 μg/L ($r > 0.999$), bias was $\leq \pm 10\%$, intra- and inter-day coefficients of variation (imprecision) were $\leq 8.1\%$, and the limit of quantification was 10 μg/L for both perhexiline and hydroxyperhexiline. The assay is being used successfully in clinical practice to enhance the safe and effective use of perhexiline.

1. Introduction

Perhexiline (Fig. 1) is an anti-anginal agent that was first marketed in the 1970s. It is an effective treatment for severe angina but a significant incidence of severe hepatotoxicity and neurotoxicity led to a rapid decline in its use within the first few years [1]. Although many countries worldwide had removed perhexiline from the market in the 1980s, perhexiline continued to be prescribed in Australia and New Zealand for the treatment of patients with intractable angina who were refractory or intolerant to other conventional therapy, or who are not suitable candidates for coronary bypass surgery [1–3]. Because of the low therapeutic index and the observation that serious toxicity was concentration-dependent [4–6], drug concentration monitoring became a standard adjunct to therapy. The need for this was reinforced by later evidence that perhexiline metabolism was subject to genetic polymorphism (CYP2D6) [7–9], and that slow metabolisers for this enzyme were more likely to experience toxicity. Yet another cause for dose-concentration–effect problems was the presence of saturable (non-linear) metabolism [10].

Perhexiline is metabolised primarily to a pair of geometric isomers of the mono-hydroxy metabolite (Fig. 1), *cis*-hydroxyperhexiline and *trans*-hydroxyperhexiline. Hydroxylation of perhexiline to *cis*-hydroxyperhexiline is the major metabolic pathway controlled by cytochrome P450 2D6 (CYP2D6) [7–9]. The *cis*-hydroxyperhexiline/perhexiline concentration ratio can be used to identify poor metabolisers shortly after commencing perhexiline therapy and hence avoid unnecessary drug toxicity by appropriate dose reduction [2]. Routine monitoring of plasma perhexiline and *cis*-hydroxyperhexiline has allowed safer use of the drug in increasing numbers of patients in Australia and New Zealand, and may extend to greater use of this drug in other countries.

Various analytical methods for measuring perhexiline and *cis*-hydroxyperhexiline in human plasma have been developed including high-performance liquid chromatography (HPLC) with fluorescence [2] or UV detection [11] and gas chromatography (GC) [12]. Services providing perhexiline therapeutic monitoring have most commonly used HPLC methods [13]. Because perhexiline lacks absorption within the UV spectrum, the sample preparation for HPLC methods requires derivatization for fluorescence or UV detection and multiple liquid–liquid extraction steps, which is reagent- and time-consuming. In recent years, HPLC with tandem mass spectrometric detection (LC–MS/MS) has been demonstrated to be a powerful technique for the quantitative determination of drugs and metabolites in biological fluids. This technique can provide high selectivity and simplification of both sample extraction procedures

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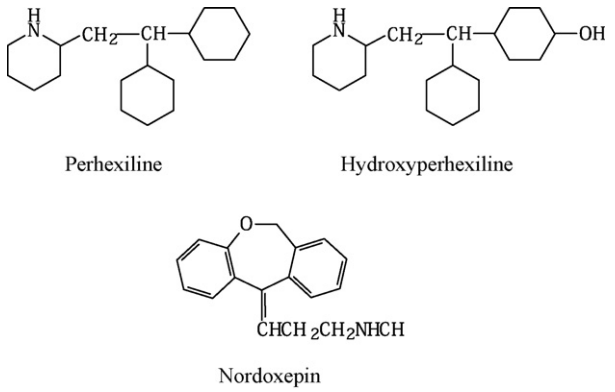

Fig. 1. Chemical structures of perhexiline, hydroxyperhexiline and nordoxepin.

and chromatography. Recently, Beck et al. [14] reported a simple liquid chromatography/mass spectrometry (LC–MS) method for determination of perhexiline and hydroxyperhexiline using protein precipitation for sample preparation. Compared with LC–MS, LC–MS/MS can achieve higher specificity and sensitivity by utilizing collision-induced dissociation while monitoring unique precursor-to-product ion transitions. The aim of the current work was to develop and validate a rapid, simple, specific, sensitive, robust and reliable LC–MS/MS method for the determination of perhexiline and *cis*-hydroxyperhexiline in human plasma, suitable for use during routine drug monitoring. An additional aim was to use a small volume of plasma and simple sample preparation without losing specificity and sensitivity.

2. Experimental

2.1. Materials

Perhexiline maleate and nordoxepin (Fig. 1) were purchased from Sigma Co. (Australia). *Cis*-hydroxyperhexiline was kindly donated by Dr Benedetta Sallustio (Adelaide, Australia). The *cis*-hydroxyperhexiline reference material contained 2.2% *trans*-hydroxyperhexiline. HPLC grade acetonitrile, methanol and formic acid were purchased from BDH (Poole, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). The human plasma used as the assay blank and for the preparation of standards was obtained from New Zealand Blood Services (Christchurch, New Zealand).

2.2. Instrumentation and analytical conditions

The LC–MS/MS system consisted of a Shimadzu LC-20AD HPLC system (Shimadzu Corporation, Kyoto, Japan) interfaced with a 3200 Q TRAP® mass spectrometer (Applied Biosystems, Foster City, Canada) equipped with a TurboIonSpray® source. Analyst software (Applied Biosystems, Foster City, Canada) was used to control equipment, to coordinate data acquisition, and to analyse data. Perhexiline, *cis*-hydroxyperhexiline and the internal standard nordoxepine were separated under gradient elution using a Luna Phenyl-Hexyl 3 μm, 50 mm × 2.0 mm internal diameter analytical column equipped with a Phenyl 4.0 mm × 2.0 mm internal diameter guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of solvent A (0.05% formic acid) and solvent B (methanol). The flow rate was set at 0.3 mL/min. The initial condition was 80% solvent A and 20% solvent B. A linear gradient was performed with mobile phase B increasing from 20 to 90% within 1 min. After 3 min, the mobile phase was returned to the initial condition and re-equilibrated for 2 min. The total analysis time was 5 min.

The mass spectrometer was operated in the positive ion mode with curtain gas, Gas 1 and Gas 2 flow rates of 20, 45 and 60 psi, respectively. The ion spray voltage was 5000 V and the source temperature was 500 °C. Data acquisition was performed via multiple reaction monitoring (MRM). The ions representing the $[M+H]^+$ species for perhexiline, *cis*-hydroxyperhexiline and the internal standard nordoxepin were selected in the first mass spectrometer (mass analyser, MS1) and dissociated with nitrogen gas to form specific product ions, which were subsequently monitored by the second mass spectrometer (mass analyser, MS2). The optimised precursor-to-product ion transitions monitored for perhexiline, *cis*-hydroxyperhexiline and nordoxepin were m/z 278.3 → 95.2 with declustering potential (DP) 56 V and collision energy (CE) 37 V, m/z 294.3 → 95.2 with DP 56 V and CE 43 V, and m/z 266.1 → 107.1 with DP 36 V and CE 29 V, respectively.

2.3. Standards

A standard stock solution of perhexiline (1.0 mg/mL) was prepared by dissolving 14.2 mg of perhexiline maleate in 10 mL of methanol. A standard stock solution of *cis*-hydroxyperhexiline (1.0 mg/mL) was prepared by dissolving 10 mg of *cis*-hydroxyperhexiline in 10 mL of methanol. Two sets of the same standard stock solutions were prepared for plasma calibration curves and for plasma quality control (QC) samples, respectively. The plasma calibration curves of perhexiline and *cis*-hydroxyperhexiline were constructed by spiking drug-free human plasma with standard solutions, giving a calibration range of 10–2000 μg/L for both perhexiline and *cis*-hydroxyperhexiline. The stock internal standard nordoxepin solution (1.0 mg/L) was prepared by dissolving 10 mg of nordoxepin in 10 mL of methanol. A working solution of the internal standard (250 μg/L) was prepared by diluting 2.5 μL of the stock solution to 10 mL with water. Perhexiline and *cis*-hydroxyperhexiline plasma quality control (QC) standards were prepared in single 5 mL aliquots in concentrations of 10, 40, 100, 500 and 2000 μg/L and stored at −30 °C until analysed.

2.4. Sample preparation

Nordoxepin the internal standard, 50 μL of 250 μg/L, was added to 50 μL of each of blank, standard, quality control or patient plasma samples. The mixture was vortexed briefly and 200 μL of acetonitrile added to precipitate the proteins. After centrifugation at 15,000 × *g* for 5 min, a 50 μL aliquot of clear supernatant was mixed with 200 μL of 0.05% formic acid and transferred to the autosampler 96 well plate. A volume of 10 μL was injected into the LC–MS/MS system.

2.5. Validation

The standard curves were the plot of the peak area ratios (analyte/internal standard) of perhexiline and *cis*-hydroxyperhexiline versus the corresponding concentrations of perhexiline and *cis*-hydroxyperhexiline. The linearity of the standard curves was evaluated using $1/x$-weighted linear regression analysis. To evaluate the assay recoveries and matrix effects, three sets of standards were prepared using a modification of the method of Matuszewski et al. [15] for both perhexiline and *cis*-hydroxyperhexiline at concentrations of 40, 100, 500 and 2000 μg/L, and nordoxepin at 250 μg/L, the concentration used in the assay. The first set was prepared in plasma from six different sources (six samples at each concentration), the second set in after-protein precipitation of blank plasma extracts from the same six different sources as in first set, and the third set in mobile phase. Absolute recoveries at each concentration were measured by comparing the peak area

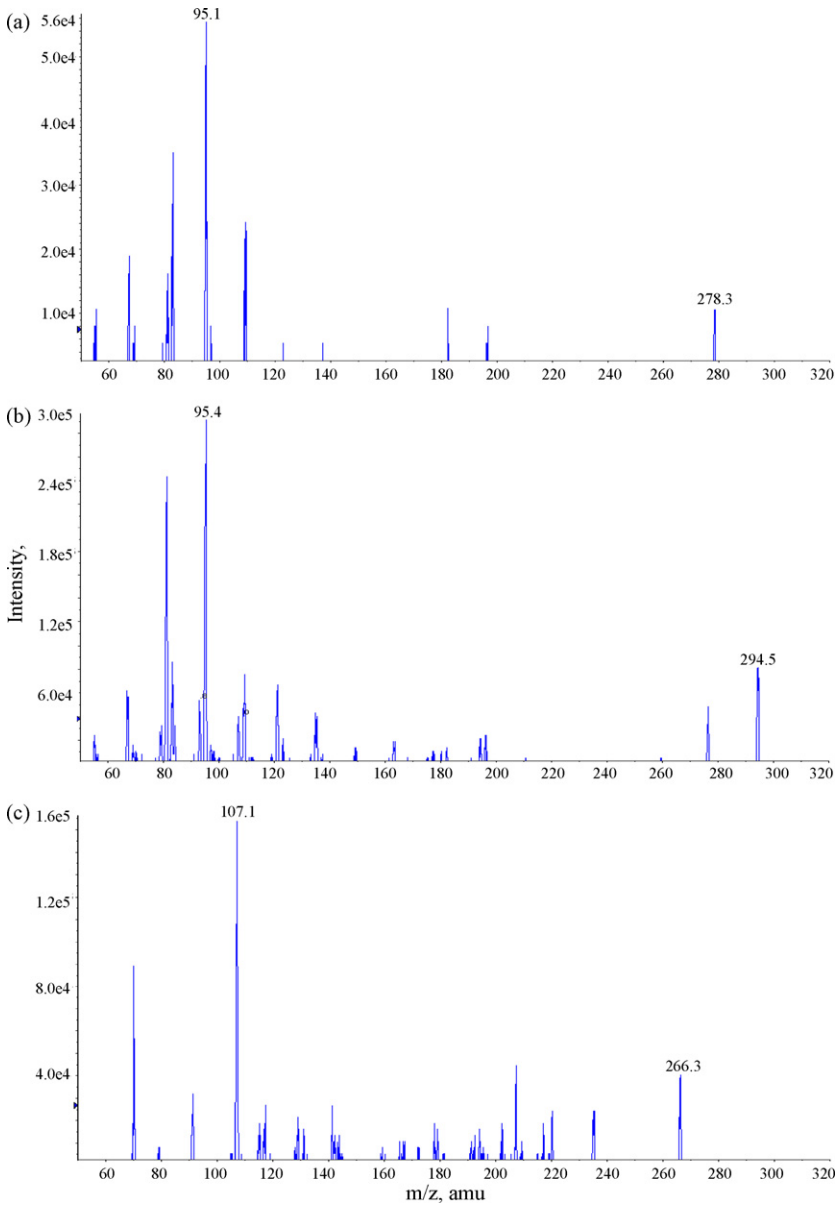

Fig. 2. Product ion mass spectra of $[M+H]^+$ for (a) perhexiline, (b) *cis*-hydroxyperhexiline and (c) nordoxepin.

of perhexiline, *cis*-hydroxyperhexiline and the internal standard in plasma standards to those in the spiked after-protein precipitation blank plasma extracts at the corresponding concentrations ($n = 6$) [absolute recovery = (peak area of analyte from the spiked plasma sample)/(peak area of analyte from the spiked after-protein precipitation blank plasma extract sample) × 100%]. The matrix effects were assessed by comparing the peak area of perhexiline, *cis*-hydroxyperhexiline and the internal standard from the spiked after-protein precipitation blank plasma extracts with the response of standard solution at the same concentration in the mobile phase ($n = 6$). Quality control was assessed by analysis of six samples at each concentration on the same day (intra-day) and of one sample at each concentration on six different days (inter-day). Bias was determined as the measured minus the actual concentration, expressed as a percentage of the actual concentration. Imprecision was measured as intra- and inter-day coefficients of variation. The limit of quantification for this assay was defined as the lowest concentration of perhexiline and *cis*-hydroxyperhexiline that could be detected with acceptable accuracy and precision ($n = 6$) (according to the US Food and Drug Administration guidance for bioanalytical method validation, the mean value determined at the lowest concentration should not deviate by more than 20% of the actual value, and the precision determined at the lowest concentration should not exceed 20% of the coefficients of variation [16]).

The effects of freezing and thawing on the concentrations of perhexiline and hydroxyperhexiline were studied using QC samples at 10, 40, 100, 500 and 2000 μg/L, which were subjected to four freeze–thaw cycles before analysis. The stability of plasma QC samples at −30 °C was evaluated by concentration analysis at weekly intervals for 10 months. The stability of the stock standard solutions of perhexiline and hydroxyperhexiline at 4 °C for 10 months was evaluated by comparing the response with that of the freshly prepared standard solutions. The stability of the processed samples at 4 °C (the temperature of the autosampler) for 3 days was evaluated by comparing the results with the original results. In all cases, the perhexiline and hydroxyperhexiline were considered to be stable as long as degradation was <10% of the concentration at day 0.

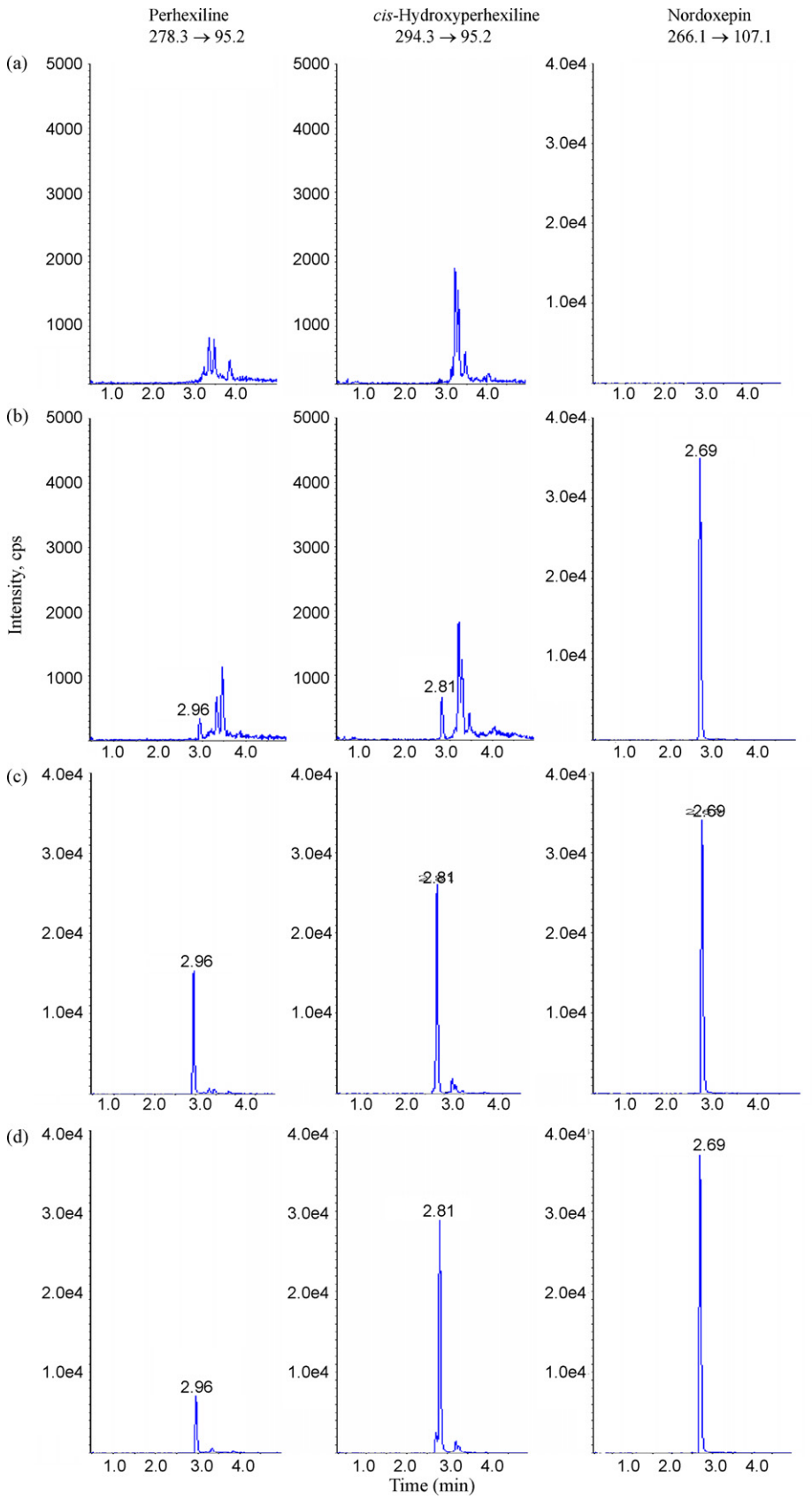

Fig. 3. Representative MRM chromatograms of (a) blank plasma, (b) plasma sample spiked with perhexiline and *cis*-hydroxyperhexiline at 10 μg/L, (c) plasma sample spiked with perhexiline and *cis*-hydroxyperhexiline at 500 μg/L and (d) plasma sample from a patient on perhexiline (perhexiline concentration = 212 μg/L and *cis*-hydroxyperhexiline = 549 μg/L).

Table 1
Intra-day assay variance of the determination of perhexiline and hydroxyperhexiline in plasma ($n = 6$).

Sample	Concentration spiked (μg/L)	Concentration found (μg/L) (mean ± SD)	Bias (%)	Imprecision CV (%)
Perhexiline				
LOQ	10	10.9 ± 0.80	9.2	7.3
QC1	40	40.7 ± 1.6	1.7	4.0
QC2	100	96 ± 5.6	−3.8	5.8
QC3	500	486 ± 11	−2.7	2.3
QC4	2000	2048 ± 37	2.4	1.8
Hydroxyperhexiline				
LOQ	10	9.12 ± 0.76	−8.9	8.4
QC1	40	40.6 ± 1.5	1.6	3.6
QC2	100	99 ± 2.2	−0.9	2.3
QC3	500	525 ± 9.6	4.9	1.8
QC4	2000	2082 ± 57	4.1	2.7

3. Results and discussion

3.1. Mass spectrometry and chromatography

The MS/MS parameters were optimised to produce maximum responses for perhexiline, *cis*-hydroxyperhexiline and the internal standard nordoxepin using electrospray ionisation in the positive ion mode. The protonated molecular ions $[M+H]^+$ were m/z 278.3 for perhexiline, m/z 294.3 for *cis*-hydroxyperhexiline and m/z 266.1 for nordoxepin, respectively. The product ion spectra of $[M+H]^+$ for three compounds are shown in Fig. 2. The transitions yielding the most abundant product ions were 278.3 → 95.2 for perhexiline, 294.3 → 95.2 for *cis*-hydroxyperhexiline and 266.1 → 107.1 for nordoxepin.

Perhexiline, *cis*-hydroxyperhexiline and the internal standard were separated from matrix components using a Phenomenex Luna Phenyl-Hexyl column and a mobile phase consisting of 0.05% formic acid and methanol. Because perhexiline and its hydroxylated metabolite vary widely in polarity, separation was time consuming under isocratic elution. Gradient elution was therefore chosen to obtain better resolution and to enhance separation efficiency. Under the chromatographic conditions employed, the retention times were approximately 2.69, 2.81, and 2.96 min for the internal standard, *cis*-hydroxyperhexiline and perhexiline, respectively (Fig. 3). Because the *cis*-hydroxyperhexiline reference material contained 2.2% *trans*-hydroxyperhexiline, the reference material produced a minor *trans*-hydroxyperhexiline peak which appeared just before the *cis*-hydroxyperhexiline peak with identical precursor-to-product ion transitions to *cis*-hydroxyperhexiline. The baseline separation for *cis*-hydroxyperhexiline and *trans*-hydroxyperhexiline could not be achieved completely, but there was no significant influence on the quantification of *cis*-hydroxyperhexiline. Blank plasma samples from more than six different sources of the same matrix were tested for interference, and perhexiline, *cis*-hydroxyperhexiline and the internal standard peaks were free of interference from any other peaks present in the plasma blanks (Fig. 3). No carry-over was observed by injecting an extract of blank plasma immediately following the highest calibration standard.

3.2. Sample preparation

The higher sensitivity of the LC–MS/MS technique allowed us to use a very small volume of plasma (50 μL) for the quantification of perhexiline and *cis*-hydroxyperhexiline in plasma. Protein precipitation is the simplest and most rapid method of plasma sample preparation for the measurement of drug concentrations. To find the most efficient precipitant for sample preparation, three widely used precipitating agents (acetonitrile, methanol and perchloric acid) were compared. Precipitation with acetonitrile was the best for sample clean-up, with a 1:4 ratio of acetonitrile to plasma being optimal. Perhexiline, *cis*-hydroxyperhexiline and the internal standard were free of interference from endogenous compounds in the plasma. To ensure long term performance, the guard column cartridge was changed every 200–300 injections. The analytical column demonstrated no deterioration of performance after more than 2000 injections.

3.3. Method validation

Plasma standard curves of perhexiline and *cis*-hydroxyperhexiline were linear ($r > 0.999$) over the concentration range of 10–2000 μg/L. The range well encapsulates the therapeutic range of perhexiline (150–600 μg/L in our laboratory). The intercept with the y-axis was not significantly different from zero. The typical standard curves were as follows: $y = 0.948x + 2.85 \times 10^{-6}$ ($r = 0.9995$) for perhexiline and $y = 1.37x + 6.4 \times 10^{-6}$ ($r = 0.9997$) for *cis*-hydroxyperhexiline, where y represents the ratio of the analyte peak area to that of the internal standard and x represents the plasma concentration of the analyte. The lower limit of quan-

Table 2
Inter-day assay variance of the determination of perhexiline and hydroxyperhexiline in plasma ($n = 6$).

Sample	Concentration spiked (μg/L)	Concentration found (μg/L) (mean ± SD)	Bias (%)	Imprecision CV (%)
Perhexiline				
LOQ	10	11.0 ± 0.89	10	8.1
QC1	40	42.2 ± 1.3	5.4	3.2
QC2	100	105 ± 4.7	4.7	4.5
QC3	500	498 ± 17.2	−0.5	3.5
QC4	2000	1928 ± 95.8	−3.6	5.0
Hydroxyperhexiline				
LOQ	10	11.0 ± 0.89	10	8.1
QC1	40	42.3 ± 1.9	5.8	4.4
QC2	100	104 ± 3.0	4.0	2.9
QC3	500	486 ± 9.8	−2.8	2.0
QC4	2000	1830 ± 66.3	−8.5	3.2

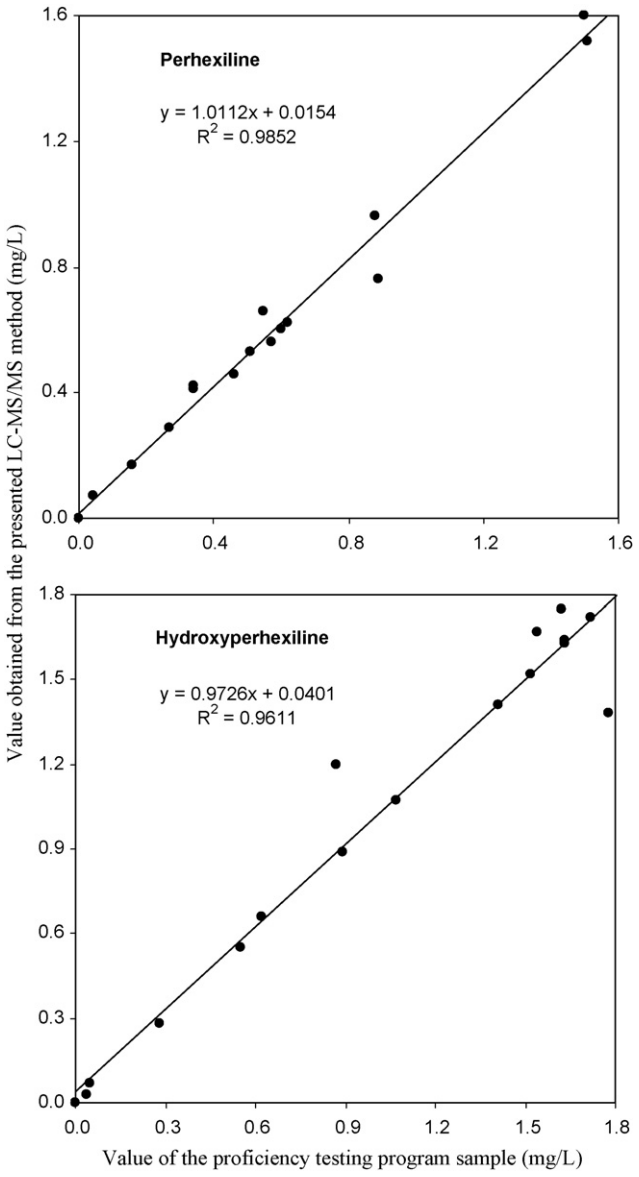

Fig. 4. Correlation between the values of perhexiline and hydroxyperhexiline obtained using the presented LC–MS/MS method and the values of the proficiency testing program samples.

tification (LLOQ) for both perhexiline and *cis*-hydroxyperhexiline was around 10 μg/L in plasma, at which the mean values were within ±10% of the spiked values and the intra- and inter-day coefficients of variation were <8.5% (Tables 1 and 2). There was no constant direction to the bias (i.e. + or −) for plasma QC samples and the mean values were within ±5% of the spiked values. Imprecision was small, as indicated by both intra- and inter-day coefficients of variation of <5.0% at all concentrations from 40 to 2000 μg/L (Tables 1 and 2). The absolute recoveries of perhexiline and *cis*-hydroxyperhexiline at concentrations of 40, 100, 500 and 2000 μg/L were similar and consistent, with the mean values >95%. The absolute recovery of the internal standard nordoxepin at the concentration employed was 82%.

The matrix effects were assessed by comparing the response of perhexiline, *cis*-hydroxyperhexiline and the internal standard from the spiked after-protein precipitation blank plasma extracts with the response of standard solution at the same concentration in the mobile phase [matrix effect = (peak area of analyte spiked in protein precipitated blank plasma)/(peak area of analyte spiked in mobile phase) × 100%]. A value of 100% indicates that the responses in the mobile phase and in protein precipitated plasma were the same and no absolute matrix effect is observed. A value of >100% indicates an ionisation enhancement and a value of <100% indicates an ionisation suppression. The matrix effects (mean ± SD%) determined at concentrations of 40, 100, 500 and 2000 μg/L for perhexiline were 99.6 ± 5.5, 108 ± 5.9, 98.2 ± 5.1 and 106 ± 8.2% respectively, and for *cis*-hydroxyperhexiline were 106 ± 4.1, 113 ± 4.3, 105 ± 4.9 and 106 ± 8.8%, respectively. The matrix effect for the internal standard nordoxepine was 94 ± 5.0%. The results showed that there were no significant matrix effects.

Perhexiline and hydroxyperhexiline were found to be stable in plasma for at least four freeze–thaw cycles when stored at −30 °C. The plasma QC samples at concentrations of 10, 40, 100, 500 and 2000 μg/L were stable for at least 10 months at −30 °C. The stock standard solutions perhexiline and hydroxyperhexiline were shown to remain stable for at least 10 months at 4 °C. The processed samples were stable for at least 3 days at 4 °C.

3.4. Application of the assays

The method presented is currently being used in our laboratory service to monitor the concentrations of perhexiline and its metabolite hydroxyperhexiline in plasma for patients on perhexiline therapy. To ensure the accuracy and reproducibility of the method, we have participated in a monthly interlaboratory proficiency testing program of perhexiline therapeutic monitoring services which is organised by the Department of Clinical Pharmacology, The Queen Elizabeth Hospital, Woodville, South Australia. The monthly returned reports have shown the performance of this method for the analysis of perhexiline and *cis*-hydroxyperhexiline in plasma has always been acceptable (Fig. 4).

4. Conclusions

A validated LC–MS/MS method for the determination of perhexiline and its metabolite *cis*-hydroxyperhexiline has been described. The method has proven to be rapid, sensitive, specific, accurate and precise, and is currently being used in routine clinical service to monitor the plasma concentrations of perhexiline and *cis*-hydroxyperhexiline in patients on perhexiline therapy.

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