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Determination of the 4-monohydroxy metabolites of perhexiline in human plasma, urine and liver microsomes by liquid chromatography

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Abstract

The use of perhexiline (PHX) is limited by hepatic and neurological toxicity associated with elevated concentrations in plasma that are the result of polymorphism of the cytochrome P450 2D6 isoform (CYP2D6). PHX is cleared by hepatic oxidation that produces three 4-monohydroxy metabolites: *cis*-OH-PHX, *trans1*-OH-PHX and *trans2*-OH-PHX. The current study describes an HPLC-fluorescent method utilising pre-column derivatization with dansyl chloride. Following derivatization, the metabolites were resolved on a C18 column with a gradient elution using a mobile phase composed of methanol and water. The method described is suitable for the quantification of the metabolites in human plasma and urine following clinical doses and for kinetic studies using human liver microsomes. The method demonstrates sufficient sensitivity, accuracy and precision between 5.0 and 0.01, 50.0 and 0.2 and 1.0 and 0.005 mg/l in human plasma, urine and liver microsomes, respectively, with intra-assay coefficients of variation and bias <15%, except at the lowest limit of quantification (<20%). The inter-assay coefficients of variation and bias were <15%. The application of this method to plasma and urine samples of five CYP2D6 extensive metaboliser (EM) patients at steady state with respect to PHX dosing determined that the mean (±S.D.) renal clearances of *trans1*-OH-PHX and *cis*-OH-PHX were 1.58 ± 0.35 and 0.16 ± 0.06 l/h, respectively. The mean (±S.D.) dose recovered in urine as free and glucuronidated 4-monohydroxy PHX metabolites was 20.6 ± 11.6%.

Keywords: HPLC; Liver microsomes; Metabolites; Perhexiline; Pharmacokinetics

1. Introduction

The efficacy of perhexiline (PHX) for anti-anginal therapy has been demonstrated when used alone [1,2], when used in patients receiving beta-blockers [3] and in patients on maximal antianginal therapy [4,5]. More recently, PHX has also been demonstrated to be beneficial in chronic heart failure [6]. Its use has been limited by its potential for causing severe hepatic and neurological toxicity associated with elevated plasma PHX concentrations [4,7] and polymorphism of cytochrome P450 2D6 (CYP2D6) [8,9] that results in very large interpatient pharmacokinetic variability [10–12]. As a consequence, therapeutic drug monitoring is essential in order to maintain plasma PHX concentrations within the range of 0.15–0.60 mg/l and achieve therapeutic efficacy whilst minimising the risk of significant toxicity [4,5].

PHX is systemically cleared by hydroxylation at C-4 of the cyclo-hexyl moieties, forming a single *cis*-OH-PHX metabolite (M1) and two *trans*-OH-PHX metabolites, *trans1*-OH-PHX (M3) and *trans2*-OH-PHX (Fig. 1) [7,13,14]. Secondary metabolism forms several 4,4′-dihydroxy metabolites as well as glucuronide conjugates [7,14]. The primary metabolites found in plasma are *cis*-OH-PHX and *trans1*-OH-PHX [14], except in CYP2D6 poor metabolisers (PM) who have a profoundly impaired capacity to form *cis*-OH-PHX [12,15,16–18]. Using a method developed previously in this laboratory, over 4000 patient samples per year are routinely used to phenotype with respect to CYP2D6 metabolism from the ratio of *cis*-OH-PHX to PHX concentrations in plasma [12]. Phenotyping serves a two-fold purpose: it permits the early identification of PM and it

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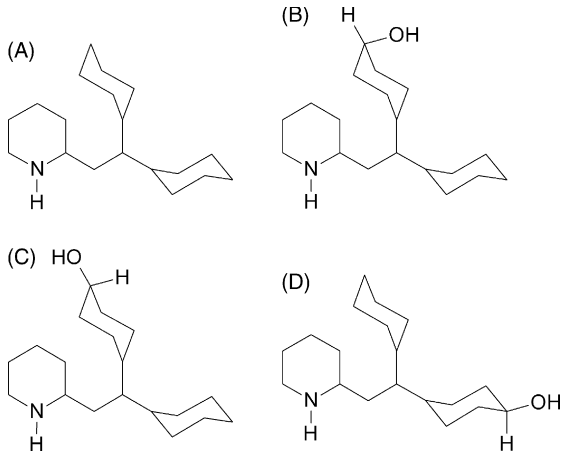

Fig. 1. Chemical structures of perhexiline (A) and its three 4-monohydroxy metabolites, *cis*-OH-PHX (B), *trans1*-OH-PHX (C) and *trans2*-OH-PHX (D).

allows an estimate of the clearance of PHX and therefore dosage requirements by CYP2D6 extensive metabolisers (EM). However, use of the *cis*-OH-PHX to PHX plasma metabolic ratio assumes there is relatively little interindividual variability in the clearance of *cis*-OH-PHX compared to PHX [12].

Published reports regarding the capacity of PM to form *trans1*-OH-PHX are conflicting; either no difference between EM and PM [7,16] or a reduced formation of this metabolite by PM [15,17,18]. No investigation of *trans2*-OH-PHX has been reported since its initial characterisation [14], although the majority of the clearance of PHX in PM is likely to be via the formation of this metabolite and *trans1*-OH-PHX.

The current study describes a validated HPLC-fluorescent method utilising pre-column derivatization with dansyl chloride. It is the first that adequately resolves the three 4-monohydroxy metabolites of PHX for their quantification in human plasma, urine and liver microsomes, and has sufficient sensitivity for pharmacokinetic and enzymatic studies.

2. Experimental

2.1. Chemicals

The *cis*-OH-PHX reference compound was supplied by Marion Merrell Dow (Kansas City, KS, USA). The *trans1*- and *trans2*-OH-PHX reference compounds were supplied by Sigma Pharmaceuticals (South Croydon, Vic., Australia). They were characterised by NMR, GC–MS and HPLC–UV and were of 97% or greater purity. Stock solutions containing 100 mg/l of *cis*-OH-PHX and 100 mg/l each of *trans1*- and *trans2*-OH-PHX were prepared in 10% methanol with 0.1 M HCl. Working standards were prepared in 0.1 M HCl and stored at −20 °C between assays and were stable upon reanalysis after six months (CV and bias < 15%). Perhexiline maleate, dansyl chloride and β-glucuronidase (*Helix pomatia*) were purchased from Sigma Chemical Company (St Louis, MO, USA). All other reagents and chemicals were obtained from commercial sources and were of analytical grade.

2.2. Urine sample preparation

Hydrolysis of 4-monohydroxy PHX glucuronides in urine samples was studied using β-glucuronidase concentrations up to 10,000 U/ml for 8–48 h. A 24 h incubation with 6000 U/ml of β-glucuronidase at 37 °C was sufficient for complete hydrolysis. A stock solution of β-glucuronidase (7500 U/ml) was freshly prepared in 0.2 M sodium acetate buffer (pH 5) prior to use in each incubation. For the determination of total metabolite concentrations (free and glucuronidated), 400 μl of β-glucuronidase stock was added to 100 μl urine samples. The incubations were stopped by rapid cooling on ice and 100 μl of this mixture added to 400 μl of 0.1 M HCl prior to extraction. Urine samples for the determination of free metabolite concentrations, calibration standards and quality control samples were prepared similarly on ice but were not incubated.

2.3. Microsome sample preparation

A human liver sample from a genotyped [19] *CYP2D6* EM (*CYP2D6*1/*4)* was obtained during partial hepatectomy from a patient who had given their written informed consent for their tissue to be used. The donor had normal clinical chemistry and haematology measurements prior to surgery and the tissue sample was normal based on gross morphology. The sample was stored at −80 °C. Human liver microsomes were prepared by differential centrifugation of liver homogenate [20] and stored in buffer at −80 °C until use. Microsomal incubations were linear with time up to 60 min and with microsomal protein concentrations up to 1.0 mg/ml for PHX concentrations of 2 μM (the approximate upper limit of the PHX therapeutic range in plasma). Accordingly, microsomal incubations were performed at 37 °C in a shaking water bath for 30 min in 0.1 M phosphate buffer (pH 7.4) containing human liver microsomal protein (0.25 mg/ml), perhexiline maleate (2 μM, added as a stock dissolved in methanol to produce a final methanol concentration of 0.5%, v/v) and an NADPH generating system composed of 1 mM NADP, 5 mM isocitrate, 1 U/ml isocitrate dehydrogenase type IV and 5 mM $MgCl_2$, for a total incubation volume of 2 ml. The incubations were stopped by rapid cooling on ice and immediately extracted.

2.4. Sample extraction procedure

A 500 μl aliquot of sample (plasma, treated urine, microsomes, calibrators and controls) was made basic with 50 μl of 2 M NaOH before the addition of 4 ml of a solution of 30% dichloromethane and 70% *n*-hexane. This mixture was shaken on a horizontal mixer at 100 oscillations per minute for 10 min, followed by centrifugation at 1200 × *g* for 15 min. The organic layer was separated and dried and the residue was derivatized with dansyl chloride. Derivatization was studied over 20–120 min using dansyl chloride concentrations of 5–25 mM in acetone and $NaHCO_3$ concentrations of 0.1–0.5 M. A 60 min incubation at 37 °C using 200 μl of freshly prepared 15 mM dansyl chloride in acetone and 200 μl of 0.3 M $NaHCO_3$ produced sufficient sensitivity and reproducibility. Following incubation,

3 ml of *n*-hexane was added and vortex mixed for 5 min before centrifuging at 1200 × *g* for 3 min. The organic layer was separated and dried and the residue reconstituted in 150 μl of a solution of 20% glass distilled water and 80% methanol, of which 100 μl was injected onto the HPLC column.

2.5. Chromatography

Chromatography was performed on an Agilent 1100 series HPLC (Agilent Technologies, Forest Hill, Vic., Australia) operated by Chemstation for LC 3D software. The hardware consisted of a model G1322A degasser, a model G1311A pump operating at 1 ml/min, a model G1313A autosampler, and a model G1321A fluorescence detector with excitation and emission wavelengths of 360 and 470 nm, respectively. The two *trans*-OH-PHX metabolites and *cis*-OH-PHX were resolved with a Merck Purospher RP-18E column (5 μm, 125 mm × 4 mm) at 30 °C and a mobile phase composed of 80% methanol and 20% glass distilled water for the first 14 min, at which time the methanol was increased to 100% for the remaining 8 min of each sample run time in order to hasten the elution of PHX.

2.6. Method validation

Concentrations of *cis*-, *trans1*- and *trans2*-OH-PHX were calculated from their respective peak areas. Calibration samples were prepared by spiking drug-free human plasma, urine or microsomal samples with *cis*-, *trans1*- and *trans2*-OH-PHX working standards. The calibration curves for *trans1*-OH-PHX, *trans2*-OH-PHX and *cis*-OH-PHX were over concentration ranges of 5.0–0.01 mg/l for plasma, 50.0–0.20 mg/l for urine and 1.0–0.005 mg/l for liver microsomes and constructed using unweighted linear regression. Precision and accuracy were evaluated using the coefficient of variation (CV) and the bias of the measured concentration versus the known spiked concentration, respectively. The method was validated within a single run by assaying six replicates of the top and bottom calibrators and six replicates of each of the quality control concentrations. Interassay quality control was assessed in analytical runs by analysing aliquots from three separately prepared control pools each for plasma, urine and liver microsomes, spiked with *cis*-, *trans1*- and *trans2*-OH-PHX and representing low, medium and high concentrations of analytes. The quality control concentrations were 0.06, 0.3 and 1.5 mg/l for plasma, 0.6, 3.0 and 15.0 mg/l for urine and 0.015, 0.075 and 0.4 mg/l for liver microsomes. A CV and bias of ±15% was considered acceptable, except at the lowest limit of quantification (LLOQ: 0.01 mg/l for plasma, 0.2 mg/l for urine, 0.005 mg/l for liver microsomes), where ±20% was accepted [21]. Due to the lack of UV absorbance and fluorescence of the underivatized 4-monohydroxy PHX metabolites and the unavailability of pure derivatized metabolites, it was not possible to determine the absolute extraction efficiencies of the method. Nevertheless, the relative recovery of each metabolite from plasma, urine (β-glucuronidase concentration of 6000 U/ml) and microsomes was determined by analysing six replicates of each quality control sample and the peak areas compared against those from six replicates of each non-extracted standard. Drugs commonly coadministered to patients receiving treatment with PHX were screened for possible chromatographic interference by analysis of plasma and urine from patients admitted to the Cardiology Unit of The Queen Elizabeth Hospital who were not taking PHX.

2.7. Clinical study

Approval was obtained from the Ethics of Human Research Committee of The Queen Elizabeth Hospital to analyse blood specimens received for routine PHX monitoring over a 3-month period with respect to the concentrations of the 4-monohydroxy metabolites of PHX. Ten patients phenotyped as CYP2D6 PM [12] and at steady-state with respect to PHX dosing were identified and their trough concentrations of the 4-monohydroxy metabolites of PHX measured in plasma. Ethics approval was also obtained for a pharmacokinetic study. Five patients phenotyped as CYP2D6 EM and one as a CYP2D6 PM [12] and at steady-state with respect to PHX dosing were identified and gave written informed consent to participate. For the EM patients, blood samples were drawn from an indwelling venous catheter at times 0, 0.5, 1, 2, 4, 8 and 12 h. Patency of the catheter was maintained by instilling 1.5 ml of heparinized saline (15 U heparin) after each sample was drawn. The first 1.5 ml of blood drawn was discarded to prevent dilution by the heparinized saline. Three of the patients were taking PHX maleate once daily and had a subsequent sample drawn at 24 h by venepuncture. Samples were collected in EDTA tubes, centrifuged immediately and the plasma collected. All the urine was collected over the course of the dosing interval. For the PM patient, blood samples were drawn by venepuncture at times 0, 8, 24, 72, 120 and 168 h. Because the patient took 50 and 100 mg of PHX maleate on alternating weeks, blood samples were also drawn at times 176, 192, 240, 288 and 336 h. Samples were collected in EDTA tubes, centrifuged immediately and the plasma collected. Plasma and urine samples were stored at −20 °C and were stable upon reanalysis after 6 months (CV and bias <15%). Plasma PHX concentrations were determined by a previously published method [12]. Creatinine clearance was calculated according to the method of Cockcroft and Gault [22]. The areas under the *cis*-, *trans1*- and *trans2*-OH-PHX concentration–time curves (AUC) were calculated by the linear trapezoidal method. Renal clearance was calculated as the mass of metabolite recovered over the dosing interval divided by its AUC in plasma for the same dosing interval.

3. Results and discussion

3.1. Chromatography

The 4-monohydroxy metabolites of PHX have very poor UV absorbance and fluorescence and require derivatization to enhance their detection by conventional HPLC. This was achieved by reaction with dansyl chloride to form highly fluorogenic dansyl derivatives.

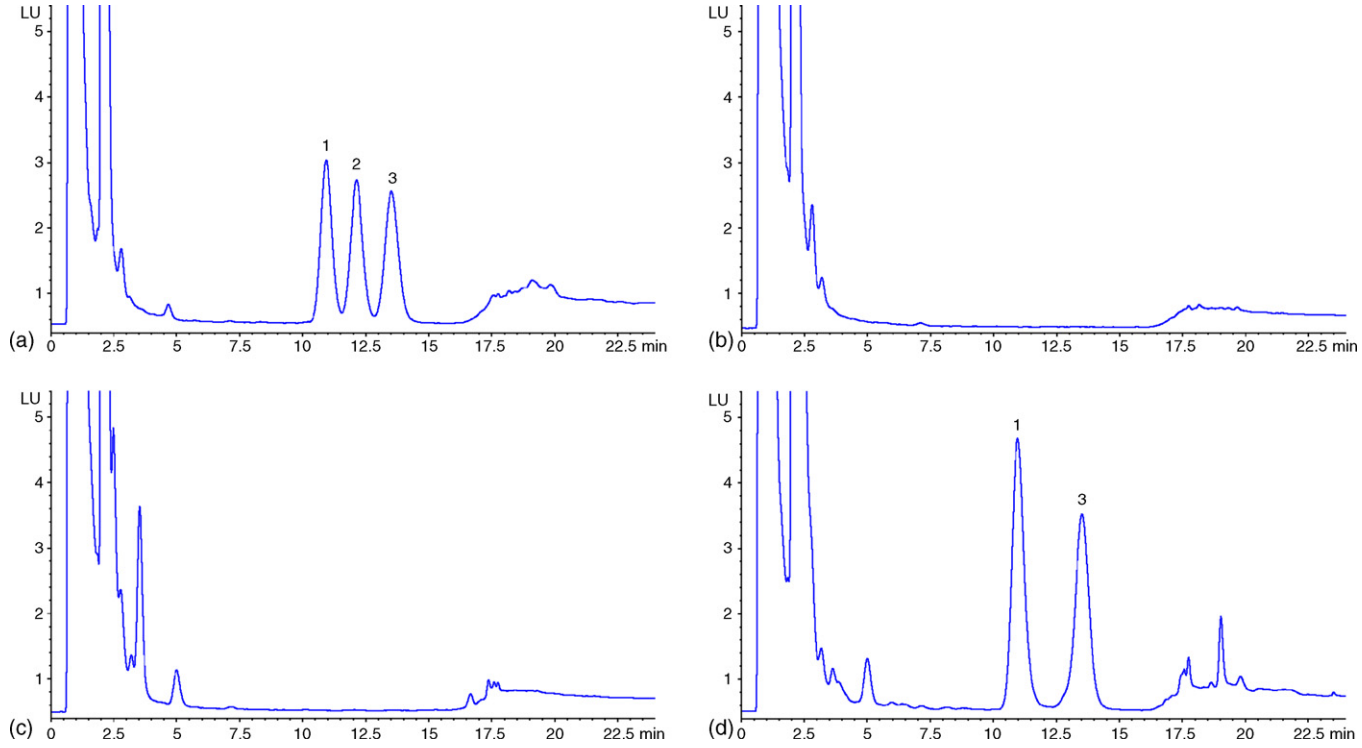

Fig. 2. Representative chromatograms of blank urine spiked with 10 mg/l each of the *trans1*-, *trans2*- and *cis*-OH-perhexiline reference compounds (a), unincubated blank urine with β-glucuronidase (6000 U/ml) (b), the same sample as (b) following a 24 h incubation at 37 °C (c), and an incubated urine specimen with β-glucuronidase (6000 U/ml) from a CYP2D6 extensive metaboliser containing 17.8 mg/l of *trans1*-OH-perhexiline and 14.3 mg/l of *cis*-OH-perhexiline (d). Peaks 1–3 correspond to *trans1*-OH-perhexiline, *trans2*-OH-perhexiline and *cis*-OH-perhexiline, respectively.

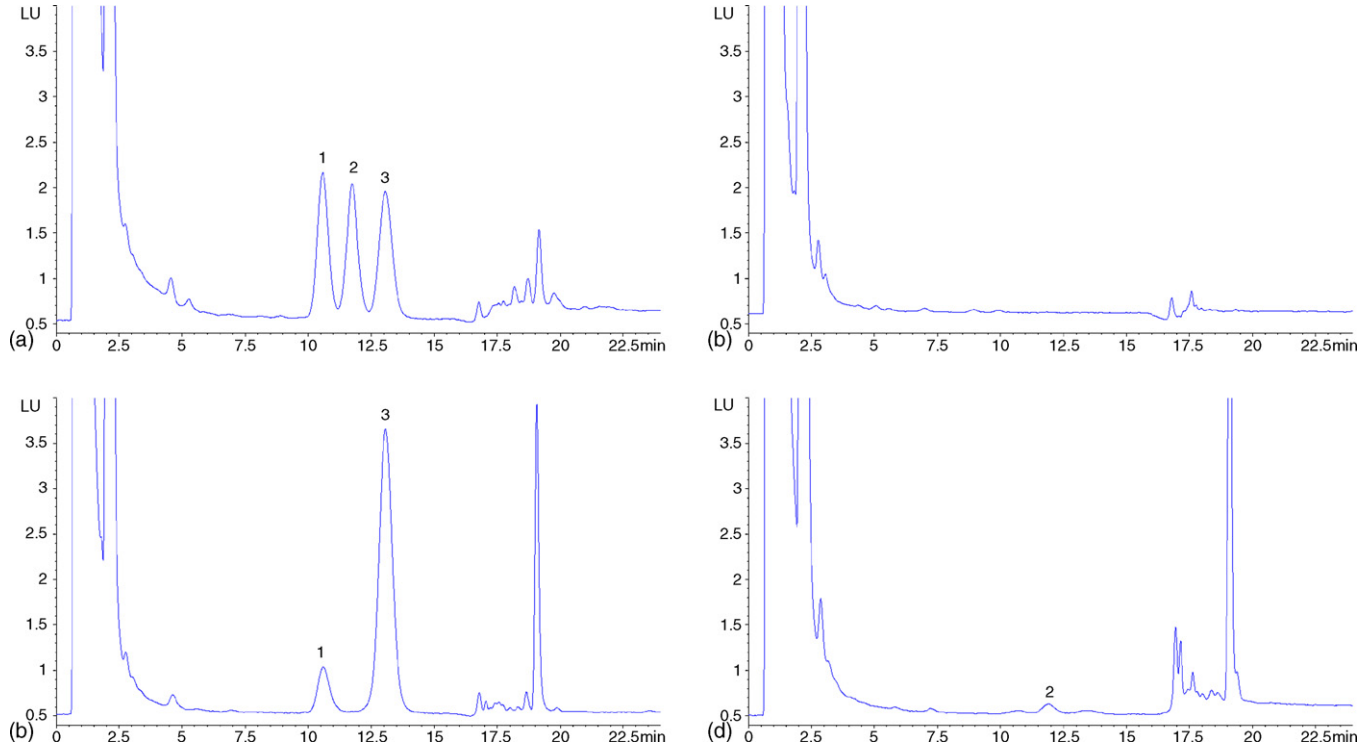

Fig. 3. Representative chromatograms of blank plasma spiked with 0.5 mg/l each of the *trans1*-, *trans2*- and *cis*-OH-perhexiline reference compounds (a), blank plasma (b), a plasma specimen containing 0.13 mg/l of *trans1*-OH-perhexiline and 0.87 mg/l of *cis*-OH-perhexiline from a CYP2D6 extensive metaboliser patient receiving treatment with perhexiline (c), and a plasma specimen containing 0.02 mg/l of *trans2*-OH-perhexiline from a CYP2D6 poor metaboliser patient receiving treatment with perhexiline (d). Peaks 1–3 correspond to *trans1*-OH-perhexiline, *trans2*-OH-perhexiline and *cis*-OH-perhexiline, respectively.

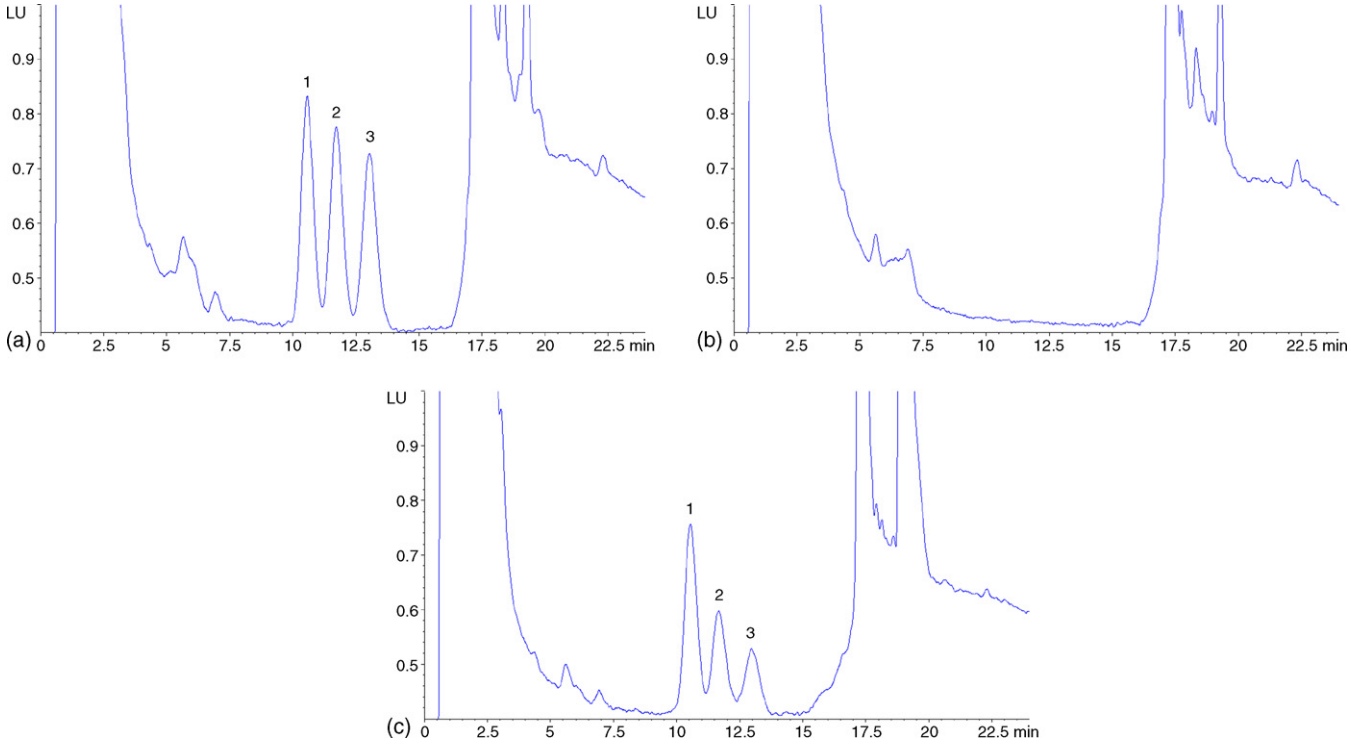

Fig. 4. Representative chromatograms of liver microsomes spiked with 0.05 mg/l each of the *trans1*-, *trans2*- and *cis*-OH-perhexiline reference compounds (a), a drug free microsomal incubation (b), and a microsomal incubation specimen from a CYP2D6 extensive metaboliser incubated with 2 μM of perhexiline and containing 0.035 mg/l (0.12 μM) of *trans1*-OH-perhexiline, 0.019 mg/l (0.06 μM) of *trans2*-OH-perhexiline and 0.014 mg/l (0.05 μM)of *cis*-OH-perhexiline (c). Peaks 1–3 correspond to *trans1*-OH-perhexiline, *trans2*-OH-perhexiline and *cis*-OH-perhexiline, respectively.

Several compounds were evaluated for use as an internal standard. Hexadiline, a dehydro derivative of PHX used routinely by this laboratory for quantification of *cis*-OH-PHX and PHX for therapeutic drug monitoring [12], proved unsuitable due to the significantly greater polarity of the analytes. The retention times for prenylamine, fendiline and pipradrol were practical, although they proved to be unacceptable with respect to precision. Due to the lack of other commercially available compounds structurally similar to the 4-monohydroxy metabolites of PHX, it was necessary to validate the assay without an internal standard. The retention times for *trans1*-, *trans2*- and *cis*-OH-PHX were approximately 10.7, 11.9 and 13.2 min, respectively (Figs. 2a, 3a, 4a). There was no chromatographic interference with any of the peaks of interest from endogenous compounds in urine before or after incubation with β-glucuronidase (Fig. 2b and c, respectively), from endogenous compounds in plasma (Fig. 3b), from endogenous compounds in liver microsomes or the NADPH generating system (Fig. 4b), or from PHX (retention time approximately 19.1 min).

3.2. Method validation

Linear relationships were obtained between response and concentration, with correlation coefficients (r^2) of 0.991–0.999 for urine ($n = 6$), 0.988–0.999 for plasma ($n = 6$) and 0.994–1.000 for liver microsomes ($n = 6$). The inter- and intra-assay accuracy and precision were within acceptable limits [21] and are presented in Table 1 (urine), Table 2 (plasma) and Table 3 (liver microsomes). The relative extraction efficiency of the 4-monohydroxy metabolites of PHX was in the range of 69–79% for plasma, 61–72% for urine (β-glucuronidase concentration of 6000 U/ml) and 88–94% for liver microsomes. No interference from drugs coadministered for ischaemic heart disease or unrelated conditions or their metabolites was detected in urine or plasma (Table 4). This demonstrates that these drugs and their metabolites will not interfere with the determination of the 4-monohydroxy metabolites of PHX in urine or plasma from patients being treated with PHX.

Table 1
Bias and precision data for the quantification of *cis*-4-monohydroxyperhexiline, *trans1*-4-monohydroxyperhexiline and *trans2*-4-monohydroxyperhexiline in urine specimens

Target (mg/l)	*trans1*-OH-PHX		*trans2*-OH-PHX		*cis*-OH-PHX	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Intra-assay ($n = 6$)						
50.0	5.8	−1.7	5.8	−1.6	4.5	−1.4
15.0	5.1	−0.2	5.8	−0.3	5.6	−0.1
3.0	6.6	13.5	9.1	13.9	5.7	11.1
0.6	8.3	2.0	4.9	13.5	10.3	8.3
0.2	12.5	13.6	11.3	13.8	12.2	11.1
Inter-assay ($n = 6$)						
15.0	6.8	5.8	7.2	4.4	6.2	3.4
3.0	5.6	3.4	6.1	2.6	6.8	−1.8
0.6	8.1	0.8	4.7	1.8	6.6	0.8

Table 2
Bias and precision data for the quantification of *cis*-4-monohydroxyperhexiline, *trans1*-4-monohydroxyperhexiline and *trans2*-4-monohydroxyperhexiline in plasma specimens

Target (mg/l)	*trans1*-OH-PHX		*trans2*-OH-PHX		*cis*-OH-PHX	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Intra-assay ($n=6$)						
5.00	9.9	4.2	10.1	4.2	9.2	2.8
1.50	10.2	−4.2	10.4	−4.0	8.4	−2.7
0.30	5.9	3.9	5.6	3.5	4.8	−6.3
0.06	7.7	−11.9	7.8	−11.6	6.4	−0.3
0.01	18.3	−9.2	15.9	6.6	18.2	−19.8
Inter-assay ($n=6$)						
1.50	8.2	6.4	6.3	4.6	9.4	4.1
0.30	8.1	5.2	8.2	4.4	5.4	5.3
0.06	9.6	3.7	10.0	5.6	9.5	2.6

3.3. Detection of the 4-monohydroxy metabolites of perhexiline in urine, plasma and liver microsome incubations

The primary 4-monohydroxy PHX metabolite recovered in the urine as free metabolite and as glucuronide conjugate was *trans1*-OH-PHX (Fig. 2d), and accounted for a mean (±S.D.) dose recovery of 9.4 ± 5.1 and 1.8 ± 1.1%, respectively (Table 5). This finding is in contrast to other research that found *cis*-OH-PHX in greater concentrations in urine [7,15–18], although all of these studies except one [7] used single doses of PHX that may have minimised the saturation of CYP2D6-mediated *cis*-monohydroxylation observed with repeated PHX dosing [23]. Free and glucuronidated *cis*-OH-PHX accounted for a mean (±S.D.) dose recovery of 8.2 ± 5.2 and 1.0 ± 0.6%, respectively (Table 5). The mean (±S.D.) percentage of the dose recovered as free and glucuronidated 4-monohydroxy perhexiline over a dosing interval was 20.6 ± 11.6%. This is consistent with the findings of two other studies conducted in patients at steady-state [7,14]. Wright et al. [14] recovered a further 11–37% of a dose as free 4,4′-dihydroxy perhexiline, and formation of these and the 4-monohydroxy metabolites accounted for up to 75% of dose recovery. The mean (±S.D.) renal clearance of *trans1*-OH-PHX and *cis*-OH-PHX, calculated from free metabolites recovered in urine, was 1.58 ± 0.35 and 0.16 ± 0.06 l/h, respectively (Table 5).

Table 3
Bias and precision data for the quantification of *cis*-4-monohydroxyperhexiline, *trans1*-4-monohydroxyperhexiline and *trans2*-4-monohydroxyperhexiline in liver microsome specimens

Target (mg/l)	*trans1*-OH-PHX		*trans2*-OH-PHX		*cis*-OH-PHX	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Intra-assay ($n=6$)						
1.000	3.1	2.2	3.0	−0.7	3.0	0.4
0.400	5.7	1.8	5.9	1.9	5.1	1.9
0.075	3.6	3.7	4.0	3.1	4.0	2.9
0.015	4.8	14.4	9.7	14.7	7.4	13.2
0.005	10.0	16.4	9.1	10.1	11.0	4.3
Inter-assay ($n=6$)						
0.400	2.9	1.6	2.9	1.2	3.9	−2.7
0.075	7.4	−2.0	7.8	−2.5	7.6	−8.4
0.015	9.4	−3.6	8.6	−5.5	11.3	−13.3

Table 4
Drugs and their metabolites screened and found to be free from chromatographic interference in the analysis of the 4-monohydoxy metabolites of perhexiline in urine[a] and plasma[b]

Acetazolamide[b]	Fentanyl[a]	Pantoprazole[b]
Allopurinol[b]	Finasteride[a]	Paracetamol[a,b]
Amiloride[b]	Flucloxacillin[b]	Pergolide[a]
Amiodarone[b]	Fluconazole[b]	Perindopril[a,b]
Amitriptyline[b]	Fluticasone[b]	Pravastatin[a,b]
Amoxycillin[a,b]	Frusemide[a,b]	Prazosin[a]
Amphotericin[b]	G-CSF[b]	Prednisolone[a,b]
Aspirin[a,b]	Gemfibrozil[b]	Prochlorperazine[b]
Atenolol[a,b]	Gentamicin[a,b]	Promethazine[a,b]
Atorvastatin[a,b]	Gliclazide[a,b]	Propranolol[b]
Azithromycin[b]	Glipizide[a,b]	Quetiapine[b]
Betaxolol[b]	Glyceryl trinitrate[a,b]	Quinapril[b]
Brinzolamide[b]	Haloperidol[b]	Quinine[b]
Busulphan[b]	Heparin[b]	Ramipril[b]
Cabergoline[a,b]	Hydrochlorothiazide[b]	Ranitidine[a,b]
Candesartan[a,b]	Hyoscine butylbromide[b]	Risperidone[a,b]
Carbidopa[a,b]	Ibuprofen[b]	Roxithromycin[a,b]
Carvedilol[a]	Indapamide[a,b]	Salbutamol[a,b]
Cefotaxime[b]	Irbesartan[a]	Salmeterol[b]
Ceftazidime[b]	Isosorbide dinitrate[a,b]	Simvastatin[a,b]
Ceftriaxone[a]	Isosorbide mononitrate[a,b]	Soltalol[a]
Cephalexin[b]	Itraconazole[b]	Spironolactone[b]
Ciprofloxacin[b]	Latanoprost[b]	Sulfamethoxazole[b]
Citalopram[a]	Levodopa[a,b]	Sulfasalazine[a]
Clavulanic acid[a,b]	Lisinopril[a]	Telmisartan[b]
Clonazepam[a]	Lithium[b]	Temazepam[b]
Clonidine[a,b]	Lorazepam[a,b]	Thyroxine[b]
Clopidogrel[a,b]	Meloxicam[b]	Ticarcillin[b]
Codeine[a,b]	Memantine[b]	Timolol[b]
Dexamethasone[b]	Metformin[a,b]	Tiotropium[b]
Digoxin[a,b]	Metoclopramide[a,b]	Tramadol[a,b]
Diltiazem[b]	Metoprolol[a,b]	Tranexamic acid[b]
Domperidone[a,b]	Metronidazole[a,b]	Trimethoprim[a,b]
Donepezil[b]	Mometasone[b]	Tropisetron[b]
Enoxaparin[a]	Morphine[b]	Vancomycin[a,b]
Ergocalciferol[b]	Nicotine[b]	Venlafaxine[b]
Ergotamine[a]	Nitrazepam[b]	Verapamil[a,b]
Escitalopram[b]	Omeprazole[a,b]	Voriconazole[b]
Famciclovir[b]	Oxazepam[b]	Warfarin[a,b]

The concentrations of 4-monohydroxy PHX metabolites in the ten PM plasma samples received for routine monitoring were very low. The primary metabolite detected was *trans2*-OH-PHX (Fig. 3d), with a mean (±S.D.) concentration of 0.020 ± 0.007 mg/l. *Trans1*-OH-PHX and *cis*-OH-PHX were detected, but were below the LLOQ in seven and four instances, respectively. Similarly, *trans2*-OH-PHX was the only metabolite detected within the LLOQ over the sampling period for the PM subject of the pharmacokinetic study (Fig. 5c). In contrast, *cis*-OH-PHX and *trans1*-OH-PHX were the primary metabolites in the EM plasma samples (Fig. 3c), with the concentration of *cis*-OH-PHX greater than that of *trans1*-OH-PHX at all time points in the dosing interval (Fig. 5a and b). Because the EM and PM patients had similar dose-corrected plasma PHX concentrations (data not shown), yet *trans1*-OH-PHX was only a primary

Table 5
Extensive metaboliser patient characteristics, renal clearances and dose recoveries of the 4-monohydroxy metabolites of perhexiline

Subject	Age (years)	Sex	*cis*-OH-PHX: perhexiline metabolic ratio	Creatinine clearance (l/h)	AUC (mg h/l)			Renal clearance (l/h)		
					trans1-OH-PHX	*trans2*-OH-PHX	*cis*-OH-PHX	*trans1*-OH-PHX	*trans2*-OH-PHX	*cis*-OH-PHX
EM1	80	M	11.7	0.9	3.8	–	29.4	1.20	–	0.13
EM2	76	M	3.6	2.7	6.4	–	61.2	2.14	–	0.21
EM3	77	M	0.8	4.7	4.1	0.6	17.7	1.64	0.73	0.24
EM4	77	M	10.3	1.5	3.6	–	44.1	1.49	–	0.12
EM5	89	F	7.9	1.6	3.4	–	41.6	1.45	–	0.10

Subject	Free metabolite recovered (% of dose)			Glucuronidated metabolite recovered (% of dose)		
	trans1-OH-PHX	*trans2*-OH-PHX	*cis*-OH-PHX	*trans1*-OH-PHX	*trans2*-OH-PHX	*cis*-OH-PHX
EM1	6.1	<LOQ	5.1	1.1	<LOQ	0.7
EM2	18.3	<LOQ	17.4	3.6	<LOQ	1.9
EM3	8.9	0.6	5.7	1.7	0.1	0.4
EM4	7.1	<LOQ	7.3	0.8	<LOQ	1.4
EM5	6.7	<LOQ	5.5	1.8	<LOQ	0.8

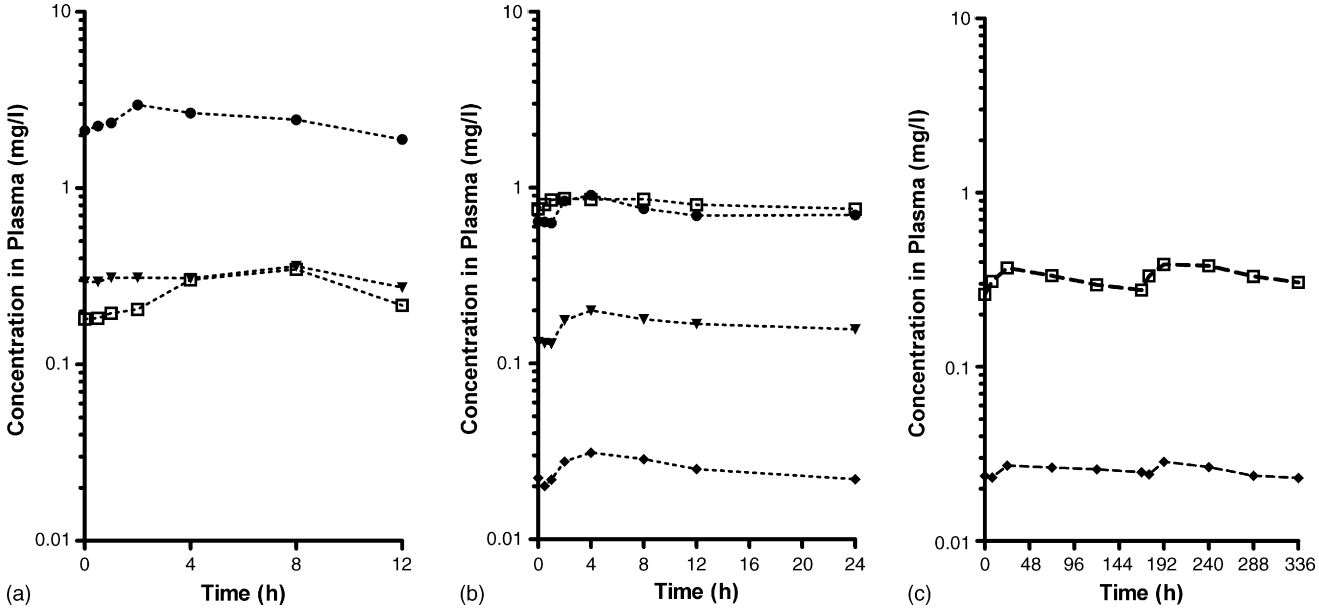

Fig. 5. Plasma perhexiline (squares), *trans1*-OH-PHX (triangles), *trans2*-OH-PHX (diamonds) and *cis*-OH-PHX (circles) concentration–time profiles in one poor and two extensive metabolisers of perhexiline at steady-state with respect to perhexiline dosing. EM1 was maintained on 100 mg of perhexiline maleate twice per day (a), EM3 was maintained on 100 mg of perhexiline maleate once per day (b) and PM1 was maintained on 50 and 100 mg of perhexiline maleate on alternating weeks (c). Perhexiline was administered after the 0 h sample was drawn and was administered again to PM1 after the 168 h sample was drawn.

metabolite in the plasma of the EM patients, clearance of PHX by the formation of *trans1*-OH-PHX is likely to be mediated primarily by CYP2D6. *Trans2*-OH-PHX was above the LOQ in the plasma of one EM patient only (Fig. 5b). This patient (EM3) also had the lowest CYP2D6 activity as measured by the *cis*-OH-PHX to PHX metabolic ratio (Table 5). Similarly, Beck et al. [13] detected both *trans*-OH-PHX metabolites in PM plasma samples, yet only one was present in the samples of EM patients. This suggests that *trans2*-OH-PHX is a substrate of CYP2D6. All three of the 4-monohydroxy PHX metabolites were detected in a microsomal incubation prepared with liver tissue from an EM (Fig. 4c).

4. Conclusion

In summary, we report the first reversed-phase HPLC-fluorescent method providing sufficient resolution for the quantification in urine, plasma and liver microsomes of the three 4-monohydroxy metabolites of PHX in humans, *trans1*-OH-PHX, *trans2*-OH-PHX and *cis*-OH-PHX. The method described

was validated over a wide range of concentrations, suitable for CYP2D6 EM and PM, with acceptable precision, accuracy and specificity necessary for complex plasma and urine samples.

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