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Determination of perhexiline and hydroxyperhexiline in plasma by liquid chromatography–mass spectrometry

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Abstract

A method for the quantitative determination of perhexiline and its main hydroxylated metabolites in human plasma, based on liquid chromatography–mass spectrometry (LC–MS), was developed. The method used protein precipitation with acetonitrile followed by dilution with water and subsequent direct injection of the extract into the LC–MS system. Hexadiline was used as internal standard and the intra-assay coefficients of variation were ≤5% for perhexiline and *cis*-hydroxyperhexiline over the target concentration range in patients. The lower limits of quantification were 0.005 mg/l for perhexiline and 0.015 mg/l for *cis*-hydroxyperhexiline, and the measuring ranges were from 0.05 to 3.0 and from 0.2 to 6.0 mg/l, respectively. The method was compared with an established HPLC method with fluorescence detection and the correlation between the methods was close to 1 for both compounds. The predominant form of hydroxyperhexiline in 87% of the patient samples was found to be one of the diastereomeric pairs of *cis*-hydroxyperhexiline. In patients not forming this metabolite, *trans*-hydroxyperhexiline could be detected. We conclude that the present LC–MS method is suitable for use in a clinical routine laboratory.

Keywords: Perhexiline; Hydroxyperhexiline

1. Introduction

Perhexiline (Fig. 1) is a drug that has found use in the pharmacotherapy of angina pectoris and acute coronary syndromes that do not respond to conventional therapy [1]. Due to its potential to cause toxicity and large inter-individual pharmacokinetic variability, it is mandatory to individualize the dosing of perhexiline [2–4]. In clinical practice, individual dosing may be guided by measurements of plasma perhexiline concentrations according to the principles of therapeutic drug monitoring (TDM) [5]. A target window of 0.15–0.6 mg/l has been established for plasma perhexiline at steady-state [4,6].

Perhexiline is metabolized into two primary mono-hydroxy metabolites (Fig. 1), *cis*-hydroxyperhexiline and *trans*-hydroxyperhexiline, both of which may exist as diastereomeric pairs [7]. Recently, routine TDM recommendations have incorporated measurements of *cis*-hydroxyperhexiline in order to better subgroup patients as poor, extensive or ultra-rapid metabolizers [8]. Recommendations regarding optimal daily doses for these subgroups of patients have been proposed [8].

So far HPLC with fluorescence detection (HPLC/FL) has been most commonly used in TDM laboratories, but gas chromatography (GC) has also been applied [9]. The HPLC methods have used sample preparation with liquid/liquid extraction and precolumn derivatization of both perhexiline and hydroxyperhexilines in order to produce fluorescent products for detection [10]. Both HPLC and GC procedures are capable of simultaneous quantification of perhexiline and hydroxyperhexilines in human plasma [8,10,11].

The development of liquid chromatography–mass spectrometry (LC–MS) in recent years has established a new powerful technique, including routine clinical analyses in TDM laboratories [12]. The possibility of applying a simple and rapid sample preparation in combination with LC–MS has proved to be of great value for the TDM laboratory. Since

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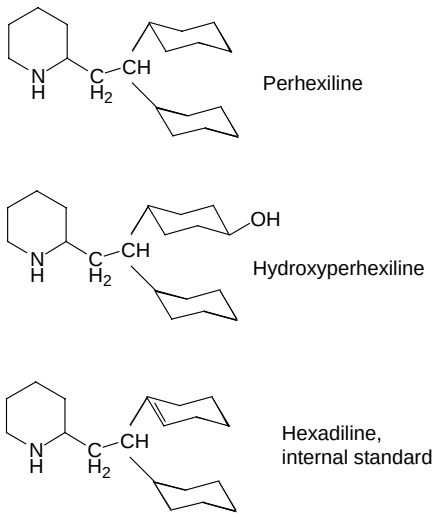

Fig. 1. Chemical structures of the analytes perhexiline and hydroxyperhexiline. The structure of hexadiline (internal standard) is not conclusively established regarding the exact position of the double bond.

perhexiline and the hydroxyperhexilines contain functionalities suitable for MS detection, we undertook to investigate the potential use of LC–MS for quantitative determination of these substances in human plasma. Perhexiline is presently being introduced for therapy in Sweden and a TDM service was required before this could be started.

2. Experimental

2.1. Chemicals

Perhexiline maleate was obtained from Sigma Co. (Australia) and a stock solution was prepared in 50% ethanol in water at a concentration of 20 mg/l (free base), which was stored at −20 °C. A stock solution of hydroxyperhexiline (Merrell Dow Pharmaceuticals, OH, USA) was prepared in 10% methanol with 100 mM HCl (1:10) at a concentration of 100 mg/l (free base), which was stored at −20 °C. The hydroxyperhexiline reference material consisted of 97% *cis*- and 3% *trans*-hydroxyperhexiline. A hexadiline (Fig. 1, Merrell Dow Pharmaceuticals) stock solution was prepared in 1 mM HCl at a concentration of 50 mg/l. A working solution of hexadiline was prepared at a concentration of 5 mg/l by dilution with1 mM HCl. Both solutions of hexadiline were stored at −20 °C. Acetonitrile of HPLC grade was obtained from JT Baker (Holland), and formic acid of analytical quality from Merck GmbH (Germany).

2.2. Sample preparation procedure

A 200 μl aliquot of plasma was mixed with 50 μl of hexadiline (internal standard) working solution (250 ng) and 350 μl of acetonitrile in a glass test-tube. The sample was mixed vigorously for 1 min and centrifuged at 1000 × *g* for 5 min. An aliqout of 100 μl of the resulting supernatant was transferred to a 250 μl glass autosampler vial together with 100 μl of ultra-pure water.

2.3. LC–MS analysis

A volume of 5 μl was injected into an Agilent 1100 MSD LC–MS system (Agilent Technologies, CA, USA). The system was equipped with an electrospray interface, a dual LC pump, degasser, column thermostat and an autosampler. A 50 mm × 2.1 mm Zorbax SB-phenyl column, particle size 5 μm (Agilent Technologies), with a 10 mm × 2.1 mm Hy-purity C4 guard column (particle size 5 μm) was used (ThermoFinnigan Co., CA, USA). The mobile phase was pumped at a flow of 300 μl/min and a linear binary gradient of 0–100% B was used, where A consisted of 20% acetonitrile in 50 mM formic acid and B of 60% acetonitrile in 50 mM formic acid. The instrument operated in the positive ion SIM mode with a fragmentor voltage of 70 V for *m*/*z* 278 (perhexiline) and *m*/*z* 294 (hydroxyperhexiline) and 250 V for *m*/*z* 276 (hexadiline, hydroxyperhexiline). The dwell time was 192 ms, drying gas flow rate10 μl/min, drying gas temperature 350 °C, and the nebulizer pressure was 330 kPa.

2.4. Quantification

Calibrator samples were prepared in calf serum from stock solutions (see above). The concentrations (free base) were from 0.05 to 3.0 mg/l for perhexiline and from 0.2 to 6.0 mg/l for hydroxyperhexiline. The prepared calibrators were stored at −20 °C. Quantification was achieved by using peak area ratios between the analyte and internal standard. Calibration graphs were constructed by using linear regression. For hexadiline (internal standard) the second eluting peak (see below) was used in the quantification. Four hydroxyperhexiline peaks were observed in the reference material (Fig. 2) consistent with both *cis*- and *trans*-hydroxyperhexiline diastereomers. The two later eluting peaks corresponded to *cis*-hydroxyperhexiline and accounted for 96% of the total hydroxyperhexiline peak areas, consistent with previously established purity of the reference material obtained by LC–MS and NMR (Sigma Pharmaceuticals, Australia). The combined area for the two (later eluting) *cis*-hydroxyperhexiline peaks was used in the calibration.

2.5. Method comparison

A total of 30 randomly selected clinical specimens were used for comparison of the LC–MS method with an established HPLC/FL method [8]. Analyses of calibration standard and quality control samples from the HPLC/FL laboratory were also used to ensure similar calibration levels of the two methods.

3. Results

3.1. Chromatography

The optimal chromatographic system for perhexiline was obtained using a phenyl column packing, which was preferred over C_{18} and cyano packing (Fig. 2). The internal standard, which is a dehydro derivative of perhexiline, produced two equal peaks in accordance with the existence of two chiral centers. In the procedure, these two peaks are almost completely separated and the later eluting peak was selected for quantification. In order to include measurements of hydroxyperhexiline in the assay, a gradient had to be used for elution because of a significant difference in polarity between the compounds. The reference material produced four peaks with identical mass spectra in the chromatogram of which two *cis*-hydroxyperhexiline peaks were dominant (Fig. 2). The minor pair of peaks (*trans*-hydroxyperhexiline) constituted 4% of the total as calculated from peak areas. A representative chromatogram obtained from the analysis of a patient specimen is shown in Fig. 3.

3.2. Method validation

Linear relationships were obtained between response and concentration for perhexiline in the range of 0.05–3.0 mg/l and for hydroxyperhexiline in the range of 0.2–6.0 mg/l. The correlation coefficients (r) were 0.997–0.999 and 0.994–0.999, respectively ($n = 7$). The lower limits of quantification defined, as the response with a signal to noise ratio of 10 were 0.005 mg/l for perhexiline and 0.015 mg/l for *cis*-hydroxyperhexiline. The within-series coefficient of variation (CV) was determined at two levels for each substance (Table 1). For both compounds, the CVs were ≤5% at all concentrations tested. Calibrators were prepared in calf serum and this was found to be no different from human serum. The recovery of perhexiline and hydroxyperhexiline in the sample preparation was >95%.

Table 1
Precision and accuracy of perhexiline and hydroxyperhexiline determination

Substance	Weighed-in concentration (mg/l)	Observed concentration (mg/l)	Bias (%)	CV (%)	*N*
Perhexiline	0.20	0.20	0	3.5	10
Perhexiline	1.0	1.1	10	5.0	10
Hydroxyperhexiline	0.40	0.40	0	1.7	10
Hydroxyperhexiline	2.0	2.1	5	4.3	10

3.3. Method comparison

A method comparison was performed with an HPLC/FL method for perhexiline and hydroxyperhexiline measurement in human plasma. The comparison was performed using authentic routine patient samples collected randomly to cover a wide concentration range. For perhexiline ($r^2 = 0.981$), the two methods gave almost identical results with no outliers, but for hydroxyperhexiline ($r^2 = 0.951$) the data were more variable, possibly due to a lower precision in *cis*-hydroxyperhexiline quantification (Fig. 4).

3.4. Metabolite isomers in patients

The pattern of hydroxyperhexiline peaks in patient samples differed from the reference material (Figs. 2 and 3).

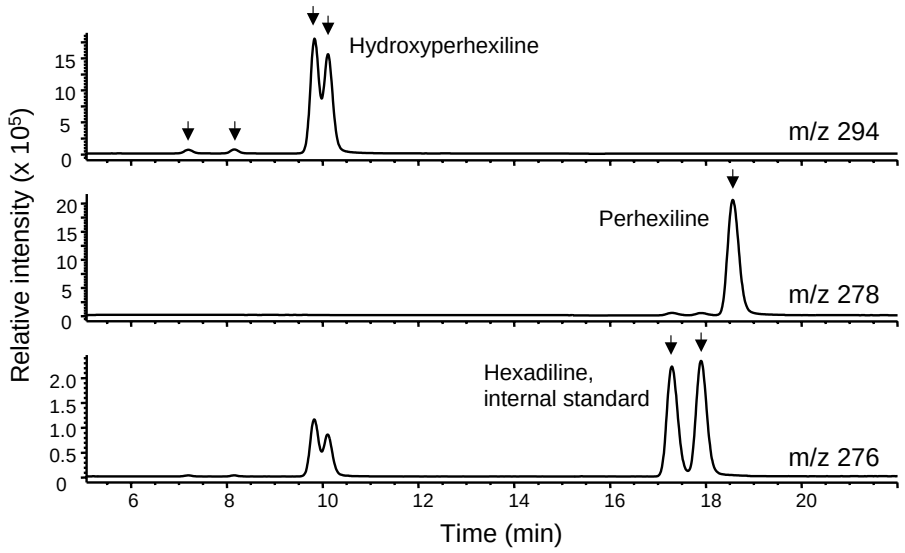

Fig. 2. Chromatogram obtained from the LC–MS analysis of an extract from a plasma standard containing 1000 ng/ml of perhexiline and 2000 ng/ml of hydroxyperhexiline. The chromatogram at *m*/*z* 294 shows the four peaks (marked with arrows) obtained for hydroxyperhexiline. These four peaks have identical mass spectra demonstrating that they represent hydroxyperhexiline isomers. The first pair of peaks represents *trans*-hydroxyperhexiline isomers and the major form *cis*-hydroxyperhexiline isomers (second pair of peaks) and constitutes 96% of the total peak area. The internal standard hexadiline was monitored at *m*/*z* 276 and also gave two peaks (marked with arrows) in accordance with the presence of two chiral centers in molecule.

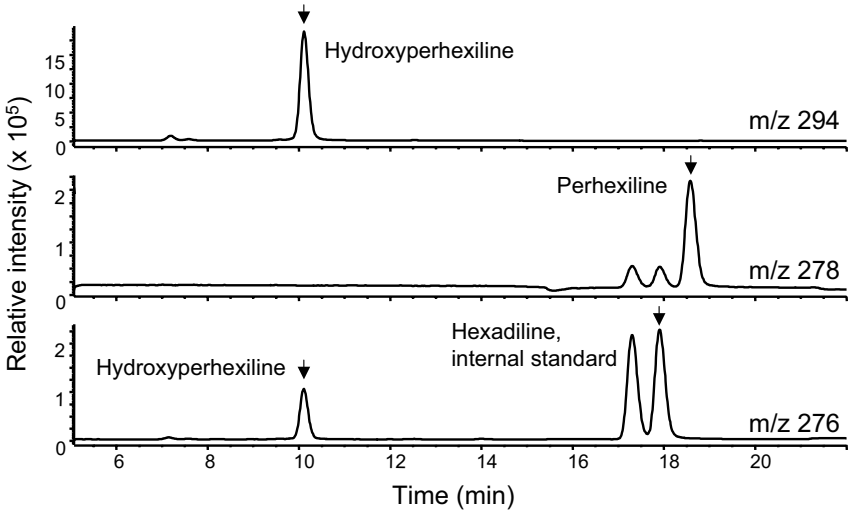

Fig. 3. Chromatogram obtained from the LC–MS analysis of an extract from a patient plasma sample. The sample was found to contain 120 ng/ml of perhexiline and 1800 ng/ml of *cis*-hydroxyperhexiline.

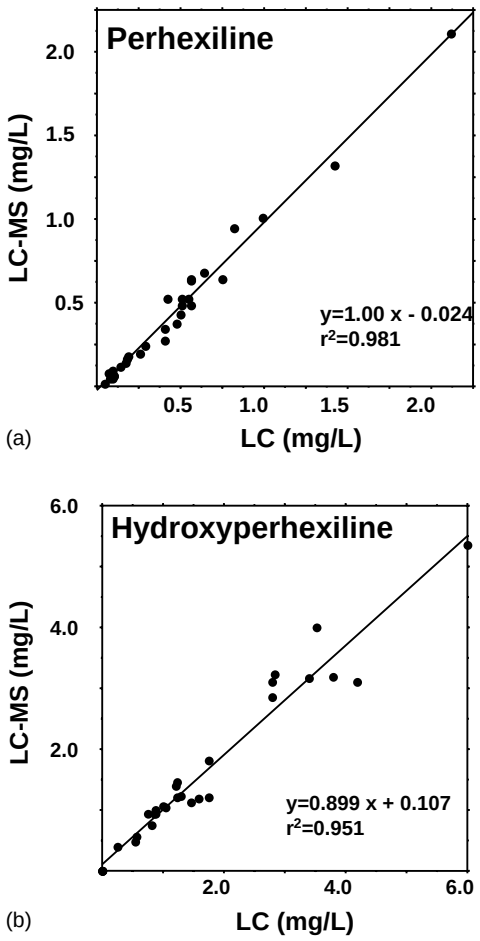

Fig. 4. Correlation between results obtained from the analysis of perhexiline (a) and hydroxyperhexiline (b) with the presented LC–MS method and a reference LC method.

In 26 out of 30 patients (87%), only one of the *cis*-hydroxyperhexiline forms (the later eluting) accounted for the majority of metabolite present (Fig. 5). No evidence was observed for the presence of the other *cis*-hydroxyperhexiline form (the first eluting) in any patient sample (<2%). In addition, the first eluting *trans*-hydroxyperhexiline diastereomer accounted for about 5% (by area ratio) of the dominant *cis*-hydroxyperhexiline. Both *trans*-hydroxyperhexiline diastereomer pairs could, however, be observed in the four patients who did not have any detectable *cis*-hydroxyperhexiline (Fig. 5). The identity

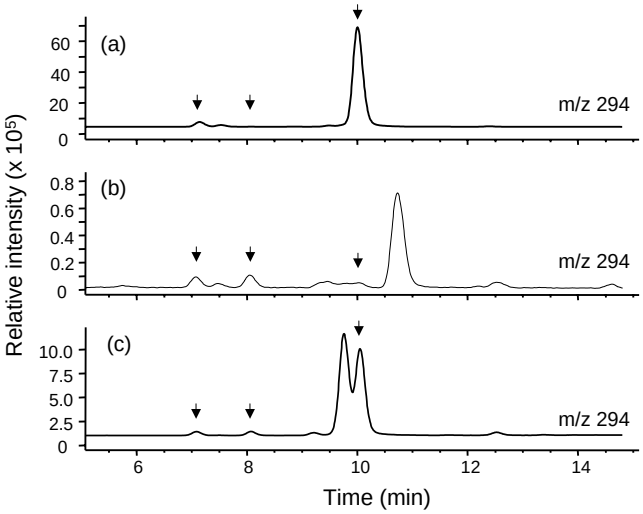

Fig. 5. Chromatogram obtained from the LC–MS analysis of extracts from a patient sample with the highest level of *cis*-hydroxyperhexiline (a), a patient plasma sample found not to contain *cis*-hydroxyperhexiline (b), and a calibrator sample (c). In patients in whom no *cis*-hydroxyperhexiline (i.e. the major metabolite form) could be detected, trace amounts of the minor trans form (both peaks) were present. In patients with *cis*-hydroxyperhexiline formation, only one of the peaks representing *trans*-hydroxyperhexiline was present.

of the *trans*-hydroxyperhexiline in the patient samples was demonstrated by co-elution using extracts from patient specimens with and without spiked reference material, and by the presence of peaks at the qualifier ion *m*/*z* 276.

4. Discussion

The results obtained in this study demonstrate that LC–MS is a suitable technique for accurate quantification of perhexiline and hydroxyperhexiline metabolites in human plasma. The technique allowed for a simple sample preparation procedure with no loss of analytes to give a robust method, which is suitable in a clinical routine laboratory. The practical consequence of this simplified preparation procedure is a 4 h shorter time before start of the chromatographic run. This will enable a shorter turn-around-time, which is of value in clinical use. The quantification was based on the use of the same internal standard (hexadiline) as commonly used in other published methods. Although this internal standard functioned well and gave acceptable results regarding both precision and accuracy, a number of drawbacks of using this compound should be mentioned. Firstly, the substance is not commercially available, secondly it resolves into two peaks (diastereomeric pairs), which must be resolved in the chromatographic system, and thirdly it is of suitable polarity for perhexiline, but not for hydroxyperhexiline metabolites. The great difference in polarity between the analytes necessitated a gradient elution. The composition of the gradient was partly determined also by the need for resolution of hydroxyperhexiline and hexadiline isomers. These requirements resulted in a rather long run time. Since all patient samples contained only one of the isomer peaks of *cis*-hydroxyperhexiline diastereomers the need for chromatographic separation could be compromized for a more rapid assay time. However, a change in internal standard must also be made to allow for this. In this respect, the simpler preparation procedure used, and especially the lack of a derivatization step, will allow for a greater freedom in the selection of internal standard.

An accurate quantification of the hydroxyperhexiline isomers depends on accurate characterization of pure reference substance. As predicted by the diastereomeric nature of the *cis*- and *trans*-isomers, the reference substance gave four peaks in the chromatogram. All four peaks produced identical mass spectra demonstrating the identity as hydroxyperhexiline isomers. The major form was the *cis*-isomer and the minor was the *trans*-isomer, as previously established [8]. Based on peak area comparison, *cis*-hydroxyperhexiline constituted 96% of the “pure” substance material. One of the *cis*-hydroxyperhexiline diastereomer pairs dominated in patient specimens, but one of the *trans*-hydroxyperhexiline diastereomer pairs was also present. However, in the four patient specimens where no evidence of formation of *cis*-hydroxyperhexiline was obtained, peaks representing both *trans*-hydroxyperhexiline diastereomers were observed. The enzymology of the formation of these isomers as well as their biological significance is being investigated.

In conclusion, the use of LC–MS enabled the development of a simple procedure suitable for the routine quantification of perhexiline and its *cis*-hydroxyperhexiline metabolite. LC–MS provides significant potential for TDM laboratories to offer high quality analytical service with rapid turn-around times. This is essential for the clinical use of drugs, such as perhexiline, which have marked toxicity when overdosed.

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