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Sensitive and specific liquid chromatographic–tandem mass spectrometric assay for barnidipine in human plasma

M. Pawula[a,*], D. Watson[a], T. Teramura[b], T. Watanabe[b], S. Higuchi[b], K.N. Cheng[a]

[a]*Huntingdon Life Sciences Ltd., Huntingdon, Cambridgeshire, PE18 6ES, UK*
[b]*Drug Metabolism Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 1–8, Azusawa 1-Chome, Itabashi-ku, Tokyo 174, Japan*

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Abstract

A sensitive and specific LC–MS–MS assay has been developed and validated for barnidipine (1-benzyl-3-pyrrolidinyl)methyl-2,6-dimethyl-4(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate). The assay involves a simple and rapid solid-phase extraction procedure. Sample analysis was on a Spherisorb S3ODS2 100 mm×2 mm I.D. column, with a Finnigan TSQ 7000 mass spectrometer, using an electrospray interface and selective reaction monitoring (SRM). The intra- and inter-day precision and accuracy, determined as the coefficient of variation and relative error, respectively, were 11.8% or less. The limit of quantitation was 0.03 ng/ml, and the calibration was linear between 0.03 and 3.0 ng/ml. The method has been used successfully for the measurement of over two thousand human plasma samples from pharmacokinetic clinical trials.

Keywords: LC–MS–MS; Barnidipine

1. Introduction

Barnidipine hydrochloride (I) is an extremely potent calcium antagonist with high selectivity for the 1,4-dihydropyridine binding site, with a long duration of action [1]. Due to the potency of the drug, the dosage regimes proposed for its therapeutic use in hypertension are anticipated to be associated with low concentrations in the plasma compartment (ca. 1 ng/ml) [2,3]. Therefore, the analysis of plasma samples requires an assay with a very low limit of quantification for use in obtaining pharmacokinetic data from clinical trials.

A GC negative ion chemical ionisation MS method has previously been established for the determination of barnidipine with high sensitivity (LOQ 30 pg/ml) [4]. Compounds with 1,4-dihydropyridine structure have been known to oxidise to their corresponding pyridine analogues in the GC injector, GC column or transfer line between GC and MS [5–8]. The established GC method for the analysis of barnidipine used a fused-silica capillary GC column directly introduced into the MS ion source (minimising oxidation), and a stable isotope labelled [$^2H_4$]-barnidipine as an internal standard, to determine the unchanged barnidipine specifically [4]. Similar approaches have been used during the analysis of other dihydropyridine based drugs to minimise oxidation, such as using a solid injector (amlodipine) [9] and a fused-silica capillary column (MPC-1304) [10]. The thermal degradation of barnidipine and other dihydropyridine drugs can also be overcome by

*Corresponding author.

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using a HPLC method (and this appears to be the most suitable technique in terms of specificity) [11–16], however, the sensitivity had not been comparable to GC and GC–MS methods [4,9,17]. This paper describes the development of an improved assay method which has been achieved by liquid chromatography on a narrow bore column and using on-line electrospray MS–MS detection (selective reaction monitoring mode). Furthermore, the LC–MS–MS method requires a simple one-step solid-phase extraction procedure, whereas the GC–MS methods involve long, laborious and complicated liquid–liquid extraction procedures [4,9].

2. Experimental

2.1. Chemicals

Barnidipine hydrochloride, the internal standard ([$^2H_4$]-barnidipine hydrochloride), metabolite M1 and metabolite M8 were synthesised in Yamanouchi Pharmaceutical Co. Ltd., Japan. The structural formulae of these compounds are shown in Fig. 1. Blank human control plasma (heparinised) for the preparation of calibration standards and quality control (QC) samples was obtained from Charterhouse Clinical Research Unit, London, UK. Super purity water (Romil Ltd., Cambridge, UK), Far UV acetonitrile, triethylamine (BDH, Poole, UK), tetrahydrofuran (Rathburns, Walkerburn, UK), methanol and glacial acetic acid (Fisons, Loughborough, UK) were all HPLC grade and used as purchased.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters 616 pump, 600 controller and 717 autosampler (Waters, Milford, MA, USA), with a Spherisorb ODS 2 (3 μm) 100 mm×2 mm I.D. analytical column (Phase Separations Ltd, Deeside, UK). The HPLC system

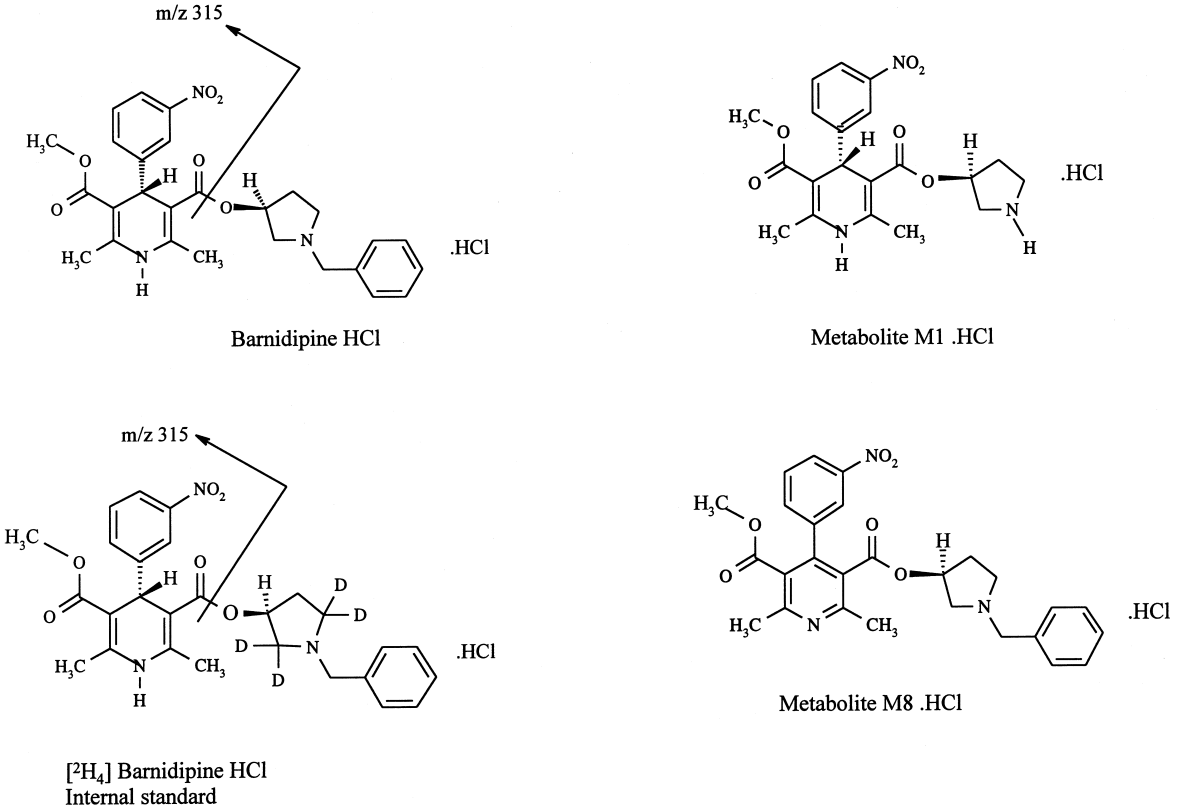

Fig. 1. Structures of barnidipine, its metabolites and the internal standard.

was operated isocratically at a flow-rate of 0.25 ml/min, and the mobile phase consisted of 0.01% formic acid (aqueous)–tetrahydrofuran–acetonitrile (10:30:60, v/v).

A Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan, San Jose, CA, USA) was used in API mode with an electrospray interface in the positive ion mode. Ions monitored in the selective reaction monitoring mode were m/z 492 (precursor ion) to m/z 315 (product ion) for barnidipine, and m/z 496 (precursor ion) to m/z 315 (product ion) for the internal standard [$^2H_4$]-barnidipine. The mass resolution measured at m/z 492 (Q1) was approximately 1000, and at m/z 315 (Q3) was 600 (mass resolution defined as $M/\Delta M$: peak width at half height). Argon was used as the collision gas, and the collision energy used was set at 30 eV. Other conditions were set as follows; sheath gas at 70 p.s.i. (1 p.s.i.=6894.76 Pa), auxiliary gas 15 units, and collision gas at 3 mTorr, heated capillary temperature at 200°C.

2.3. Preparation of stock solutions, calibration standards and quality control samples

Primary stock solutions of barnidipine hydrochloride were prepared from separate weighings for calibration standards and quality control samples. The primary stock solutions were prepared in methanol, and the subsequent working stock solutions were prepared by dilution with acetonitrile. The internal standard [$^2H_4$]-barnidipine hydrochloride, metabolite M1 and metabolite M8 were prepared in methanol, and then diluted with acetonitrile. All solutions were stored at ca. 4°C when not in use, for up to 1 month.

Calibration standards (range 0.03–3.0 ng/ml) were prepared by spiking an appropriate amount of concentrated barnidipine hydrochloride stock solutions into blank control plasma. Quality control samples were prepared in blank control plasma at concentrations of 0.03, 0.25 and 2.5 ng/ml of barnidipine, and stored at ca. −20°C until required for analysis.

2.4. Sample preparation and extraction procedure

Calibration standards, plasma and QC samples (1 ml) were spiked with 20 μl of [$^2H_4$]-barnidipine solution (50 ng/ml), and 1 ml of ammonium acetate buffer (0.05 *M*, pH 4), vortex mixed and centrifuged at ca. 2700 *g* for 10 min. The sample was applied to a C_{18} (100 mg/ml) Bond Elut solid-phase extraction cartridge (Anachem, Luton, UK), which had been preconditioned with 2×1 ml methanol and 2×1 ml ammonium acetate buffer (0.05 *M*, pH 4). The cartridge was then washed with 2×1 ml of the same buffer. The cartridge was air dried for 2 min, followed by elution with 2×1 ml of 1% triethylamine in acetonitrile. The eluates were evaporated to dryness under nitrogen, at 37°C, and the residue reconstituted in 150 μl of acetonitrile. A 100-μl portion of the reconstituted sample was injected onto the analytical column and eluted with a run-time of ca. 2 min.

2.5. Data analysis

Data were acquired using ICIS 8.1.1 mass spectrometry software (Finnigan MAT, San Jose, CA, USA). Regression and data analysis was performed using VAL 1.2 software (written in house at Huntingdon Life Sciences Ltd., Cambs, UK).

2.6. Assay validation

2.6.1. Linearity

Calibration standards (range=0.03–3.0 ng/ml, at seven concentrations) were extracted and assayed. A linear model was used to fit the concentration/peak area ratio data using weighted ($1/c^2$) least squares linear regression.

2.6.2. Precision and accuracy

Spiked QC samples (n=6) at each of three concentrations (0.03, 0.25 and 2.5 ng/ml) of barnidipine were assayed to determine repeatability and accuracy, by comparing calculated values against nominal concentrations. The inter-day precision and accuracy was assessed on three separate occasions.

2.6.3. Extraction recovery

The absolute recovery (extraction efficiency) of barnidipine through the extraction procedure was determined at low and high concentrations (0.03 and 2.5 ng/ml), by adding known amounts of barnidipine to human plasma prior to extraction. The responses of these samples were compared to those in which barnidipine was added after extraction (n=6).

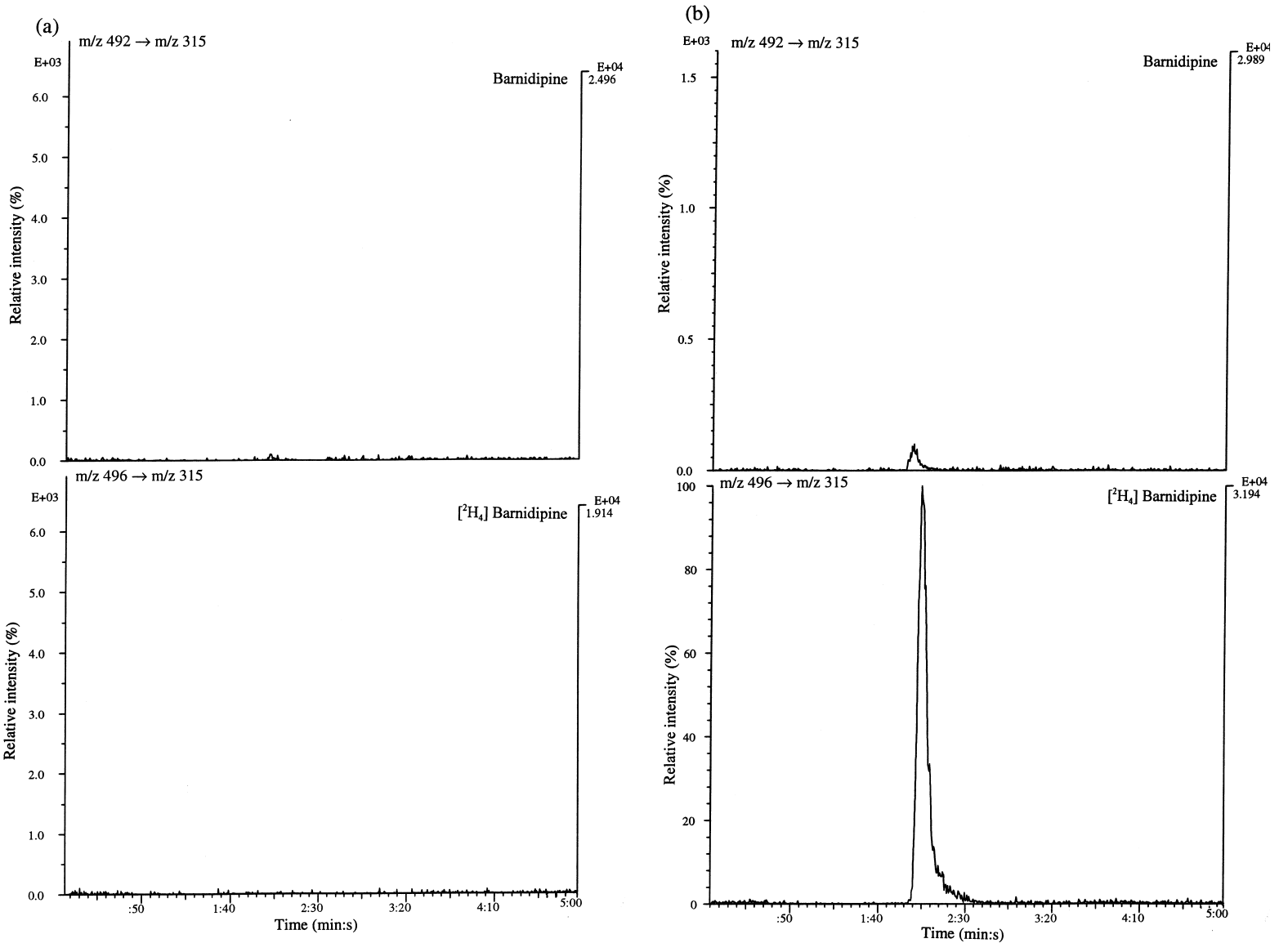

Fig. 2. Representative SRM chromatograms of extracted plasma samples: (a) blank plasma, (b) plasma spiked with 0.03 ng/ml barnidipine, (c) plasma spiked with 3.0 ng/ml barnidipine, (d) plasma sample from a volunteer at 12 h after dosing.

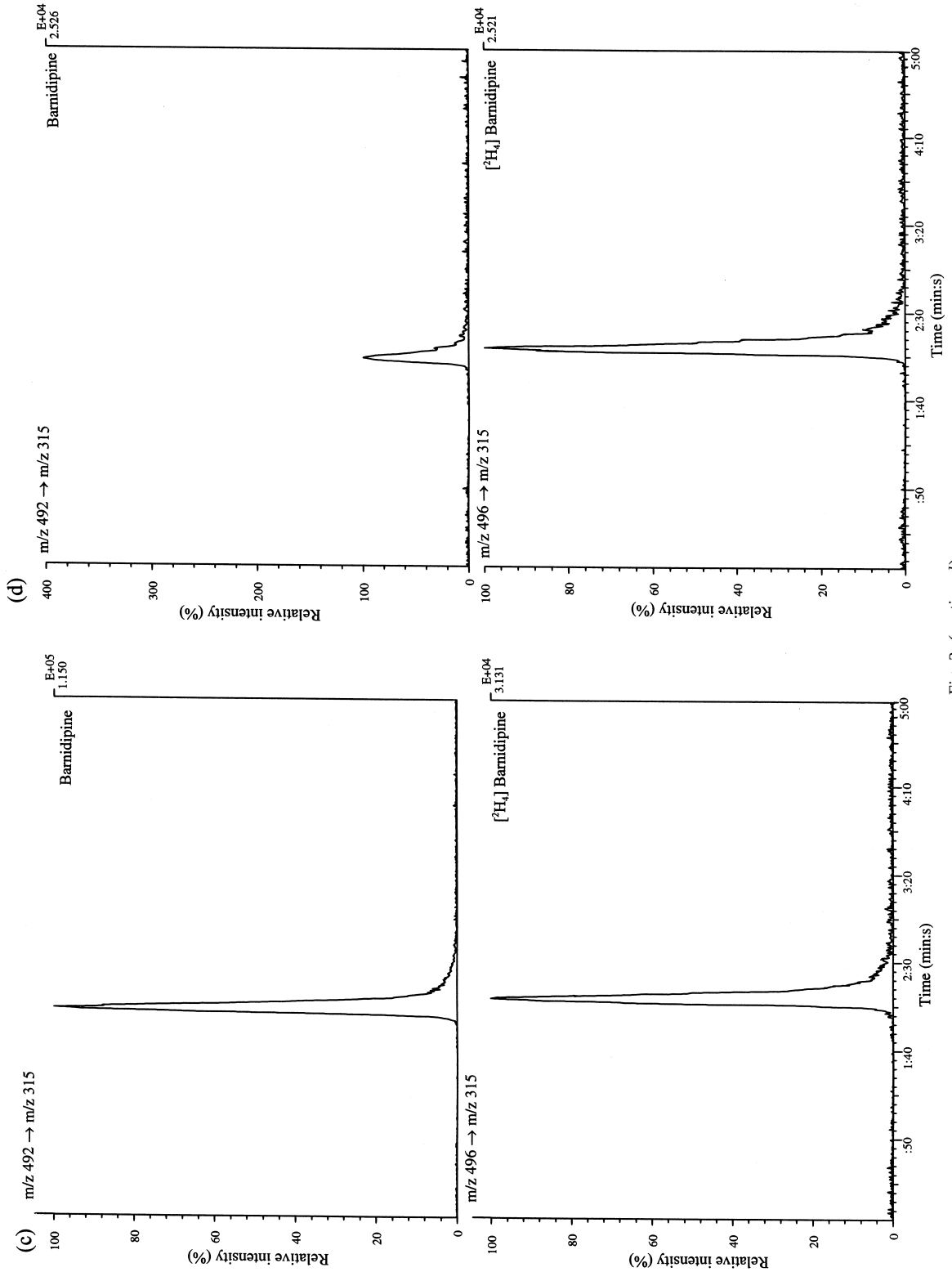

Fig. 2. (*continued*)

2.6.4. Specificity

Specificity was assessed by extracting samples of six different batches of blank control plasma, and comparing these with control plasma spiked with barnidipine (0.25 ng/ml), [$^2H_4$]-barnidipine (2 ng/ml), metabolite M1 and metabolite M8 (both 100 ng/ml). The chromatograms were also visually inspected for interfering chromatographic peaks from endogenous substances.

2.6.5. Stability

The stability of barnidipine was assessed at low and high concentrations (0.03 ng/ml and 2.5 ng/ml) after storage at ambient temperature ca. 22°C, frozen at ca. −20°C for 2 years, and after three freeze/thaw cycles.

2.6.6. Dilution

The effect of dilution was assessed by preparing a control plasma sample fortified with barnidipine at a concentration of 15 ng/ml. This sample was diluted 1 in 10 with blank control plasma, and the concentration of barnidipine found in replicate samples ($n=6$) was compared to the nominal concentration.

3. Results and discussion

3.1. Linearity and lower limit of quantitation

Fig. 2 shows representative chromatograms of (a) blank plasma, (b) plasma spiked with 0.03 ng/ml barnidipine, (c) plasma spiked with 3.0 ng/ml barnidipine and (d) a plasma sample from a volunteer at 12 h after barnidipine hydrochloride administration. The relationship between peak area ratio of barnidipine to internal standard and concentration of barnidipine in human plasma was linear ($r \geqslant 0.995$) over the calibration range 0.03 to 2.5 ng/ml. A plot (Fig. 3) of the Studentised residuals for the least squares linear regression analysis, indicated that the variance associated with the response was homogenous when a $1/c^2$ weighting function was used.

The lower limit of quantitation (LLOQ) of the assay was 0.03 ng/ml (the lowest calibration point)

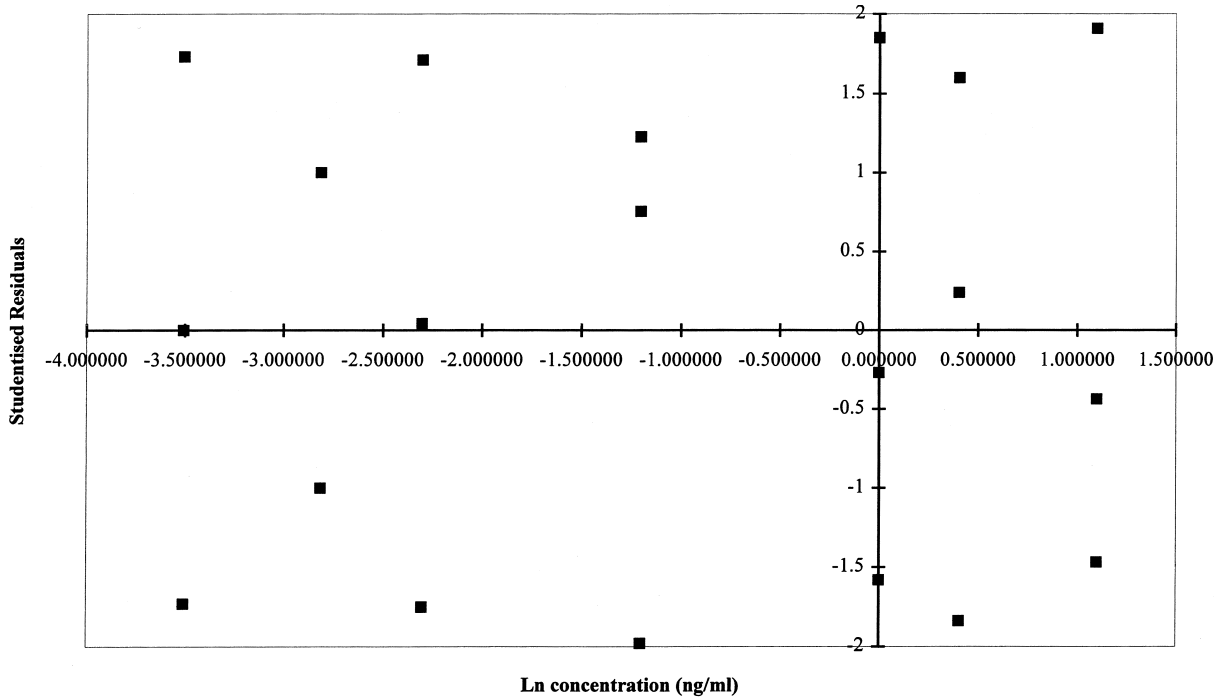

Fig. 3. Studentised residuals plot for the $1/c^2$ weighted calibration curve regression during validation ($n=3$, at seven concentrations).

using 1 ml of plasma. At this level, the inter-day precision on three occasions was 11.8% and the accuracy (expressed as % bias) was −2.2% (Table 2). Fig. 4 gives an indication of the signal to noise ratio at the LLOQ concentration (same chromatogram as Fig. 2b for barnidipine only, with an expanded scale).

3.2. Precision and accuracy

The intra-day (repeatability) and inter-day precision and accuracy (expressed as the coefficient of variation and % bias, respectively) measurements of the assay for barnidipine in human plasma using quality control samples are presented in Table 1. The values for inter-day precision were less than or equal to 11.8%, and the inter-day accuracy was ±2.2% or less. Inter-day precision and accuracy measurements were also determined for the calibration standards, as shown in Table 2. The values for inter-day precision were 13.5% or less, and the inter-day accuracy ±10.0% or less.

Table 1
Intra- and inter-day precision and accuracy of quality control samples

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	C.V. (%)	Bias (%)	*n*
Intra-day				
0.03	0.027	5.7	−11.7	6
0.03	0.032	8.8	6.7	6
0.03	0.030	6.6	−1.3	5
0.25	0.272	5.1	8.6	6
0.25	0.265	3.2	5.9	5
0.25	0.231	4.3	−7.6	6
2.50	2.511	1.6	0.4	6
2.50	2.546	2.7	1.9	6
2.50	2.312	3.3	−7.5	6
Inter-day[a]				
0.03	0.029	11.8	−2.2	17
0.25	0.255	9.6	2.1	17
2.50	2.456	5.7	−1.7	18

[a] Inter-day precision was estimated using a one-way analysis of variance (ANOVA), using SAS 6.11. (SAS®).

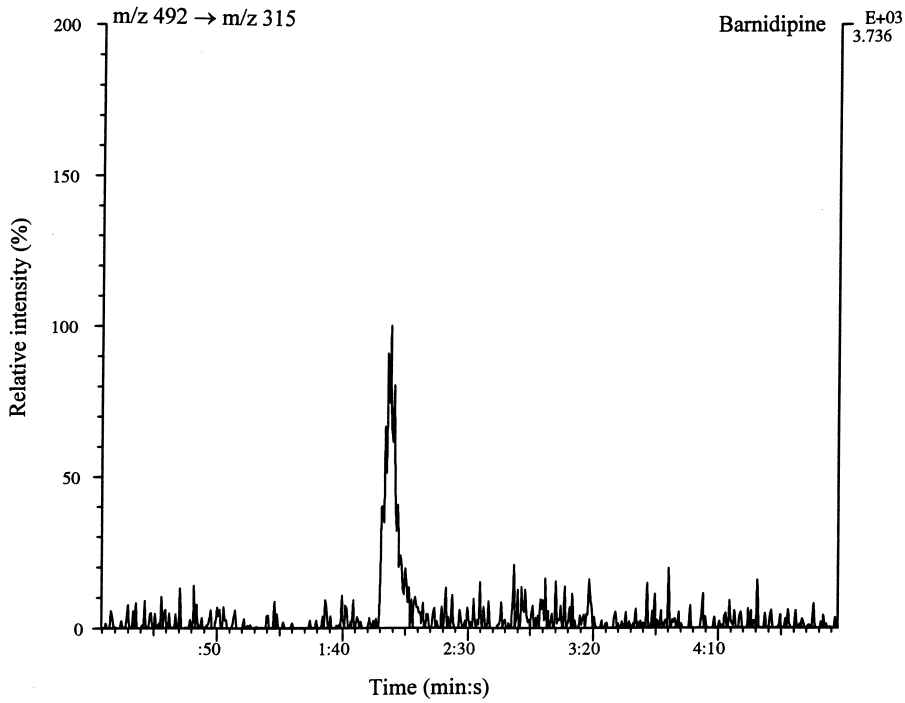

Fig. 4. SRM chromatogram to show signal-to-noise ratio at 0.03 ng/ml barnidipine (extracted spiked plasma sample).

Table 2
Inter-day precision and accuracy of calibration standards

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	C.V. (%)	Bias (%)	*n*
0.030	0.029	3.4	−3.3	3
0.060	0.066	4.3	10.0	2
0.100	0.100	13.5	−0.3	3
0.300	0.296	11.1	−1.4	3
1.000	1.018	1.7	1.8	3
1.500	1.493	2.9	−0.5	3
3.000	2.903	8.4	−3.3	3

3.3. Extraction recovery

The mean recovery (extraction efficiency) of barnidipine from human plasma was 99% (±6%, $n=5$) at 0.03 ng/ml and 87% (±2%, $n=5$) at 2.5 ng/ml. The recovery of the internal standard, [$^2H_4$]-barnidipine, was also assessed and found to be 93% (±7%, $n=5$) at 2.0 ng/ml.

3.4. Specificity

Despite the use of a simple extraction procedure in the assay, high specificity was achieved by mass spectrometry when monitoring the protonated molecules corresponding to the analyte (barnidipine) and its internal standard. Specificity of the assay was further enhanced in the selective reaction monitoring (SRM) mode where only the unique dissociations of the precursor ions to their respective product ions were monitored. No constituents of human plasma from six different individuals were found to interfere with the assay. A large excess of metabolite M8 (100 ng/ml) produced a chromatographically separated response in the barnidipine chromatogram (Fig. 5). No response was observed for metabolite M1.

3.5. Stability

Barnidipine was shown to be stable in human plasma at ambient temperature (ca. 22°C) for up to 2 h, when frozen at ca. −20°C for up to 2 years and following 3 freeze/thaw cycles (Table 3).

3.6. Effect of dilution

The concentration of barnidipine found in control samples following a ten-fold dilution with blank control plasma was in good agreement (C.V. 3.8%, Bias 11.0%, $n=5$) with the theoretical spiked and diluted concentration. This effectively extended the concentration range of the samples which could be analysed by this method ten-fold (i.e. up to 30 ng/ml).

4. Clinical application

This assay has been successfully applied to the quantitation of barnidipine in four separate pharmacokinetic studies (2161 samples). Volunteers were orally administered varying doses of barnidipine hydrochloride in a capsule formulation with sustained release granules, and samples were taken predose, and 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 24

Table 3
Stability of quality control samples

Time of storage (temperature)	Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	C.V. (%)	Recovery of mean concentration found (%)
0 h	0.030	0.031	19.7	103
	2.500	2.567	1.9	103
2 h	0.030	0.030	17.0	99
(ca. 22°C)	2.500	2.610	7.9	104
Freeze/thaw (3×)	0.030	0.027	15.4	91
(ca. −20°C)	2.500	2.462	7.3	98
2 years	0.030	0.028	8.8	94
(ca. −20°C)	2.500	2.308	9.1	92

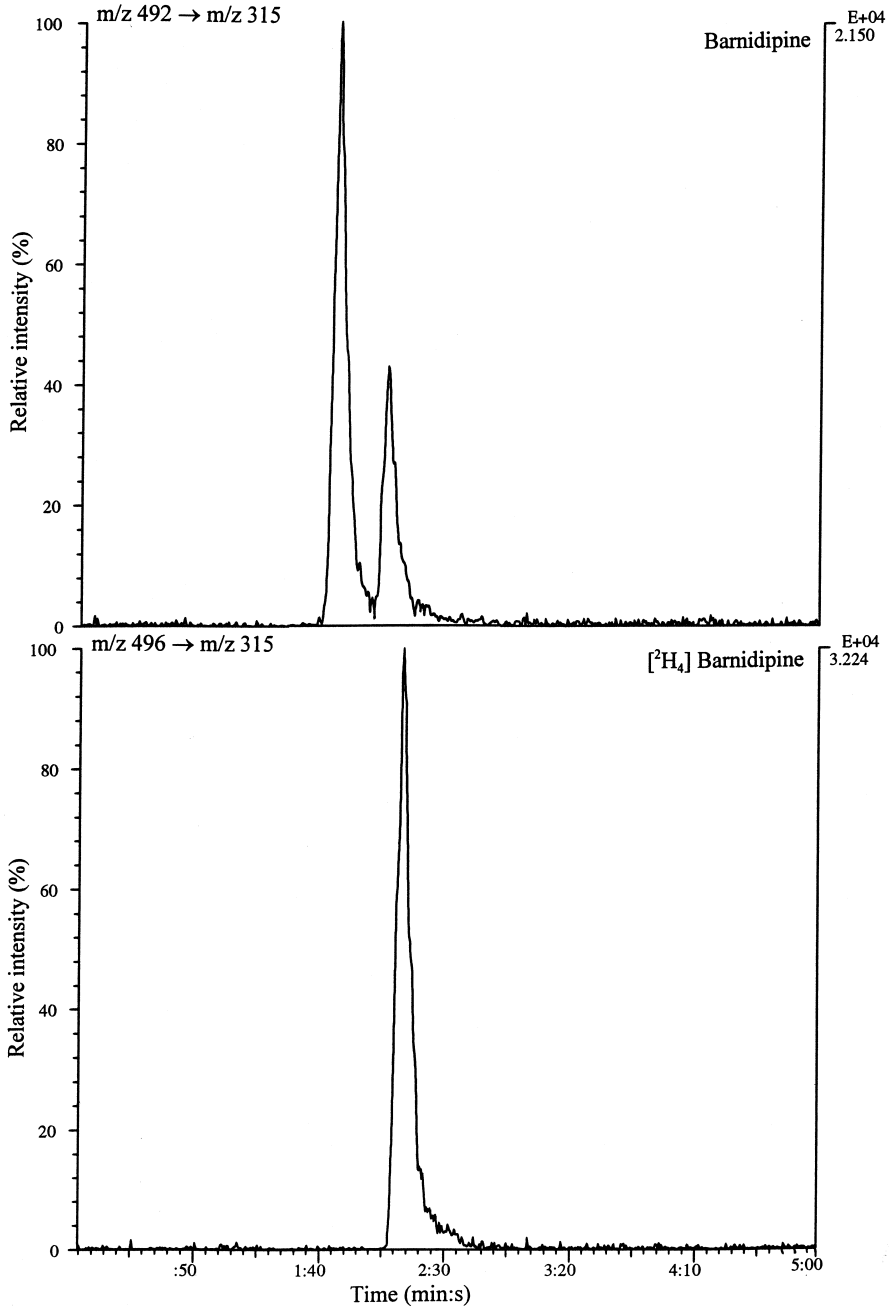

Fig. 5. Specificity check SRM chromatogram showing separation of metabolite M8 from barnidipine in extracted human plasma (0.25 ng/ml barnidipine, 100 ng/ml metabolite M8).

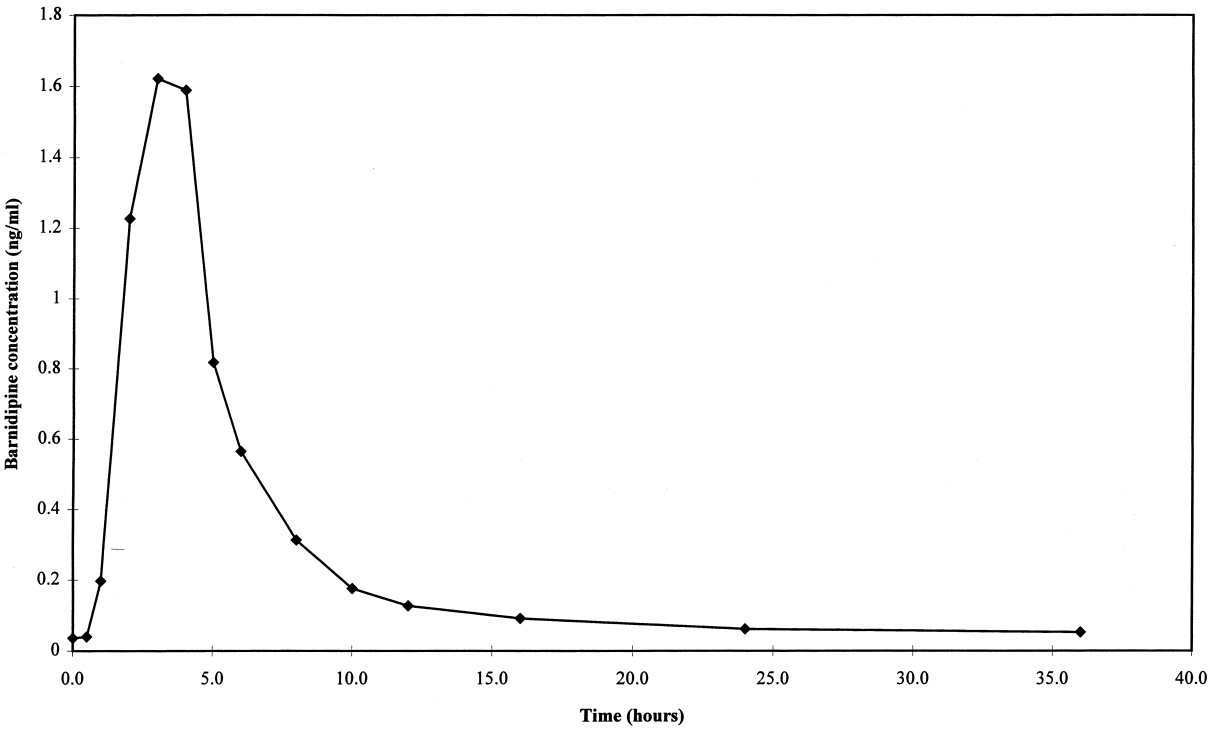

Fig. 6. Pharmacokinetic profile of a volunteer after oral dosing with barnidipine hydrochloride.

and 36 h post-dose. The combined quality control results from the four studies are presented in Table 4, and show that the precision and accuracy obtained during the day to day running of the method (4 months) was very similar to that of the validation. Fig. 6 shows a typical concentration–time curve of barnidipine hydrochloride in a volunteer after an oral dose of the drug.

Table 4
Inter-day precision and accuracy of quality control samples

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	C.V. (%)	Bias (%)	*n*
0.03	0.029	11.1	−2.2	69
0.25	0.249	8.1	−0.3	69
2.50	2.403	7.1	−3.9	71

5. Conclusions

A highly sensitive and specific LC–MS–MS method for the direct determination of barnidipine in human plasma has been developed and validated, down to a lower quantification limit of 0.03 ng/ml, which is the same sensitivity as the previously established GC–NICI-MS method. Validation experiments have shown that the assay has very good precision and accuracy over a wide concentration range (0.03–3.0 ng/ml), and no interferences caused by endogenous compounds were observed.

This simple, rapid and robust assay will enable the complete processing of large sample batches on a daily basis, and so give the high throughput required to quantify barnidipine in over two thousand plasma samples from clinical trials.

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