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High-performance liquid chromatography–electrospray ionization mass spectrometry method for the measurement of moclobemide and two metabolites in plasma

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Abstract

A rapid and sensitive liquid chromatography–electrospray ionisation mass spectrometry (HPLC–ESI-MS) assay has been developed for the measurement of moclobemide and metabolites, Ro12-5637 and Ro12-8095, in human plasma. Sample preparation (0.5 ml plasma) involves solid-phase extraction using C_{18} cartridges. A Nova-Pak phenyl column (Waters, 4 μm, 150×2 mm I.D.) was employed for analyte separation with a mixture of 0.2 *M* ammonium formate buffer, pH 3.57 and acetonitrile as the mobile phase. The within- and between-day precisions of the assay were <18% and the limit of quantification for all analytes was 0.01 μg/ml. The total run-time was 6 min. The method described was used to measure moclobemide, Ro12-5637 and Ro12-8095 in human plasma following an oral 300 mg dose.

Keywords: Moclobemide

1. Introduction

The reversible and selective inhibitor of monoamine oxidase A (MAO-A), moclobemide, is widely prescribed for the treatment of depression [1]. It is rapidly absorbed from the gastrointestinal tract [2] and undergoes extensive metabolism with less than 1% of an oral dose being recovered in the urine as the parent compound [3]. To date, 19 metabolites of moclobemide have been identified in urine, which together account for approximately 65% of an oral dose [3]. The principal pathways of moclobemide metabolism involve C- and N-oxidation of its morpholine ring to yield its two major metabolites in plasma, Ro12-8095 and Ro12-5637, respectively (Fig. 1). Moclobemide C-oxidation is partially mediated by the human liver cytochrome P450 2C19 (CYP2C19) [4]. This enzyme is subject to a polymorphism in drug metabolism [5]. Ro12-8095 has no MAO-A or -B inhibitory activity whilst Ro12-5637 is a weak inhibitor of MAO-A [6].

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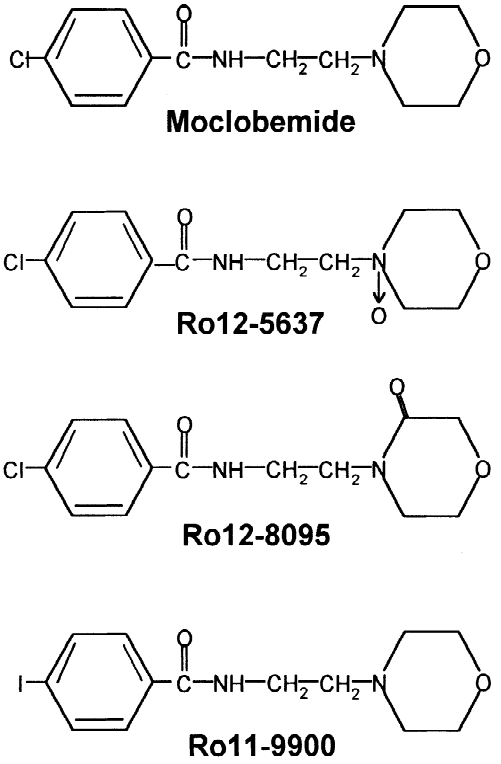

Fig. 1. The structures of moclobemide, its two major metabolites in plasma, Ro12-5637 and Ro12-8095, and the internal standard (I.S.), Ro11-9900.

Few analytical techniques have been published for measuring moclobemide and metabolites in biological fluids. Currently, three gas chromatographic methods have been described for monitoring plasma concentrations of moclobemide alone [7–9]. These methods have employed nitrogen-selective detection [7,8] and mass spectrometry (MS) [9]. To our knowledge only one analytical technique to measure moclobemide and its two major metabolites in human plasma has been published. This high-performance liquid chromatographic (HPLC) technique, which employs UV detection (240 nm), has been used in most moclobemide pharmacokinetic studies [10,2]. The method uses solid-phase Extrelut 1 glass columns packed with keiselguhr (natural diatomaceous earth) to extract moclobemide and metabolites from plasma [10]. The analyte recovery is high (83%), however, these columns are expensive. This prompted our group to develop a more cost-efficient extraction technique, utilising Bond Elut C_{18} cartridges.

The method of Geschke et al. [10] separates analytes using a Spherisorb C_6 column with a mobile phase containing a mixture of acetonitrile–phosphate buffer adjusted to pH 3.9. These chromatographic conditions were not cost-effective for the measurement of moclobemide, Ro12-5637 and Ro12-8095 in a large number of plasma samples extracted using the technique described in this paper due to inadequate robustness of the HPLC column. Other HPLC conditions were tested but none provided sufficient selectivity for moclobemide and metabolites. Selectivity was achieved by using MS rather than UV absorption as the detection system.

This paper describes a HPLC technique in combination with electrospray ionisation (ESI) MS for the measurement of moclobemide, Ro12-5637 and Ro12-8095 in human plasma. This assay utilises inexpensive solid-phase extraction (SPE) cartridges, has a high analyte recovery, a low limit of quantification and detection and a short final run time (6 min). This new technique provides for faster analytical times than previously reported methods.

2. Experimental

2.1. Chemicals and reagents

Moclobemide (M_r 268.75), Ro12-5637 (M_r 284.7), Ro12-8095 (M_r 282.7) and Ro11-9900 (I.S., internal standard, M_r 360.19) were supplied by Roche Products (Sydney, Australia). Acetonitrile and methanol were HPLC grade and purchased from Mallinckrodt Baker (Selby-Biolab, Sydney, Australia). Ammonium formate, formic acid and phosphate salts were all analytical grade and purchased from Ajax (Hazelbrook, Australia). All solutions and buffers were prepared in deionised water (Milli-Q; Millipore, Bedford, MA, USA).

2.2. Liquid chromatographic conditions

The mobile phase, which consisted of 0.2 *M* ammonium formate buffer (pH 3.57)–acetonitrile

(55:45, v/v), was pumped using an LC-10AT pump [Shimadzu Scientific Instruments (Oceania), Rydalmere, Australia], at a flow-rate of 0.2 ml/min, through an Opti-guard C_8 column (1.5 cm×1 mm I.D.) (Optimize Technologies, Alpha Resources Australia, Thornleigh, Australia) and a Nova-Pak phenyl column (60 Å, 4 μm, 150×2 mm I.D.) (Waters, Milford, MA, USA). The back pressure was 78 kg/cm^2 when the mass spectrometer was attached. An SIL-10AD VP Autoinjector (Shimadzu Scientific Instruments) was used to inject the samples. The system was controlled using an SCL-10A VP System Controller (Shimadzu Scientific Instruments).

2.3. Mass spectrometric conditions

Mass spectrometric detection was performed using a liquid chromatograph mass spectrometer LCMS-QP8000 (Shimadzu Scientific Instruments) employing an ESI detector in selected ion monitoring (SIM) mode. The optimal conditions for analyte detection were achieved in positive-ion mode, with a CDL temperature of 230°C, a CDL voltage of −5 V, deflector voltages of 45 V, a probe voltage of 3 keV, nebuliser gas flow of 4.5 l/min and a detector voltage of 1.7 keV. Moclobemide, Ro12-5637, Ro12-8095 and I.S. were monitored at the following mass-to-charge ratios (m/z) of $[M+H]^+$; 269.05, 285.00, 283.05 and 361.05, respectively. The MS data were analysed using LCMS Workstation CLASS-8000 software (Shimadzu Scientific Instruments). During assay development the atmospheric pressure chemical ionisation (APCI) interface was also tested for its ability to detect the analytes. The APCI detector was used in SIM mode and the optimal conditions were obtained at an APCI probe temperature of 330°C, a CDL temperature of 230°C, a probe voltage of 4 keV, CDL voltage of −5 V and deflector voltages of 50 V.

2.4. Preparation of stock solutions, working solutions and plasma calibration standards

Stock solutions for moclobemide, Ro12-5637, Ro12-8095 and I.S. of 1 mg/ml were all prepared in 100% methanol and stored at −20°C in 10 ml polypropylene tubes (Sarstedt Australia, Technology Park, Australia). A 10 μg/ml working solution of I.S. was prepared in water and stored at 4°C. For calibration standards, six working solutions containing a mix of moclobemide and metabolites, were prepared in acetonitrile–water (1:1, v/v) and stored at 4°C. Calibration curves were prepared in duplicate on the day of an analytical run by adding 10 μl of the working solutions to 0.5 ml of human drug-free plasma. The final plasma standard concentrations ranged from 0.01 to 5.20 μg/ml for moclobemide ($n=6$), 0.01 to 0.60 μg/ml for Ro12-5637 ($n=5$) and 0.01 to 2.12 μg/ml for Ro12-8095 ($n=5$).

Quality control (QC) samples were prepared in the same way as standards by adding 10 μl of working solutions to 0.5 ml of drug-free plasma. The working solutions used were prepared from different analyte stock solutions to those used to prepare the calibration standards. QC samples were extracted in duplicate with each analytical run and the analyte concentrations are listed in Table 2.

2.5. Sample preparation

In 10 ml polypropylene tubes, 0.5 ml plasma (unknowns and standards), 20 μl of I.S. (10 μg/ml) and 2 ml of 0.05 *M* phosphate buffer (pH 8.30) were vortex-mixed and then centrifuged for 7 min at 1750 *g* to remove any precipitated solids. Supernatants were loaded onto Bond Elut C_{18} SPE cartridges (Varian Australia, Sydney, Australia) that were preconditioned with 2 ml 100% methanol followed by 2 ml of 0.05 *M* phosphate buffer. A vacuum (<40 kPa) was applied to the Vac Elut system (Varian Australia). Cartridges were washed with 2 ml of water followed by 2 ml of methanol–water (20:80, v/v) and allowed to dry under vacuum for 5 min (>40 kPa). The analytes were eluted from the column in two washes of 0.5 ml of 100% methanol and collected into 1.7 ml polypropylene centrifuge tubes (Sarstedt Australia). The samples were dried using a Speedivac Rotary Evaporator (Savant Instruments, Farmingdale, NY, USA). For reconstitution of dried extracts, 50 μl of mobile phase was added, vortex-mixed and centrifuged at 6800 *g* for 10 min. Supernatants (45 μl) were transferred to polypropylene insert vials (Alltech, Deerfield, IL, USA) and 0.2–10 μl was injected onto the LC column.

2.6. Assay validation: linearity, precision, accuracy, recovery and specificity

Human drug-free plasma and calibration samples were extracted in duplicate with each analytical run. Standard curves were estimated by least-squares linear regression of analyte concentration versus the ratio of the peak areas of analyte to internal standard without weighting for Ro12-5637 and using a weighting of $1/y^2$ for both moclobemide and Ro12-8095. Concentrations of the analytes in the unknown samples and QCs were calculated from the regression lines. Unknown samples, with analyte concentrations higher than the standard curve range, were diluted 1:4 with drug-free plasma and reassayed.

For assay validation calibration standards were extracted in quadruplicate on 3 separate days. The recovery of analytes was assessed for the calibration standards on a single occasion. The peak areas of analytes added to extracts of drug-free plasma were measured and compared with the peak area means of calibration standards and expressed as a percentage. One-way analysis of variance (ANOVA) was used to estimate the between-day precision for each calibration concentration using the run day as the classification variable [11] (SYSTAT, Version 7.0). The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentrations across analytical run days were obtained. The between-day precision was calculated for each calibration standard as follows where n is the number of replicates within each analytical run:

$$\text{Between-day precision} = \frac{[(\text{DayMS} - \text{ErrMS})/n]^{0.5}}{\text{GM}} \times 100\% \quad (1)$$

The within-day precision was calculated for each calibration standard as follows:

$$\text{Within-day precision} = \frac{(\text{ErrMS})^{0.5}}{\text{GM}} \times 100\% \quad (2)$$

The accuracy was assessed for each calibration standard as the ratio of the grand mean of the observed concentrations to the actual concentrations expressed as a percentage.

The between-day precision was also assessed by determining the coefficients of variation for the QC samples that were analysed in duplicate on 11 different occasions. The signal-to-noise ratio was determined using the LCMS Workstation CLASS-8000 software (Shimadzu Scientific Instruments).

Drug-free plasma samples from 31 healthy individuals were tested for potential interference from endogenous plasma peaks at the retention times (t_R) of interest.

2.7. Pharmacokinetic study

This assay was developed for a pharmacokinetic study to assess the relationship between *CYP2C19* genotype and moclobemide disposition. A single 300 mg dose of moclobemide was given to healthy volunteers and blood samples taken for 34 h.

3. Results and discussion

3.1. LC separation and solid-phase extraction

Using this SPE technique the average recoveries achieved over the concentration ranges measured were greater than 87% for all analytes, as given in Table 1. The recoveries of moclobemide, Ro12-5637 and Ro12-8095 ranged from 92 to 120%, 87 to 106% and 92 to 102%, respectively. The mean and standard deviation of I.S. recovery were 93.4±2.6%. These analyte recoveries were higher than reported for keiselguhr columns [10] (moclobemide; 83%, Ro12-5637; 73% and Ro12-8095; 86%).

Representative chromatograms of drug-free plasma, drug-free plasma spiked with moclobemide, metabolites and internal standard and a plasma sample collected 1.27 h after a 300 mg oral dose of moclobemide are shown in Fig. 2A–C, respectively. The retention times for Ro12-8095, Ro12-5637, moclobemide and I.S. were 2.65, 3.29, 4.06 and 4.75 min, respectively, with a final run time of 6 min. The short run time allowed for more rapid sample throughput than the published liquid chromatography technique, which requires a total run time of 17 min [10]. No interfering endogenous plasma peaks were observed at retention times of interest in the 31 drug-free plasma samples tested.

Using the current extraction technique Spherisorb

Table 1
The accuracy, within- and between-day precision and recovery data for the measurement of moclobemide, Ro12-5637 and Ro12-8095 in human plasma

Nominal concentration (μg/ml)	Measured concentration (μg/ml)	Recovery (%)	Within-day precision (%)	Between-day precision (%)	Accuracy (%)
Moclobemide					
0.010	0.011	120	15.8	3.3	106
0.10	0.11	97.3	3.2	3.5	104
0.52	0.57	93.8	3.5	4.5	110
1.04	1.04	91.6	3.1	2.2	99.5
2.00	2.00	99.3	4.5	0.8	96.0
5.10	4.88	95.2	2.2	3.8	96.0
Ro12-5637					
0.010	0.010	106	16.7	5.3	100
0.050	0.050	94.0	5.5	4.1	100
0.10	0.11	86.7	5.2	0.4	105
0.25	0.25	88.8	2.8	0.7	99.2
0.60	0.59	98.2	5.4	4.6	98.5
Ro12-8095					
0.011	0.011	97.4	11.7	3.7	104
0.053	0.048	96.9	6.6	10.4	90.6
0.21	0.22	93.1	5.8	6.5	105
0.53	0.60	91.5	3.1	4.3	113
1.06	1.11	102	6.4	12.8	105
2.12	2.20	98.0	6.3	6.3	104

C_6 columns, used by Geschke et al. [10], could only be used for as few as three analytical runs (approximately 50 plasma extracts injected onto the column each occasion) as a result of greatly increased back-pressure and compromised column selectivity. In contrast, the assay development, validation and measurement of 500 plasma samples, obtained from 24 volunteers who participated in the moclobemide pharmacokinetic study, was performed using a single Nova-Pak phenyl column. This indicates that this column is considerably more robust than the C_6 Spherisorb column previously used [10].

3.2. Mass spectrometry

ESI-MS conditions were initially optimised for the quantification of moclobemide. Background subtracted scan spectrums of moclobemide, Ro12-8095, Ro12-5637 and I.S. are presented in Fig. 3. Under these conditions a predominant peak for moclobemide corresponded with the m/z of 269.05 $[M+H]^+$. The I.S., Ro12-8095 and Ro12-5637 also provided abundant $[M+H]^+$ signals at m/z 361.05, 283.05 and 285.00, respectively. Other ions, $[M+Na]^+$, $[M+K]^+$ and $[M+Na+CH_3CN]^+$, appeared in the mass spectrum of Ro12-8095 with m/z values of 305, 321 and 346, respectively. Because of the isotope distribution of elemental chlorine, Ro12-8095 yielded a peak with the same m/z as Ro12-5637 (285.00). However, under the chromatographic conditions used this peak did not interfere with the quantification of either analyte.

The analytes could also be monitored using the APCI interface, however, the peak intensity and the signal-to-noise ratios were less than those achieved using ESI-MS. For the lowest calibration plasma standard (0.01 μg/ml; moclobemide, Ro12-5637 and Ro12-8095) the signal-to-noise ratios using ESI detection were 87:1 for moclobemide, 32:1 for Ro12-5637 and 7:1 for Ro12-8095. For APCI the

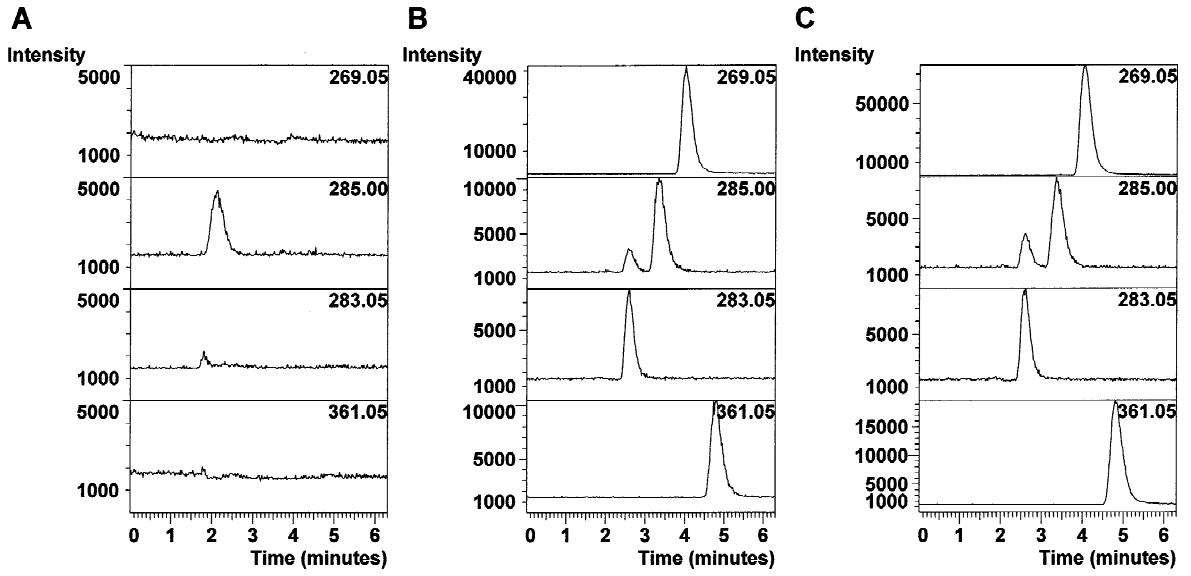

Fig. 2. Single ion monitoring (SIM) chromatograms obtained by LC–ESI-MS analysis of (A) drug-free human plasma, (B) a calibration standard consisting of 1.93 μg/ml moclobemide (m/z=269.05, t_R=4.06 min), 0.64 μg/ml Ro12-5637 (m/z=285.00, t_R=3.29 min), 1.03 μg/ml of Ro12-8095 (m/z=283.05, t_R=2.65 min) and 0.40 μg/ml internal standard (Ro11-9900 m/z=361.05, t_R=4.75 min) and (C) a plasma sample from a healthy Caucasian male (CA76) taken 1.27 h after ingesting 300 mg of moclobemide. The analyte concentrations were 2.19, 0.28, 0.69 and 0.40 μg/ml for moclobemide, Ro12-5637, Ro12-8095 and Ro11-9900, respectively.

signal-to-noise ratios for the same sample were 57:1, 4:1 and 3:1 for moclobemide, Ro12-5637 and Ro12-8095, respectively.

3.3. Validation

The technique was linear for all analytes over the concentration ranges studied: 0.01 to 5.2 μg/ml for moclobemide, 0.01 to 0.60 μg/ml for Ro12-5637 and 0.01 to 2.12 μg/ml for Ro12-8095. The means and standard deviations for the slope and *y*-intercept determined from 18 moclobemide calibration curves were 2.22±0.12 and 0.008±0.005, respectively. The slopes and *y*-intercepts for Ro12-5637 were 1.27±0.23 and −0.011±0.021, and for Ro12-8095 were 0.58±0.10 and −0.002±0.005, respectively.

The analytical techniques for moclobemide and metabolites are characterised in Tables 1 and 2. The within-day precision of all analytes was less than 20% at 0.01 μg/ml, the lowest limit of quantification, and less than 10% at the highest calibration standard. The between-day precision for moclobemide was less than 5%, for Ro12-5637 less than 5% and for Ro12-8095 less than 13%. The between-day precision was also assessed using QC samples and was less than 10% for moclobemide and less than 20% for the metabolites (Table 2). Across the concentration ranges studied the accuracies ranged from 92 to 120% for moclobemide, from 87 to 106% for Ro12-5637 and from 92 to 102% for Ro12-8095 (Table 1). The limit of quantification for all analytes was 0.01 μg/ml, which is lower than that previously reported [10]. The limits of detection, at a signal-to-noise ratio of 4:1, were 0.001, 0.002 and 0.005 μg/ml for moclobemide, Ro12-5637 and Ro12-8095, respectively.

3.4. Assay application and analyte stability

This method has been used to measure moclobemide and its metabolites in plasma samples from a single-dose pharmacokinetic study. Fig. 4 shows a representative plasma concentration–time profile for moclobemide, Ro12-5637 and Ro12-8095 in a male Caucasian volunteer (CA76) after an oral dose of 300 mg moclobemide. The maximal concentration of moclobemide (C_{max}) of CA76 was 1.86 μg/ml, which is within the range of C_{max} values

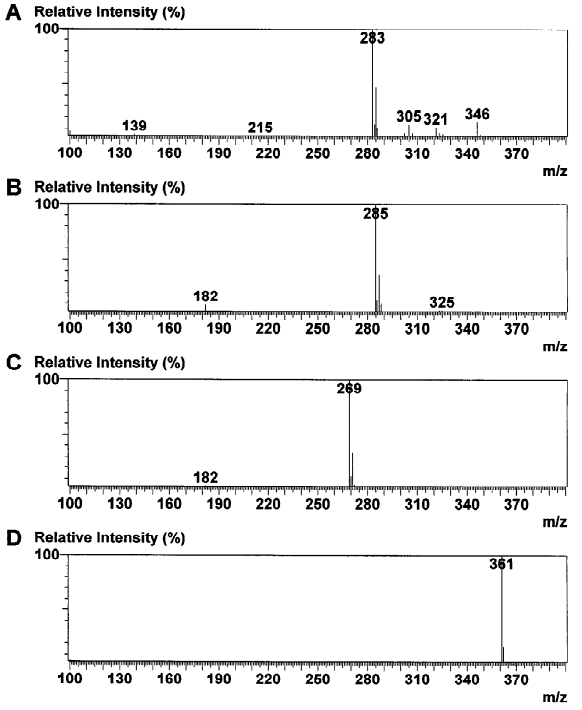

Fig. 3. Positive-ion electrospray mass spectra of (A) Ro12-8095, (B) Ro12-5637, (C) moclobemide and (D) internal standard (Ro11-9900).

(1.46–2.96 μg/ml) reported for males [2] measured by the analytical technique of Geschke et al. [10]. Moclobemide could be measured in the plasma of CA76 for 12 h post-dose, which is six-times longer than the half-life of moclobemide. The sensitivity of the assay is therefore more than adequate for accurately characterising the pharmacokinetics of moclobemide at conventional doses. Ro12-5637 and Ro12-8095 could be measured in the plasma of CA76 for 12 and 14 h post-dose, respectively.

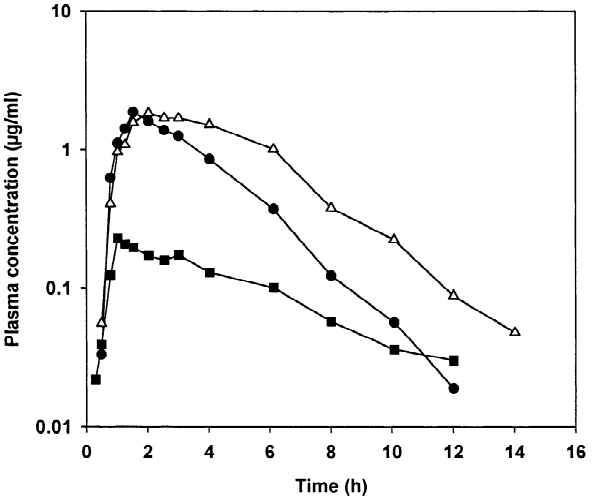

Fig. 4. Plasma concentration–time profile of moclobemide (filled circle), Ro12-5637 (filled square) and Ro12-8095 (unfilled triangle) in a healthy Caucasian male volunteer (CA76) administered 300 mg moclobemide orally.

The stability of moclobemide and metabolites in fresh and frozen plasma was not investigated in this study. It has previously been reported that moclobemide is stable in plasma stored at −20°C for up to 9 months [10].

Table 2
The between-day precision of quality control (QC) samples measured in duplicate on 11 separate days

QC sample	No. of samples	Nominal concentration (μg/ml)	Mean measured concentration (μg/ml)	Coefficient of variation (%)
Moclobemide				
QC1	22	0.040	0.041	10.1
QC2	22	0.75	0.75	5.5
QC3	22	3.00	2.74	7.7
Ro12-5637				
QC1	22	0.040	0.051	18.0
QC2	22	0.20	0.19	19.7
QC3	22	0.40	0.41	8.9
Ro12-8095				
QC1	22	0.040	0.35	19.2
QC2	22	0.30	0.29	9.0
QC3	22	0.70	0.81	9.9

4. Conclusions

A sensitive, accurate and reproducible LC–ESI-MS method has been developed for the measurement of moclobemide and its two major metabolites in human plasma, Ro12-5637 and Ro12-8095. The method utilises standard C_{18} SPE cartridges and has a higher analyte recovery ($>$87%) as well as a lower limit of quantification (0.01 μg/ml) than previously reported techniques. This method also has a shorter total run time (6 min), which allows for more rapid sample analysis. This method is robust and has been used for the quantification of moclobemide, Ro12-5637 and Ro12-8095 in plasma samples from 24 healthy volunteers of known *CYP2C19* genotype after a single 300 mg moclobemide dose.

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