Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1416–1422
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

High-performance liquid chromatographic method for the determination of moclobemide and its two major metabolites in human plasma

Anita Rakic [a,*], Branislava Miljkovic [b], Milena Pokrajac [b], Katarina Vucicevic [b]

[a] *Medicines and Medical Devices Agency of Serbia, Vojvode Stepe No. 458, Belgrade 11152, Serbia and Montenegro*
[b] *Department of Pharmacokinetics, Faculty of Pharmacy, Vojvode Stepe No. 450, Belgrade 11152, Serbia and Montenegro*

Received 17 June 2006; received in revised form 8 October 2006; accepted 10 October 2006
Available online 1 December 2006

Abstract

A selective, sensitive, and simple high-performance liquid chromatographic (HPLC) method was developed for the determination of moclobemide and its two major metabolites, Ro 12-5637 and Ro 12-8095, in human plasma. Sample preparation (0.5 ml of plasma) involved solid-phase extraction (SPE) using *Speedisk*® H_2O-Philic DVB columns. Separations were performed on a Waters XTerra™ RP18 column (5 μm, 150 mm × 4.6 mm). The mobile phase consisted of 10 mM KH_2PO_4 with 1% triethylamine (pH 3.9) and acetonitrile (83:17, v/v), and a flow-rate was 1.2 ml/min. The total run time was 13 min. UV detection was performed at 240 nm. Mean absolute recoveries were ≥90% and the limit of quantification (LOQ) for all analytes was 0.02 mg/l. Calibration curves were linear ($r > 0.995$) over a wide range of the analyte concentrations in plasma; thus, the method is suitable for different clinical studies when large variations in the drug/metabolites concentrations are observed. During a 5-day assay validation procedure the accuracy and precision were tested and proven (relative errors (RE) ≤ 13%; intra-day coefficient of variation (CV) ≤ 7%; inter-day CV ≤ 13%). Many drugs frequently used in the target patient population were evaluated for potential interference in order method selectivity to be ensured. The assay has been used in a clinical pharmacokinetic study to assess steady-state pharmacokinetics of moclobemide and two metabolites in depressive patients on mono- and combined therapy.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Moclobemide; Metabolites; Plasma; HPLC; Solid-phase extraction

1. Introduction

Moclobemide is a selective and reversible inhibitor of monoamine oxidase A (MAO-A), which is used in the treatment of depression. The drug undergoes extensive hepatic metabolism; a total of 19 metabolites has been identified in urine. However, only the unchanged drug and two products of the morpholine ring oxidation, Ro 12-8095 and Ro 12-5637, can easily be detected in plasma (Fig. 1). The N-oxide of moclobemide (Ro 12-5637) retains certain MAO-A inhibitory activity, while the other metabolite is inactive [1–3].

Several bioanalytical techniques have been published for the determination of moclobemide in human plasma [4–11], however, only three of them [8–10] provide conditions for the simultaneous measurement of the drug and its two major metabolites. All three methods involved high-performance liquid chromatography (HPLC), with either ultraviolet (UV) detection [8,9] or electrospray ionisation-mass spectrometry (ESI-MS) [10]. Although a ring-opened metabolite Ro 16-3177 was also reported to be measured with two of these techniques [8,9], this very compound is not of interest for pharmacokinetic studies, since its concentrations in human plasma are too low to be monitored for a required period of time [8,12], and the metabolite is therapeutically inactive (it was found to inhibit MAO-B in rat liver, but has no MAO-A inhibitory activity) [2,12]. Therefore, in all published pharmacokinetic studies that have been found in literature [3,13–16], only plasma concentrations of moclobemide and two metabolites, Ro 12-8095 and Ro 12-5637, have been monitored.

The first reported method for the determination of moclobemide and its metabolites in biological fluids involved liquid–liquid extraction in the sample preparation procedure. The compounds were extracted from alkalized plasma (0.5 ml) on a pre-packed glass column (Extrelut 1) with dichloromethane (two

* Corresponding author. Tel.: +381 11 395 11 45; fax: +381 11 395 11 30.
E-mail address: anita.rakic@alims.sr.gov.yu (A. Rakic).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.10.032

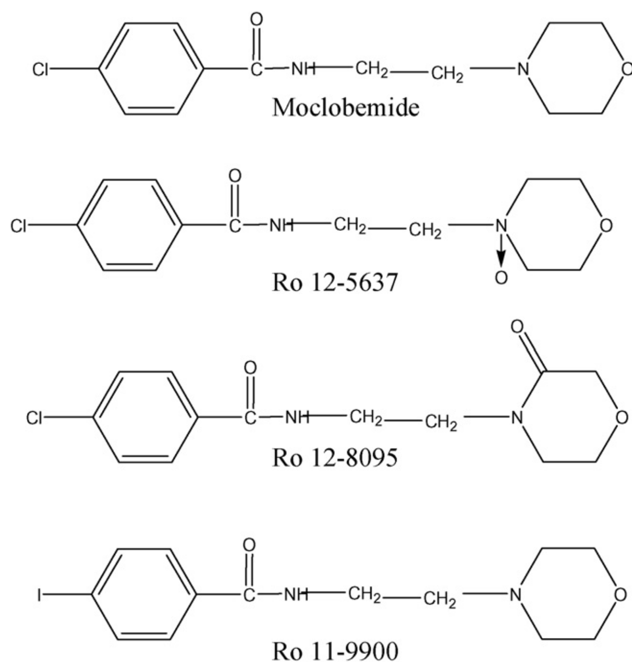

Fig. 1. The structures of moclobemide, its two major metabolites in plasma (Ro 12-5637 and Ro 12-8095), and the internal standard (Ro 11-9900).

6 ml portions). The analytes were separated using a Spherisorb C_6 column and a mobile phase that consisted of a mixture of acetonitrile–phosphate buffer (30:320, v/v), with a pH of 3.9 [8]. However, Extrelut 1 columns are expensive, and the applied extraction procedure is time-consuming.

In the other two methods [9,10], solid-phase extraction (SPE) was employed, which was based on the use of bonded-phase silica sorbents: Bakerbond CN [9] and Bond Elut C_{18} [10]. The extraction was performed from 1 ml [9] and 0.5 ml [10] of plasma and compounds were eluted from the SPE column with methanol. The recovery values were higher than those in the previously published methods [8], however, both extraction procedures required sample pre-treatment (i.e. protein precipitation and centrifugation).

This paper describes a HPLC technique with UV detection for the quantification of moclobemide and its two major metabolites in human plasma. The sample preparation involved SPE, utilising new *Speedisk*® polymer columns. Due to their specific design, columns resist clogging and ensure high throughput rates, even when samples contain solids. For that reason, it was possible to eliminate other time consuming preparation steps required with conventional SPE columns. The applied extraction procedure is simple and rapid, and the total time required for the analysis is shorter than that of the previously published methods.

2. Materials and methods

2.1. Chemicals

Moclobemide, Ro 12-5637, Ro 12-8095, and Ro 11-9900 (internal standard, IS) were kindly donated by Hoffmann-La Roche Ltd. (Basel, Switzerland). Methanol and acetonitrile (Mallinckrodt Baker B.V., Deventer, Holland) were HPLC grade. All other chemicals, including 99.5% triethylamine (Aldrich-Chemie GmbH, Steinheim, Germany), potassium dihydrogen phosphate (Renal, Budapest, Hungary), di-potassium hydrogen phosphate anhydrous (Merck, Darmstadt, Germany), and 85% orthophosphoric acid (Zorka A.D., Sabac, Serbia and Montenegro), were analytical grade. Water deionized by an "EasyPure RF" D-7033-3 (Barnstead, Iova, USA) was used for the preparation of the mobile phase and all solutions. Auromid® tablets, containing 150 mg of moclobemide were manufactured by Galenika (Belgrade, Serbia and Montenegro).

2.2. Chromatographic conditions

An Agilent 1100 Series (Agilent Technologies Deutschland Gmbh, Waldbronn, Germany) chromatographic system was used. The system was equipped with a binary pump, degasser, autosampler with thermostat, Agilent ChemStation, variable wavelength detector, and thermostatted column compartment. Separations were performed on a Waters XTerra™ RP18 column (5 μm, 150 mm × 4.6 mm). The mobile phase consisted of 10 mM KH_2PO_4 with 1% triethylamine (pH 3.9) and acetonitrile (83:17, v/v). The mobile phase flow-rate was 1.2 ml/min and the column temperature was 25 °C. The detection was performed at 240 nm.

2.3. Preparation of stock solutions, working solutions, and calibration standards

Stock solutions of moclobemide, Ro 12-5637, Ro 12-8095, and IS were all prepared in methanol (in the concentration of 1 mg/ml) and stored at 4 °C. Plasma samples were stored at −20 °C until required. Working solutions were prepared on the day of an analysis by further dilution of the stock solutions with purified water. Calibration standards were prepared by spiking drug-free plasma (0.5 ml) with 25 μl of the IS working solution (10 mg/l) and 20–50 μl of the working solutions containing a mixture of moclobemide and the metabolites in appropriate concentrations. Thus, the following concentration ranges were covered: 0.02–5.00 mg/l for moclobemide ($n = 8$), 0.02–1.00 mg/l for Ro 12-5637 ($n = 6$), and 0.02–3.00 mg/l for Ro 12-8095 ($n = 7$). Quality control (QC) samples were prepared in the same way, but using working standards made from different stock solutions.

2.4. Sample preparation

In 10 ml test tubes, 0.5 ml of plasma (standards and patients' samples), 25 μl of IS, and 2 ml of 0.05 M K_2HPO_4 (pH 8.4) were vortex-mixed and then loaded onto *Speedisk*® H_2O-Philic DVB columns (1 ml, 35 mg) (Mallinckrodt Baker, Inc., Phillipsburg, NJ, SAD), that were pre-conditioned with 1 ml of methanol, followed by 2 ml of 0.05 M K_2HPO_4. Vacuum Manifold Processing Station (P/N 5185-5754) (Agilent Technologies, Wilmington, DE, SAD) was used to draw samples through SPE columns. Cartridges were washed with 2 ml of water, followed by 2 ml of

methanol–water (30:70, v/v). Columns were let dry for 1 min, and the analytes were eluted thereafter with two portions of 0.5 ml methanol. Eluates were evaporated to dryness in a thermostatted water-bath at 40 °C under nitrogen, and reconstituted (by vortexing for 30 s) with 250 μl of the mobile phase. A volume of 100 μl was injected for the analysis.

2.5. Method optimisation

The following HPLC columns were evaluated: Waters XTerra™ RP18 (5 μm, 150 mm × 4.6 mm) ZORBAX SB-C_{18} (5 μm, 250 mm × 4.6 mm) and ZORBAX Extend-C_{18} (5 μm, 250 mm × 4.6 mm). XTerra™ RP18 was found to be the most appropriate due to the optimum separation of all the analytes and the shortest run time.

The optimisation of the sample preparation procedure included the selection of the SPE column, as well as the content of methanol in a wash solution thereafter. Two types of *Speedisk®* polymer columns were evaluated: H_2O-Philic DVB and H_2O-Philic SC-DVB. A slightly modified method proposed in the literature [17] was applied for H_2O-Philic SC-DVB columns. Plasma diluted with water was loaded onto a preconditioned column. A cartridge was washed with 1 ml 0.1N HCl, followed by 1 ml methanol. The analytes were eluted with two portions of 0.5 ml methanol–ammonium hydroxide (95:5, v/v). However, with this type of column it was not possible to isolate all analytes from plasma.

2.6. Assay validation

For assay validation, calibration standards and QC samples were prepared on 5 separate days (according to the above described procedure). The calibration curves were obtained by plotting peak area ratios (analyte/IS) versus analyte concentrations and analyzed using weighted least-squares linear regression, since heteroscedascity of the data was observed. Different weighting factors (w_i) were evaluated ($1/x^{1/2}$, $1/x$, $1/x^2$, $1/y^{1/2}$, $1/y$, and $1/y^2$) and the selection of the best one was based on the calculation of the sum of the percentage relative errors (%RE) over the whole concentration range for each weighting factor [18]. To establish the calibration models, six to eight concentration levels were studied (n) and five replicates were analysed at each level. Calibration curves were prepared each day when the patients' plasma samples were analysed as well (with no replicates). Concentrations of the analytes in QC and unknown samples were calculated from the regression lines. The recoveries, accuracy and precision of the method (intra- and inter-day) were assessed by analysis of the replicate sets of QC samples covering the entire concentration range of the calibration curves. The accuracy (expressed as %RE) and the intra-day precision (expressed as a coefficient of variation, CV) were estimated by analyzing five QC samples at each concentration on the same day. The inter-day precision (CV) was determined in the same manner, but on 5 different days. The limit of quantification (LOQ) was determined by establishing the minimum concentration of an analyte in plasma that still can be quantified with acceptable accuracy and precision under the stated experimental conditions [19]. The absolute recovery was calculated for each concentration as the mean (±S.D.) of five samples by comparing peak areas of directly injected analytes solutions with peak areas of extracted plasma samples.

Drug-free plasma from 20 healthy individuals was tested for potential endogenous interference. Furthermore, the expected concomitant drugs to be taken by the studied patient population were evaluated in order the method selectivity to be ensured. This was accomplished first by a direct injection of a drug aqueous solution in the maximum therapeutic concentration [20,21]. The drugs showing interference were further evaluated after being extracted from plasma.

Since plasma samples were stored for no longer than 1 month, the stability of the analytes in frozen plasma was not investigated. It has been reported previously that these samples are stable at −20 °C for up to 9 months [8].

2.7. Method application

The assay was developed for a pharmacokinetic study to compare steady-state pharmacokinetics of moclobemide and its metabolites in depressive patients on mono- and combined therapy with valproic acid, a well-known inhibitor of various drug metabolic enzymes [22]. The aim of this ongoing study is the evaluation of a potential pharmacokinetic interaction between the two drugs.

The analytical method has been used for the quantification of moclobemide and two metabolites in plasma during multiple oral dose administration of 150 mg moclobemide three times daily. Each patient participating in the study is monitored for 28 days, starting from the 8th day of therapy (steady-state plasma levels of moclobemide are reached approximately 1 week following dose adjustment [1]). Blood samples are collected once weekly: just before the first daily dose and 1–6 h after it.

The study was approved by the Ethics Committee of the Institute for Mental Health, and the written, informed consent was obtained from all the subjects.

3. Results and discussion

Considering the relatively hydrophilic nature of the analytes, in particular moclobemide and Ro 12-5637, the use of a C_{18} column was believed to be the best solution to prevent too early elution of the substances from the column. In addition to XTerra™ RP18, two further C_{18} columns were evaluated: ZORBAX SB-C_{18} and ZORBAX Extend-C_{18}. The optimum retention times were achieved through the mobile phase pH adjustment, the content of acetonitrile present in it and the flowrate. Triethylamine was added to the mobile phase to prevent peak tailing. The results showed that using ZORBAX SB-C_{18} it was not possible to achieve, at the same time, the separation of all analytes and an acceptable run time (the separation of Ro 12-5637 and IS was problematic and incomplete when the run time was ≤20 min). On the other hand, the separation of the investigated compounds was successfully achieved using ZORBAX Extend-C_{18}, with the optimum mobile phase consisting of

10 mM KH_2PO_4 with 1% triethylamine (pH 3.1) and acetonitrile (80:20, v/v). However, the retention time of the metabolite Ro 12-8095 was longer than the one on XTerra (13.7 min versus 11.4 min, respectively). Consequently, the total run time when using XTerra^{TM} RP18 was 2.5 min shorter and that was the reason for choosing this column for further analysis (the retention times of the other analytes on Zorbax Extend-C_{18} were comparable to those on XTerra: 4.0, 4.9, and 6.2 min for moclobemide, Ro 12-5637, and IS, respectively).

The following representative chromatograms are shown: drug-free plasma, plasma spiked with standard mixture of moclobemide and its two metabolites, and a plasma sample obtained from a patient, administered 150 mg moclobemide orally three times daily (blood was withdrawn under the steady-state conditions, 4 h following the first daily dose) (Fig. 2). The retention times of moclobemide, Ro 12-5637, Ro 12-8095, and Ro 11-9900 (IS) were: 3.9, 4.9, 11.4, and 6.5 min, respectively. The total run time was 13 min, which is approximately 4 min shorter than that previously reported for HPLC methods with UV detection [8,9]. Using this time-saving procedure, it would be possible to analyse up to 100 samples/day.

The selectivity of the method was investigated thoroughly. No interfering endogenous plasma peaks were observed at the retention times of interest in the 20 drug-free plasma samples tested. In addition, many drugs were evaluated for potential interference including several drugs frequently used in the general population (analgesics, vitamins, antibiotics, caffeine), as well as drugs frequently or occasionally used in the target patient population (valproic acid, benzodiazepines, carbamazepine, chlorpromazine, diuretics, beta-blockers and other antihypertensives) (see Table 1). Only few drugs interfered with the compounds of interest detection after direct injection into the chromatographic system (ciprofloxacin, sulfamethoxazole, hydrochlorothiazide, acetylsalicylic- and salicylic acid). They were re-evaluated after being extracted from plasma by using the described SPE procedure. The interference was proven in the case of ciprofloxacin, sulfamethoxazole and hydrochlorothiazide (the patients have not been administered these drugs during the study), while acetylsalicylic- and salicylic acid were undetectable in the final extracts. Thus, only three drugs have been found to interfere with the determination of moclobemide and its metabolites in human plasma, but none of the observed

Table 1
Retention times of the drugs analysed for interferences

Drug	Analysis from an aqueous solution (direct HPLC)		Analysis from plasma (SPE prior to HPLC)	
	Concentration (mg/l)	Retention time (min)	Concentration (mg/l)	Retention time (min)
Acetylsalicylic acid	300	3.3[a]	300	n.d.[b]
Amoxicillin	8	1.8		
Ampicillin	8	1.9		
Ascorbic acid	20	1.5		
Atenolol	1.3	1.8		
Caffeine	1.8	2.5		
Captopril	0.5	n.d.		
Carbamazepine	12	n.d.[b]		
Cefaclor	10	1.9		
Cephalexin	50	1.8		
Chlorpromazine	300	n.d.[b]		
Ciprofloxacin	8	3.8	8	3.8
Clonazepam	0.08	n.d.[b]		
Diazepam	1	n.d.[b]		
Diclofenac	3	n.d.[b]		
Enalapril	0.125	n.d.[b]		
Erythromycin	2.5	n.d.[b]		
Hydrochlorothiazide	0.4	4.3	0.4	4.3
Ibuprofen	50	n.d.[b]		
Indapamide	0.26	n.d.*		
Metamizole	20	2.9		
Metoprolol	0.1 (>1)[c]	n.d.[b] (4.1)[c]		
Naproxen	50	n.d.[b]		
Nifedipine	0.015	n.d.[b]		
Paracetamol	20	2.4		
Propranolol	0.1	n.d.[b]		
Salicylic acid	300	3.3	300	n.d.[b]
Sotalol	4	1.9		
Sulfamethoxazole	200	10.9	200	10.9
Trimethoprim	12	2.8		
Valproic acid	100	n.d.[b]		

The drugs in bold denote the ones that interfered from an aqueous solution.
[a] Underwent hydrolysis to salicylic acid, which is also its main metabolite in plasma.
[b] Not detectable under the applied conditions (during the run time of 13 min).
[c] Detectable in concentrations higher than therapheutic (>1 mg/l), with retention time of 4.1 min (it woud interfere with moclobemide determination).

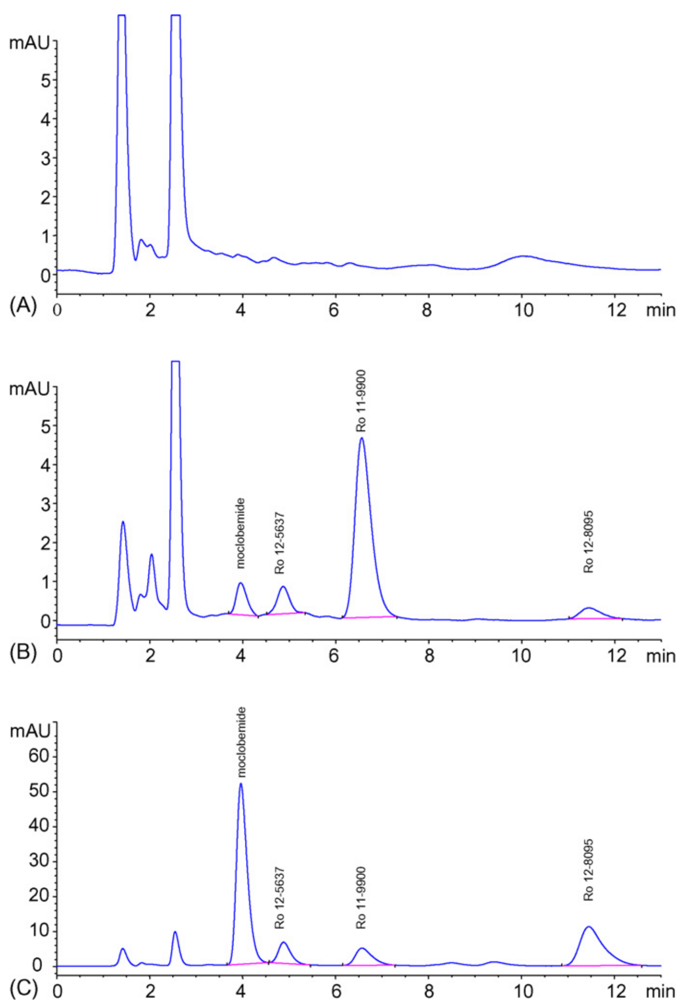

Fig. 2. Representative chromatograms of: (A) blank (drug-free) plasma, (B) plasma spiked with 0.02 mg/l of moclobemide, Ro 12-5637, and Ro 12-8095, and (C) a patient plasma sample (the analyte concentrations are 1.50, 0.19, and 0.81 mg/l for moclobemide, Ro 12-5637, and Ro 12-8095, respectively). Ro 11-9900 (IS) was added to all samples (the concentration is 0.50 mg/l).

interferences are of great importance, since these drugs are not essential in the treatment of the target patient population. In general, the interference should not be expected from lipophilic drugs (e.g. benzodiazepines, carbamazepine, lipid-soluble beta-blockers, phenothiazines) since they cannot be detected under the stated chromatographic conditions during the run time of 13 min (for example, the retention time of propranolol was 17.5 min, but the drug was detectable only in the concentrations higher than therapeutic). As can be seen from the Table 1, only relatively hydrophilic drugs, like water-soluble beta-blockers, some analgesics and antibiotics (penicillins and cephalosporins) can be detected under the described chromatographic conditions. Since the vast majority of drugs have more pronounced lipophilic properties, it was very problematic to find the one that could be used as IS. Many drugs were evaluated, but most of them had unacceptably long retention times. Only Ro 11-9900, a compound with very similar chemical structure to moclobemide, was found to be an adequate. On the other hand, the applied extraction procedure may not be appropriate for acid drugs (e.g. salicylic acid). The proven selectivity of the method is very important for its clinical application, as many depressive patients are on combined therapy.

Since concentrations of moclobemide and the metabolites can vary considerably under different conditions that are met in clinical practice (e.g. concomitant therapy with the drug metabolism inductors/inhibitors, patients with liver impairment or genetically deficient drug metabolism) [3], it was important to obtain calibration curves that would cover a wide range of the expected analytes concentrations. Therefore, the following concentration ranges were studied: 0.02–5.00 mg/l for moclobemide, 0.02–1.00 mg/l for Ro 12-5637, and 0.02–3.00 mg/l for Ro 12-8095. The suitability of linear regression as the calibration technique was checked and proved by performing the ANOVA lack-of-fit test for each analyte. The concentrations were then estimated using the lines calculated by the least-squares method at each y-value. However, using the unweighted linear regression, large relative errors occurred at the lowest part of the concentration range for all compounds (for example, the %RE on small moclobemide x-values reached 80% although r exceeded 0.999). Therefore, F-test was performed in order to check the homo-/heteroscedasticity of the data sets, and in addition, residuals ($y_{observed} - y_{predicted}$) versus concentrations were plotted. The residual plots clearly showed that the error was not randomly distributed around the concentration axis. Instead, an increase in variance as a function of concentration was observed as can be seen from Fig. 3, which presents the residual plot for moclobemide. The F-test also revealed a significant difference between the variances, when the experimental F-value for each analyte was compared to the tabled one. Thus, heteroscedasticity of the data was evident and the weighted least-square linear regression was used to fit the data. The smallest %RE sum was produced by using $1/x^2$ for moclobemide and Ro 12-5637, and $1/x$ for Ro 12-8095. The use of the most appropriate weighting factor significantly improved the accuracy of the analytical method. The following regression equations were obtained: $y = 4.35283(\pm 0.07785)x + 0.01122(\pm 0.00400)$, $r = 0.99904$ for moclobemide, $y = 4.54969(\pm 0.09227)x + 0.00233(\pm 0.00411)$, $r = 0.99918$ for Ro 12-5637, and $y = 3.89974(\pm 0.02398)x - 0.00555(\pm 0.00563)$, $r = 0.99991$ for Ro 12-8095. The

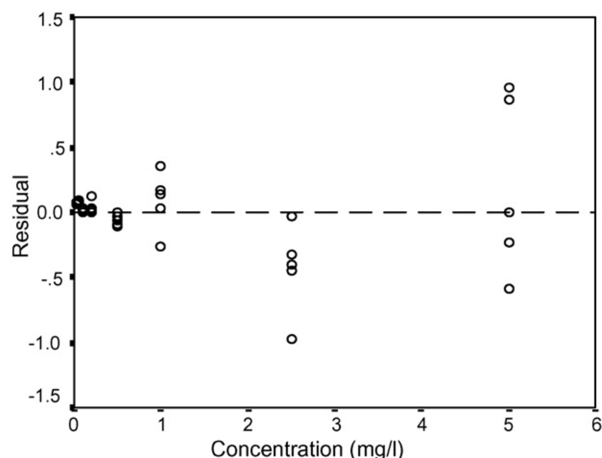

Fig. 3. Residuals plotted against moclobemide concentrations for the validation intra-day assay.

Table 2
The recovery, accuracy, intra-, and inter-day precision data for the measurement of moclobemide, Ro 12-5637, and Ro 12-8095 in human plasma ($n = 5$)

Nominal concentration (mg/l)	Recovery (%) (mean ± S.D.)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
Moclobemide				
0.02	92.10 ± 6.81	3.04	5.31	7.32
0.05	95.41 ± 5.93	−9.97	5.04	13.09
0.20	91.29 ± 1.40	5.32	5.63	7.25
0.50	93.74 ± 3.45	6.52	1.88	4.18
1.00	96.28 ± 2.12	0.16	4.56	5.06
2.50	98.23 ± 1.74	−3.19	3.15	3.33
5.00	98.93 ± 1.69	−8.68	2.96	3.84
Ro 12-5637				
0.02	85.56 ± 3.61	2.52	6.15	8.07
0.05	92.28 ± 7.53	−13.44	5.25	7.31
0.20	94.52 ± 2.49	−8.42	5.22	6.62
0.50	89.47 ± 4.86	1.70	3.90	4.62
1.00	88.07 ± 2.65	4.50	1.50	3.92
Ro 12-8095				
0.02	103.57 ± 5.60	3.45	7.08	6.79
0.05	103.75 ± 6.37	−10.66	4.00	10.05
0.20	98.68 ± 3.53	2.74	6.23	4.94
0.50	92.69 ± 2.47	4.15	2.73	5.60
1.00	99.78 ± 2.79	−2.52	2.84	4.81
3.00	100.09 ± 1.58	−0.10	3.09	4.05

recoveries, accuracy and precision of the method were assessed during a 5-day validation procedure. Assay validation parameters are shown in Table 2.

The limit of detection (LOD) for moclobemide and Ro 12-5637 was 0.005 mg/l, and 0.008 mg/l for Ro 12-8095, at a signal-to-noise ratio of 3:1. The LOQ (the lowest concentration on a calibration curve) for all the compounds was 0.02 mg/l (see Table 2). This is in the range of the LOQ values in the previously published methods (0.01–0.03 mg/l) [8–10]. However, in two HPLC methods with UV detection [8,9], the lowest concentration on calibration curves was higher than LOQ. In the method of Misztal et al. [9], where LOQ values for moclobemide and the metabolites were in the range 0.010–0.015 mg/l, the lowest concentration on the calibration curve for each analyte was 0.05 mg/l. This prevents the method from being used for the determination of the analytes concentrations in plasma lower than 0.05 mg/l that are often found in single-dose moclobemide studies, such as bioequivalence ones [11]. Thus, the HPLC–ESI-MS method [10] only can be regarded to be more sensitive (with LOQ of 0.01 mg/l). However, it has to be pointed out that although HPLC–MS technique has recently become a more commonly applied, it still cannot be considered standard equipment, especially in clinical laboratories, which is a very important practical issue.

H_2O-Philic SC-DVB columns were inappropriate for the extraction of moclobemide and its two metabolites from plasma, since the metabolite Ro 12-8095 was completely eluted from the column during the wash step with 100% methanol. Therefore, H_2O-Philic DVB columns were used. With this type of columns, the extraction of Ro 12-5637 was found to be the most critical. It was important to balance adequately the content of methanol in a wash solution, in order to get clean extracts and, at the same time, retain Ro 12-5637 on the column. The wash solvent strength was increased gradually in the range 20–40% methanol in water. A content of 30% methanol was found to be optimal. Absolute recoveries were high and reproducible for all analytes, with means and standard deviations of 95.1 ± 2.9, 90.0 ± 3.5, and 99.8 ± 4.0% found for moclobemide, Ro 12-5637, and Ro 12-8095, respectively. The mean parameter value (±S.D.) for IS was 97.6 ± 2.7%. These recovery values were higher than reported for the liquid–liquid extraction procedure (83, 73, and 86% for moclobemide, Ro 12-5637, and Ro 12-8095, respectively) [8] and comparable to the values achieved with two published SPE techniques [9,10]. However, in contrast to these SPE procedures, the extraction procedure applied in this method did not require any other sample pre-treatment, except for dilution with buffer, in order to improve flow during loading. A run time of 13 min, together with a simple and rapid sample preparation procedure, allows a more rapid sample throughput than all the methods published previously (including the HPLC–ESI-MS method [10], in which 17 min centrifugation during the sample preparation procedure significantly increases the total analysis time). In addition, SPE sorbents based on new hydrophilic polymer resins have several advantages over bonded-phase silicas, such as broad pH stability (operating pH range is 1–14) and reduced dependence on wetting prior to extraction. Namely, the effectiveness of silica sorbents is highly dependent on the presence of the activating solvent. However, during the course of running many samples, the cartridge can become dry. If the sorbent is then used, the recovery of the analyte will be low. On the contrary, the modified resins have the ability to wet with water even after drying and will still absorb effectively [13,23].

So far, a total of 125 plasma samples obtained from seven patients were analysed by using the described method. Fig. 4 shows a representative steady-state plasma concentration–time profile of moclobemide, Ro 12-5637 and Ro 12-8095 in a depres-

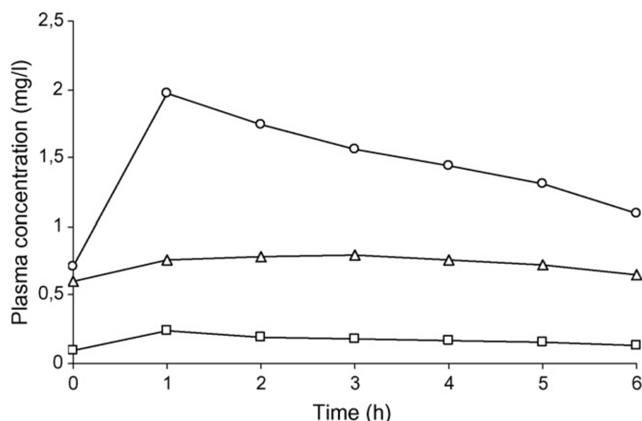

Fig. 4. A steady-state plasma concentration–time profile of moclobemide (circle), Ro 12-5637 (square), and Ro 12-8095 (triangle) during multiple oral dose administration of 150 mg moclobemide three times daily.

sive patient taking 150 mg of moclobemide orally three times daily at dosing intervals of 6, 6, and 12 h. The blood sample was taken after the first daily dose, on the 36th day of the therapy. The maximum moclobemide plasma concentration (C_{max}^{SS}) was 1.97 mg/l, while the minimum moclobemide concentration obtained just prior to the second daily dose (C_{min}^{SS}) was 1.09 mg/l. They are within the range of C_{max}^{SS} values (2.24 ± 0.72 mg/l) and C_{min}^{SS} values (0.74 ± 0.40 mg/l) reported for the same dosing regimen [15] and measured by the HPLC technique of Geschke et al. [8]. Although this method has been used for studying moclobemide pharmacokinetics under the steady-state conditions, its sensitivity is high enough to enable the application to a single-dose study (the method of Geschke et al. [8] with comparable sensitivity has been used in the most clinical pharmacokinetic studies, including single dose ones [3]).

4. Conclusion

This paper describes a rapid, selective and sensitive HPLC method with UV detection for the quantification of moclobemide and its two major metabolites in human plasma, and its applicability to pharmacokinetic studies. The method allows a high sample throughput due to the chromatographic run time of 13 min and a very simple and fast sample preparation procedure. Accurate and precise determination of moclobemide and its metabolites in plasma was possible over the wide concentration ranges studied; thus, this method can be applied to various clinical situations in which large variations in the analytes concentrations are observed. The lack of analytical interference was proven for many drugs used frequently in the target patient population, and that is what is essential for a valid measurement of compounds of interest when patients are on combined therapy. This method is time saving and economical and therefore suitable for different clinical studies and routine laboratory analyses, including drug interaction studies, therapeutic drug monitoring, and compliance assessment, where its applicability has already been proven. Also, the sensitivity of the method is high enough to enable its application to single-dose studies, such as bioequivalence ones.

Acknowledgements

We would like to acknowledge the cooperation of the Institute for Mental Health in Belgrade, and we are very grateful to our colleagues Prof. I. Timotijevic, D. Todorovic, and R.Velickovic, for conducting the present clinical study and for supplying patient plasma samples. In addition, we would like to thank our colleague I. Ivanovic (Mallinckrodt Baker, The Netherlands), for her time and willingness in helping us to understand the important principles of the solid-phase extraction technique.

References

[1] U. Bonnet, CNS Drug Rev. 9 (2003) 97–140.
[2] R. Jauch, E. Griesser, G. Oesterhelt, W. Arnold, W. Meister, W.H. Ziegler, T.W. Guentert, Acta Psychiatr. Scand. Suppl. 360 (1990) 87–90.
[3] M. Mayersohn, T.W. Guentert, Clin. Pharmacokinet. 29 (1995) 292–332.
[4] K.P. Maguire, T.R. Norman, B.M. Davies, G.D. Burrows, J. Chromatogr. 278 (1983) 429–433.
[5] J. Raaflaub, P. Haefelfinger, K.H. Trautmann, Arzneim.-Forsch. 34 (1984) 80–82.
[6] M. Gex-Fabry, A.E. Balant-Gorgia, L.P. Balant, Ther. Drug Monit. 17 (1995) 39–46.
[7] Y. Gaillard, G. Pepin, Forensic Sci. Int. 87 (1997) 239–248.
[8] R. Geschke, J. Korner, H. Eggers, J. Chromatogr. 420 (1987) 111–120.
[9] G. Misztal, R. Skibinski, M. Olajossy, B. Paw, J. Pharm. Biomed. Anal. 30 (2002) 1277–1285.
[10] J.M. Hoskins, A.S. Gross, G.M. Shenfield, L.P. Rivory, J. Chromatogr. B 754 (2001) 319–326.
[11] H. Amini, B. Shahmir, A. Ahmadiani, J. Chromatogr. B 807 (2004) 271–275.
[12] G.K. Ibister, L.P. Hackett, A.H. Dawson, I.M. Whyte, A.J. Smith, Br. J. Clin. Pharmacol. 56 (2003) 441–450.
[13] M.P. Shoerlin, M. Mayersohn, A. Korn, H. Eggers, Clin. Pharmacol. Ther. 42 (1987) 395–404.
[14] K.S. Yu, et al., Clin. Pharmacol. Ther. 69 (2001) 266–273.
[15] T.W. Guentert, G. Tucker, A. Korn, J.P. Pfefen, P. Haefelfinger, M.P. Schoerlin, Acta Psychiatr. Scand. Suppl. 360 (1990) 91–93.
[16] J. Dingemanse, N. Wood, T. Guentert, S. Oie, M. Ouwerkerk, R. Amrein, Psychopharmacology 140 (1998) 164–172.
[17] C.W. Huck, G.K. Bonn, J. Chromatogr. A 885 (2000) 51–72.
[18] A.M. Almeida, M.M. Castel-Branco, A.C. Falcao, J. Chromatogr. B 774 (2002) 215–222.
[19] FDA Guidance for Industry on Biomedical Method Validation, 2001. http://www.fda.gov.cder.guidance/index.htm.
[20] W.A. Ritchel, G.L. Kearns, Handbook of Basic Pharmacocinetics Including Clinical Applications, American Pharmaceutical Association, Washington, 1999.
[21] AHFS Drug Information, American Society of Health-System Pharmacist Inc., 2006.
[22] J. Fleming, M. Chetty, Clin. Neuropharmacol. 28 (2005) 96–101.
[23] E.M. Thurman, M.S. Mills, Solid-Phase Extraction: Principles and Practice, John Wiley & Sons Inc., New York, 1998.