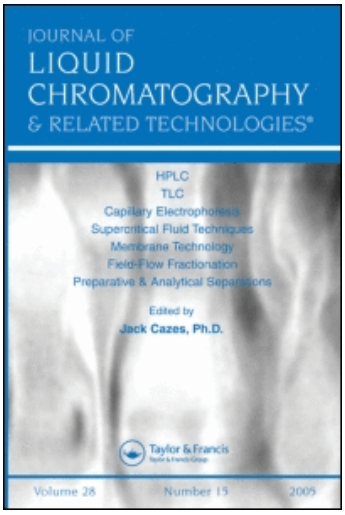


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# Determination of Moclobemide and its Metabolites in Human Plasma by SPE-HPLC-UV: Evaluation of Critical Experimental Conditions and QSRR Study

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**Abstract:** A SPE-HPLC-UV method for determination of moclobemide and its major metabolites, Ro 12-5637 and Ro 12-8095, in human plasma had been developed previously and its selectivity was evaluated against the most frequently coadministered drugs. The objective of the present work was to develop a quantitative structure retention relationship (QSRR) model capable of providing good predictions of chromatographic behaviour of other related potentially interfering drugs, based on the previously generated data, in order to further improve the clinical applicability of the existing method. Moreover, the most critical factors affecting SPE and chromatographic separations of moclobemide and its metabolites were evaluated and discussed, taking into account molecular properties of the analyzed compounds.

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**Keywords:** HPLC, Metabolites, Moclobemide, Plasma, QSRR model, SPE

## INTRODUCTION

Moclobemide [p-chloro-n-(2-morpholinoethyl)benzamide] is a selective and reversible inhibitor of monoamine oxidase type A (MAO-A). The drug is eliminated almost entirely by hepatic metabolism, with less than 1% of an oral dose being recovered in urine as the parent compound. The principal pathways of moclobemide metabolism involve C- and N-oxidation of the morpholine ring to yield its two major metabolites in plasma, Ro 12-8095 and Ro 12-5637, respectively. Ro 12-5637 retains certain MAO-A inhibitory activity, while the other metabolite is inactive.[1,2] Moclobemide has a broad spectrum of antidepressant activity. Its lack of adverse anticholinergic, cardiovascular, cognitive, and psychomotor effects makes moclobemide a particularly useful option in the elderly, or patients with cardiac disease.[3] Consequently, the target patient population frequently comprises elderly and individuals with concurrent somatic illness under concomitant treatment with other psychotropic and/or somatic medications. In a clinical study enrolling 89 depressive patients on moclobemide therapy, benzodiazepine comedication was present in a large proportion of patients (63%), while 51% of patients received at least one other drug, the most frequently encountered therapeutic classes being analgesics, drugs prescribed for cardiac illness, hypnotics other than benzodiazepines, antihypertensive agents, antibiotics, and phenothiazines.[4]



A simple, fast, and highly selective high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for the quantification of moclobemide and its two major metabolites, Ro 12-5637 and Ro 12-8095, in human plasma has been developed in our laboratory.[5] Although various analytical methods for the determination of moclobemide in biological fluids have been described in the literature, only few others provide conditions for the simultaneous measurement of the drug and its two major metabolites in human plasma.[6–9] These methods also involved HPLC with either UV detection[6,7,9] or electrospray ionisation-mass spectrometry (ESI-MS),[8] while sample preparation was performed by solid phase extraction (SPE)[6–8] or liquid liquid extraction.[9] The important advantage of the method developed by our group, however, was a very simple and rapid sample preparation (SPE using Speedisk® polymer columns) without time consuming sample pretreatment procedures required. Therefore, it allowed a more rapid sample throughput than other reported methods. In addition, these methods have been mainly used for the quantification of moclobemide and its metabolites in plasma samples from healthy volunteers, and their

application to samples from patients, who are being administered other drugs concomitantly, has not been included. Therapeutic monitoring, however, requires the availability of a single method that can be used in different clinical situations (e.g., in the presence of coadministered drugs, disease states, genetic, and other patients' characteristics as important sources of variability in plasma drug concentrations) in order to save time, cost, and effort. The most important characteristics that make an analytical method suitable for clinical application, including both routine therapeutic drug monitoring and clinical studies, are validity of the assay over a whole range of possible therapeutic concentrations, selectivity against all potential interferences (endogenous compounds from biological samples, concomitant drugs, dietary substances and supplements), simplicity, and high sample throughput. The method developed by us was designed to be suitable for a clinical pharmacokinetic study; therefore, its analytical performance was validated over a wide range of reported therapeutic concentrations,[10] and its selectivity was evaluated against 30 drugs from the most frequently coadministered therapeutic classes.[4]



Based on the obtained HPLC retention data for moclobemide, its metabolites and these 30 potentially interfering drugs,[5] quantitative structure retention relationship (QSRR) study was performed. Only 10 drugs that were detectable at the chromatograms under the applied conditions were subjected to the QSRR analysis. QSRR models have been proven to be a very helpful tool for evaluation of chromatographic behaviours of various analytes.[11] In this paper, we have correlated the obtained HPLC retention data with theoretically calculated molecular parameters of dominant structures of 10 detectable drugs, IS (Ro 11-9900), moclobemide and its metabolites (Ro 12-5637 and Ro 12-8095) at the analytical pH 3.9 (Figure 1). The objective of the study was to develop a QSRR model capable of providing good predictions of chromatographic behaviour of other related potentially interfering drugs in order to further improve the applicability of the existing method to various clinical situations. To the best of our knowledge, the presented report is the first attempt to develop a QSRR model for evaluation of interferences between moclobemide, its metabolites, and other concomitantly used drugs.

In addition, the effect of different experimental conditions on the analysis of moclobemide and its two major metabolites in human plasma were explored and the most critical factors affecting SPE and chromatographic separations were identified and discussed. Therefore, we believe that this paper could also serve as a useful guide for the development of potential new chromatographic methods for monitoring these analytes in plasma samples, reducing the time required for establishing appropriate experimental conditions.

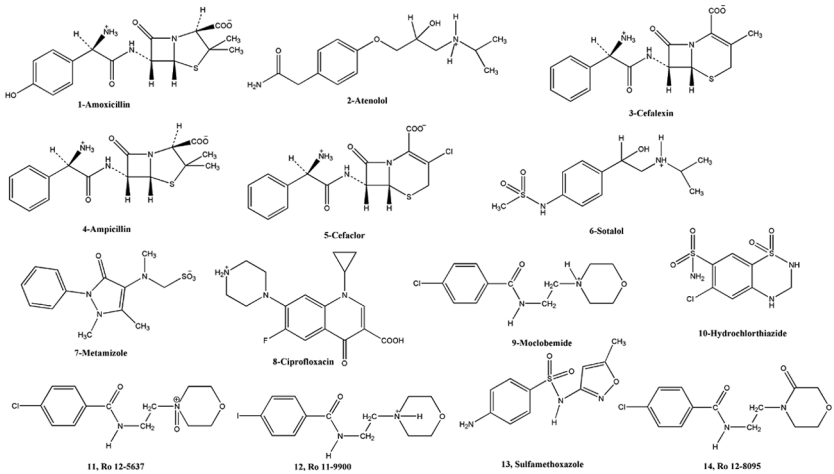


***Figure 1.*** Structural Formulas of the drugs used for the QSRR study. The depicted structures are the most dominant forms of the compounds at the analytical pH 3.9 (Marvin 4.0.5 Chemaxon).



## EXPERIMENTAL

### Chemicals and Solutions

Moclobemide, the metabolites, Ro 12-5637 and Ro 12-8095, and internal standard (IS), Ro 11-9900, 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 were of analytical grade and came from different commercial suppliers.[5] Stock solutions of moclobemide, the metabolites, and IS were all prepared in methanol (1 mg $mL^{-1}$). Working solutions were prepared by further dilution of the stock solutions with purified water.[5]

### Apparatus

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. The detection was performed at 240 nm.

## Optimisation of Chromatographic Conditions

To develop appropriate chromatographic conditions for the separation of four compounds having similar chemical structures (moclobemide, two metabolites, Ro 12-5637 and Ro 12-8095, and IS), different mobile and stationary phases were investigated.

The following HPLC columns containing octadecylsilica stationary phase were evaluated: Waters XTerra$^{TM}$ RP18 (5 μm, 150 mm × 4.6 mm), Agilent Zorbax SB-$C_{18}$ (5 μm, 250 mm × 4.6 mm), and Zorbax Extend-$C_{18}$(5 μm, 250 mm × 4.6 mm). The mobile phase consisted of a mixture of acetonitrile and phosphate buffer with 1% triethylamine. Influences of pH and percentage of acetonitrile in the mobile phase on the efficiency of chromatographic separations and retention times of the analytes were examined.

The optimum separation conditions were achieved using Waters XTerra$^{TM}$ RP18 column with the mobile phase consisted of 10 mM $KH_2PO_4$ with 1% triethylamine (ph 3.9) and acetonitrile (83:17, v/v). A flow rate was 1.2 mL min$^{-1}$ and the column temperature was 25°C.



## Optimisation of Solid Phase Extraction (SPE)

Determination of the optimum SPE conditions included selection of the appropriate extraction cartridge, sample pretreatment procedure, the volume and composition of the washing solvent, and the eluent volume. Two types of Speedisk® SPE polymer columns were evaluated: $H_2O$-Philic DVB (1 mL, 35 mg) and $H_2O$-Philic SC-DVB (1 mL, 35 mg) (Mallinckrodt Baker, Inc., Phillipsburg, NJ, SAD).

The final SPE procedure involved the use of Speedisk® $H_2O$-Philic DVB columns that were preconditioned with 1 mL methanol, followed by 2 mL 0.05 m $K_2HPO_4$ (pH 8.4). Plasma samples (0.5 mL), diluted with phosphate buffer (2 mL), were applied to the cartridges and washed with 2 mL water, followed by 2 mL methanol-water (30:70, v/v). The analytes were eluted thereafter with 1 mL methanol. Eluates were evaporated to dryness under nitrogen at 40°C, and the residue was reconstituted with 250 μL of the mobile phase. A volume of 100 μL was injected for the analysis.

## Quantitative Structure Retention Relationship (QSRR) Study

Calculation of pKa and selection of dominant forms at analytical pH 3.9 was performed for all examined compounds using the Marvin 4.0.5 Chemaxon program.[12] Geometries of the most abundant structures at

analytical pH were optimized using the MOPAC/$PM_3$ method.[13,14] The partition coefficient octanol/water (logP), pKa, distribution coefficient (logD), charge distribution, isoelectric point, polar surface area (PSA), were determined by using the Marvin 4.0.5 Chemaxon.[12] Single and multilinear regression models were developed for the data set by use of the Microsoft-Excel 2000/Regression Data Analysis and Multi-Precision Floating Point Computation for Excel (XNUMBERS.XLA-Ver. 4.7-2006).[15]

## RESULTS AND DISCUSSION

### Optimisation of the Stationary Phase

Optimisation of the stationary phase was found to be the most critical factor in the development of the appropriate chromatographic method, which would provide satisfactory separation of all the analysed compounds. Different commercial $C_{18}$ columns often show different separation selectivity, usually based on differences in silica supports or stationary phase chemistry.[16] In the present study, the use of three different $C_{18}$ columns, XTerra™ RP18, Zorbax Extend-$C_{18}$ and Zorbax SB-$C_{18}$, in combination with the same mobile phase consisting of 10 mm $KH_2PO_4$ with 1% triethylamine (pH 3.5) and acetonitrile (80:20, v/v), led to a markedly different chromatographic behaviour of the analytes (see Table 1).

Good separation of all the analysed compounds and acceptable run times were achieved by using both XTerra™ RP18 and Zorbax Extend-$C_{18}$ (Table 1). It has already been reported that most of the commercially available HPLC columns are not suitable for the chromatographic separation of moclobemide and its metabolites.[6,7,9] They gave inadequate separation and tailing peaks of the analysed compounds. These effects were probably related to the interactions of the analytes with residual silanol groups,[6,9] which have also been reported for other basic drugs. Basic analytes interact strongly with residual silanols of the silica based reversed phase columns and cause tailing peaks that are detrimental to resolution, as well as to the accuracy and precision of quantitation.[17,18]

Zorbax SB-$C_{18}$ packing is a densely covered, sterically protected $C_{18}$ silane stationary phase, specifically designed for use in low pH environments.[19] However, the results of the present study indicated that this column was not sufficiently deactivated to enable symmetrical peaks to be obtained for the analysed compounds, even with the addition of the amine modifier to the mobile phase. In addition, the metabolite Ro 12-8095 had a long retention time on this column (Table 1).

***Table 1.*** The effect of different HPLC columns application on chromatographic retention, separation and peak symmetry of moclobemide, its metabolites, Ro 12-5637 and Ro 12-8095, and IS (Ro 11-9900). Mobile phase consisted of 10 mm $KH_2PO_4$ with 1% triethylamine (pH 3.5) and acetonitrile (80:20, v/v); A flow-rate was 1 ml $min^{-1}$

| HPLC column | Compound | Retention time ($t_R$; min) | Retention factor ($k$) | Separation factor ($\alpha$) | Resolution (R) | Peak symmetry |
|---|---|---|---|---|---|---|
| Zorbax SB-$C_{18}$ | Moclobemide | 5.86 | 1.33 | – | – | 0.59 |
| Length: 250 mm | Ro 12-5637 | 8.05 | 2.21 | 1.66 | 4.78 | 0.43 |
| Stat. Phase: Sterically-protected $C_{18}$ silane, | IS | 9.34 | 2.72 | 1.23 | 2.49 | 0.58 |
| non-endcapped | Ro 12-8095 | 21.86 | 7.71 | 2.83 | 20.14 | 0.7 |
| Pore size: 80 Å | | | | | | |
| Zorbax Extend-$C_{18}$ | Moclobemide | 4.7 | 1.18 | – | – | 0.72 |
| Length: 250 mm | Ro 12-5637 | 5.71 | 1.64 | 1.4 | 4.48 | 0.7 |
| Stat. phase: propylene-bridged bidentate | IS | 7.09 | 2.28 | 1.39 | 5.18 | 0.75 |
| $C_{18}$ silane, double endcapped | Ro 12-8095 | 15.58 | 6.21 | 2.72 | 19.79 | 0.79 |
| Pore size: 80 Å | | | | | | |
| XTerra™ RP18 | Moclobemide | 3.68 | 1.14 | – | – | 0.8 |
| Length: 150 mm | Ro 12-5637 | 4.51 | 1.62 | 1.43 | 2.5 | 0.81 |
| Stat. phase: Hybrid organic-inorganic | IS | 5.65 | 2.28 | 1.41 | 2.9 | 0.87 |
| $C_{18}$ silane with embedded polar | Ro 12-8095 | 10.35 | 5.01 | 2.2 | 9.38 | 1 |
| carbamate group, endcapped | | | | | | |
| Pore size: 129 Å | | | | | | |

Zorbax Extend-$C_{18}$ packing is made by first chemically bonding a dense monolayer of propylene-bridged bidentate $C_{18}$ silane stationary phase to a specially prepared porous silica microsphere support. The combination of bidentate-$C_{18}$ and the exhaustive endcapping produces a highly hydrophobic stationary phase, which is well protected against silanophilic interactions[19] and especially suited for separating highly basic compounds that produce poor peak shapes on most columns.

XTerra™ RP18 packing material is synthesized using hybride particle technology. Hybrid particles contain both inorganic (silica) and organic (organosiloxane) components. These particles are surface bonded to attach $c_{18}$ groups, then endcapped to further reduce the concentration of residual silanols. The additional characteristic of XTerra™ RP18 is the presence of an embedded polar carbamate group; therefore, this column displays reduced retention and tailing for basic analytes.[16,17,19,20] In general, all compounds are somewhat less retained on hybrid organic-inorganic columns because of their lower surface area and carbon content in comparison to typical bonded silicas.[17] Due to these specific characteristics of the packing material and the decreased column length, use of XTerra™ RP18 resulted in the best peak symmetry and the shortest retention times for all the analytes (Table 1).



## Optimisation of the Mobile Phase

Influences of the organic modifier concentration in the mobile phase (in the ranges 20–23% and 17–20% for Zorbax Extend-$C_{18}$ and XTerra™ RP18, respectively), pH (in the range 3.0–4.0), and the mobile phase flowrate (1.0–1.5 mL $min^{-1}$) were then evaluated for the two selected stationary phases in order to further optimise retention times of the analytes and ensure separation from polar plasma endogenous compounds. The optimisation of chromatographic conditions resulted in the development of the two independent HPLC methods: the one employing XTerra™ RP18, with the optimum mobile phase consisting of 10 mm $KH_2PO_4$ with 1% triethylamine (pH 3.9) and acetonitrile (83:17, v/v), and the other employing Zorbax Extend-$C_{18}$, with the mobile phase consisting of 10 mm $KH_2PO_4$ with 1% triethylamine (pH 3.1) and acetonitrile (80:20, v/v). Both methods enabled good separation of all the analytes with no endogenous interferences, but the one employing XTerra™ RP18 was preferred for further analysis, due to the shorter analysis time (retention times on XTerra™ RP18 were 3.9, 4.9, 11.4, and 6.4 min for moclobemide, Ro 12-5637, Ro 12-8095, and IS, respectively, and the ones on Zorbax Extend-C18 were 4.0, 4.9, 13.6, and 6.2 min for moclobemide, Ro 12-5637, Ro 12-8095 and IS, respectively) (Figure 2).

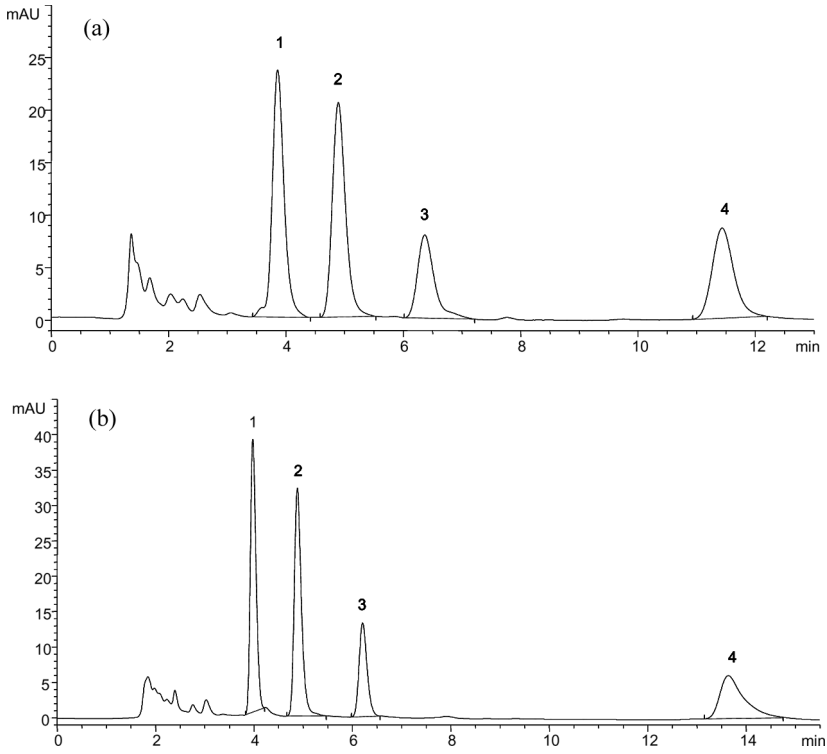


***Figure 2.*** Representative chromatograms of plasma spiked with moclobemide (1), Ro 12-5637 (2), Ro 12-8095 (4) and Ro 11-99000 (IS, 3), obtained by applying two optimised HPLC methods based on the use of Waters XTerra$^{TM}$ RP18 (a) and Agilent Zorbax Extend-$C_{18}$ columns (b). The concentration of all the analytes and IS was 0.50 mg $L^{-1}$.



## Optimisation of the Extraction Procedure

The impact of variable experimental conditions on the efficiency and selectivity of the extraction procedure was studied. Two types of Speedisk® SPE polymer columns were evaluated: the $H_2O$-Philic DVB (hydrophilic divinylbenzene column) and $H_2O$-Philic SC-DVB (hydrophilic divinylbenzene column with cation exchanger properties, containing benzylic and sulfonic acid sites). Slightly modified generic methods proposed by the manufacturer were applied for the extraction.

$H_2O$-Philic SC-DVB is a mixed mode strong cation exchanger with hydrophobic and π-π interactions. Pretreated plasma samples (using orthophosphoric acid as protein precipitant agent) were loaded onto a column that was preconditioned with 1 mL methanol, followed by

2 mL water. Washing was performed with 1 mL 0.1 N HCL, followed by 1 mL methanol, and elution with two portions of 0.5 mL methanol – ammonium hydroxide (95:5, v/v).

The performed extraction method on the $H_2O$-Philic SC-DVB column was not appropriate for the simultaneous isolation of all the examined compounds from plasma. Actually, Ro 12-8095 was not detected in the final extract because it was completely washed from the column with methanol, which was used to remove impurities. On the other hand, relatively high percentages of recovery were obtained for the other analytes (90.1, 92.8, and 94.7% for moclobemide, Ro 12-5637 and IS (Ro 11-9900), respectively). The different extraction efficiency of the analytes could be explained by differences in their acido-basic properties and charges at the analytical pH (plasma acidified with orthophosphoric acid). Since moclobemide and Ro 11-9900 are basic compounds (pKa: 6.02), in acidified plasma they have been predominantly present in the form of cations. Ro 12-5637 and Ro 12-8095 do not express significant basic/acidic character (pKa: 14.73); however, Ro 12-5637 is positively charged heterocyclic N-oxide. The positive charge of moclobemide, Ro 11-9900, and Ro 12-5637 together with neutral charge of Ro 12-8095 at the analytical pH (see Figure 1) could explain the selective elution of the neutral Ro 12-8095 from the $H_2O$-Philic SC-DVB column with methanol. Due to the combined reverse phase and cation exchange properties of this sorbent, the sample was separated into an acidic-neutral fraction (elution with methanol) and a basic/positively charged fraction (elution with methanol – ammonium hydroxide).[21]



SPE on the $H_2O$-Philic DVB column is based on hydrophobic, hydrogen bonding, and $\pi$-$\pi$ interactions with the analytes. The same sample preparation and column conditioning procedures were applied as for the $H_2O$-Philic SC-DVB. Washing was performed with water (1 mL with two portions of 0.5 mL methanol. In our study, isolation of all the analytes by the extraction method on the $H_2O$-Philic DVB column was successful.

However, the obtained extracts were not sufficiently purified and a modification of the proposed generic procedure was required. In order to choose an adequate washing solvent to enhance selectivity, elution of moclobemide and its metabolites was examined as a function of the composition of methanol-water solution (used in the second washing step), as it is shown in Table 2. The washing solvent strength was increased significantly in comparison to the generic method, and its volume was doubled. In addition, sample pretreatment procedure was also modified. Plasma samples were diluted with 2 mL phosphate buffer (0.05 M $K_2HPO_4$, pH 8.4) in order to improve flow during loading and retain moclobemide and is (Ro 11-9900) in non-ionic, molecular form, which had higher affinity for the sorbent (buffer was also used for column

***Table 2.*** Effect of washing solvent composition on the efficiency of spe of moclobemide and its two metabolites, Ro 12-5637 and Ro 12-8095, on the $H_2O$-Philic DVB column from plasma samples

| | Recovery (%) | | |
|---|---|---|---|
| Washing solvent composition | Moclobemide[a] | Ro 12-5637[a] | Ro 12-8095[a] |
| Methanol-Water, 20:80 (v/v) | 94.3 | 88.2 | 100.7 |
| Methanol-Water, 30:70 (v/v) | 92.4 | 83.6 | 99 |
| Methanol-Water, 40:60 (v/v) | 89.0 | 34.4 | 94 |

[a]Plasma concentration was $0.02\,mg\,L^{-1}$.

conditioning instead of water). The specific design of Speedisk® Polymer Columns enabled the elimination of other sample preparation steps required with the conventional SPE columns, including protein precipitation and centrifugation.

The concentration of methanol in the washing solution over the studied range was found to have the most significant effect on the extraction efficiency of Ro 12-5637. The extraction of this compound had been previously reported to be the most critical and highly dependent on the type of extraction solvent and other experimental conditions (dichloromethane, an aprotic polar solvent, had been found to be much more effective than non-polar solvents, such as chloroform and n-butyl chloride).[6] These findings can be explained by the cationic nature of Ro 12-5637, low lipophilicity (log P: 0.49) and the highest polar surface area (PSA: $65.21\,Å^2$), when it is compared with the other analytes (Table 3).

Created plots of the extraction efficiency, expressed in terms of percentage of recovery, against percentage of methanol in the mixture gave us information about the best composition for washing solvent (that containing the largest amount of methanol without eluting the drug or its metabolites). The washing solution chosen (methanol-water, 30:70 (v/v)) eliminated the endogenous compounds from the matrix without eluting the analytes to any significant extent, yielding a chromatogram free of

***Table 3.*** Physico-chemical parameters of the investigated compounds, computed by Marvin 4.0.5 ChemAxon

| Compounds | PSA [$Å^2$] | logP | pKa |
|---|---|---|---|
| Moclobemide | 42.77 | 1.10 | 6.02 |
| Ro 12-5637 | 65.21 | 0.49 | >14 |
| Ro 11-9900 (IS) | 42.77 | 1.57 | 6.02 |
| Ro 12-8095 | 58.64 | 0.29 | >14 |

interferences. Further modification of the elution step by increasing the eluent volume did not result in additionally improved extraction efficiency.

## Quantitative Structure Retention Relationship (QSRR) Study of the HPLC Retention and Separation

The methods of theoretical chemistry were employed to select the molecular properties of moclobemide, its metabolites, and 10 drugs relatively related with structure of moclobemide that are detectable at the chromatograms under the applied conditions, during the run time of 13 min (Figure 1).[5] All of the examined drugs are frequently coadministered in patients on moclobemide therapy.

QSRR models of the drugs were performed to correlate their physicochemical properties with the chromatographic retention parameter (log (t)). Multilinear regression was used as the regression method to develop the relationship between independent variables (physicochemical, electronic, and constitutional parameters) and chromatographic retention time (dependent variable). The optimum number of components (latent variables) was determined by cross validation and the model predictive ability was assessed by cross validated $r^2$ ($r^2_{cv}$, $q^2_{pre}$) and root mean square error of prediction (RMSEP).[22]

The compounds were selected with an intention of covering a wide range of the HPLC retention times (t, [min]). The correlation coefficients were determined for all calculated molecular parameters with HPLC retention times (t, [min]). The molecular properties with the highest correlation coefficients were selected for multiple linear regression study.

The partition distribution coefficient (logD), and polar surface area (PSA) of the analyzed structures expressed the strongest influence on the chromatographic retention parameter (log (t)). Multiple linear regression models, log (t) = f (PSA, logD), were obtained with $r^2 > 0.86$ and cross validation parameter, $q^2_{pre} > 0.88$. The developed QSRR approach can help in understanding the structural features that contributes to the chromatographic retention parameter (log (t)) of the other potentially interfering drugs.

The quality of the regression fits was estimated using parameters such as the regression factor (r), square of regression factor ($r^2$), adjusted square of regression factor (($R^2_{adj}$), $q^2_{pre}$, (validation $R^2$), F ratio, and P values.

One measure of performance of a model is its ability to make predictions. In this context, withhold-1 cross validation of the created models was carried out. This validation method is based on excluding one observation ($x_i$, $y_i$) from the data set and then fitting the model using the remaining $n - 1$ observations. From that estimation, the $e_{(i)}$ residual of the withheld data point ($x_i$, $y_i$), $e_{(i)} = y_{data,\,i} - \hat{y}_{model\,(i)}$ was calculated.

This procedure was repeated for i = 1, 2..., n, yielding $e_{(1)}, \ldots, e_{(n)}$. In this setting, Allen defined PRESS (Predicted Sum of Squares)[22] as:

$$PRESS = \sum_{i=1}^{n} e_{(i)}^2 \quad (1)$$

$$RMSEP = \sqrt{\frac{PRESS}{n}} \quad (2)$$

$$q_{pre}^2 = 1 - \frac{PRESS}{SSTo} \quad (3)$$

Models with $q_{pre}^2 \geq 0.6$ can be considered to have good predictive capability.[22]

The experimental data of the drugs reported in Table 4 were subjected to the QSRR study. A preliminary data analysis showed a good spread and distribution of the log (t) values, which spanned about a 1 log unit range (from 0.255 to 1.057). This was a good precondition for the derivation of meaningful models.

The quantitative regression analysis was performed to investigate the correlations between HPLC retention time (t [min] and log (t)) and the calculated constitutional, geometrical, physicochemical, and electronical properties of the optimized models. The molecular properties with the highest correlation coefficients, such as PSA and logD (Marvin 4.0.5 Chemaxon Program), were selected for multiple linear regression study. PSA is defined as the surface sum over all polar atoms, (usually oxygen and nitrogen), including also attached hydrogens. PSA is a commonly used in medicinal chemistry metric for the optimization of pharmacokinetic properties.

Created QSRR models with the two variables, PSA and logD at pH 3.9, attempted to fit the data with the corresponding regression parameters: $r^2$ (0.863), $r_{adj}^2$ (0.838), $q_{pre}^2$, RMSEP, F ratio and P values. The multivariable model succeeded the performed cross validation with $q_{pre}^2$ values 0.876 (Table 4). Thus, the obtained regression equation:

$$\log\,(t) = 0.84053 - 0.00052 \cdot \mathrm{PSA} + 0.140922 \cdot \mathrm{logD}\ (\mathrm{pH}\ 3.9) \quad (4)$$

was suggested as the optimal QSRR model for evaluation of the HPLC retention parameter log (t).

As models with $q_{pre}^2 \geq 0.6$ and low RMSEP can be considered to have good predictive capability, the created regression model ($q_{pre}^2 = 0.876$, RMSEP = 0.133) could be used for the HPLC retention time (t, [min]) evaluation of other related potentially interfering drugs. The considerable improvement in the regression parameters for the multivariate log (t) = f (PSA, logD (pH 3.9)) linear regression model over the

***Table 4.*** Regression analysis relating the two variables, polar surface area (PSA) and distribution coefficient (logD) at pH 3.9 with HPLC retention time (log (t)) Values. RMSEE – root mean square error of estimation; RMSEP–root mean square error of prediction

| Compounds | log (t) [min] | PSA [Å$^2$] | logD at pH 3.9 |
|---|---|---|---|
| Amoxicillin (1) | 0.255 | 162.71 | −3.76 |
| Atenolol (2) | 0.255 | 89.16 | −3.34 |
| Cefalexin (3) | 0.255 | 142.48 | −3.57 |
| Ampicillin (4) | 0.279 | 142.48 | −3.47 |
| Cefaclor (5) | 0.279 | 142.48 | −4.16 |
| Sotalol (6) | 0.279 | 91.39 | −3.44 |
| Metamizole (7) | 0.462 | 92.37 | −1.52 |
| Ciprofloxacin (8) | 0.58 | 77.46 | −2.02 |
| Moclobemide (9) | 0.591 | 42.77 | −1.11 |
| Hydrochlorthiazide (10) | 0.633 | 135.12 | −0.16 |
| Ro 12-5637 (11) | 0.69 | 65.21 | 0.49 |
| Ro 11-9900 (IS) (12) | 0.813 | 42.77 | −0.64 |
| Sulfamethoxazole (13) | 1.037 | 106.6 | 1.03 |
| Ro 12-8095 (14) | 1.057 | 58.64 | 0.29 |
| r, Correlation | | −0.572 | 0.927 |
| Regression equation | $\log(t) = 0.84053 - 0.00052 \cdot \mathrm{PSA} + 0.140922 \cdot \log\mathrm{D}\ (\mathrm{pH}\ 3.9)$ | | |
| $R^2$ | 0.863 | | |
| $R^2_{ADJ}$ | 0.838 | | |
| RMSEE | 0.103 | | |
| F-ratio | 34.751 | | |
| P-value | 0.00002 | | |
| $Q^2_{PRE} > 0,6$ | 0.876 | | |
| RMSEP | 0.133 | | |

corresponding univariate models (Table 4), indicated the important influence of the both selected parameters (PSA and logD (pH 3.9)) on the final HPLC log (t) values. The fitting power of the QSSR model giving the best statistics can be seen on the plots of predicted vs. observed HPLC log (t) values (Figure 3).

Therefore, in the early stages of the analysis of moclobemide and its metabolites in plasma in the presence of related potentially interfering drugs, it is becoming important to compute PSA and logD at pH 3.9 of coadministered drugs, and to use the developed QSRR model for prediction of their HPLC retention and separation behaviours. Furthermore, pKa, logP and PSA of the analyzed compounds are molecular parameters, which should be considered and compared as critical factors affecting SPE.

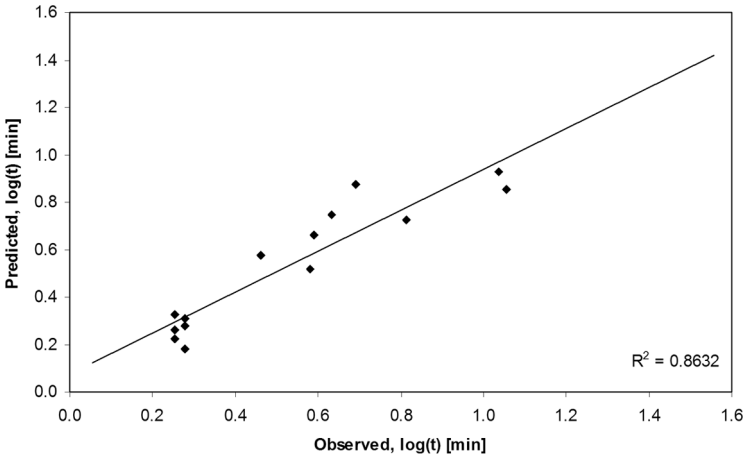


***Figure 3.*** Plot of observed vs. predicted log (t) values of the analyzed compounds. Predicted Yn-1 log (t) values are from the leave-one-out cross validation data.



## CONCLUSIONS

Optimisation of the stationary phase has been found to be the most critical factor in the development of appropriate chromatographic method, which provides satisfactory separation of moclobemide and its metabolites. A fully reacted endcapped $C_{18}$ column provides better peak shapes and improved separation resolution than non-endcapped materials. The results of the present study indicates that SPE sorbents based on mixed mode cation-exchange mechanisms are not appropriate for the simultaneous isolation of all the analytes from plasma, as metabolite Ro 12-8095 is a neutral compound and always present in non-ionic, molecular form. On the other hand, Ro 12-5637 is a positively charged heterocyclic N-oxide, with low lipophilicity (logP: 0.49) and the high polar surface area (PSA: 65.21 $Å^2$), so the hydrophobic interactions with SPE sorbents based on adsorption (reverse phase) mechanism could be weaker for this compound with a consequently lower extraction efficiency. Thus, the content of organic solvent in the washing solution has been carefully balanced (methanol-water, 30:70 (v/v)). The developed SPE-HPLC-UV method has proved to be valid for quantitative determination of moclobemide and its metabolites in human plasma over a wide range of therapeutic drug concentrations, without interferences from endogenous compounds and other commonly co-administered drugs. In addition, the developed QSRR model ($r^2 = 0.863$, $q^2_{pre} = 0.876$, RMSEP = 0.133) enables reliable prediction of the HPLC retention times for the other related potentially interfering drugs. Therefore, the use of the developed

analytical method in combination with the created QSRR model can save efforts when monitoring individuals who take several medications, which is a common clinical situation.

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