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Effects of derivatization reagents consisting of *n*-alkyl chloroformate/*n*-alcohol combinations in LC–ESI-MS/MS analysis of zwitterionic antiepileptic drugs

Nađa Kostić [a], Yannis Dotsikas [b], Anđelija Malenović [a,*], Mirjana Medenica [c]

[a] University of Belgrade, Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450, Belgrade, Serbia
[b] University of Athens, School of Pharmacy, Department of Pharmaceutical Chemistry, Panepistimioupoli Zografou, Athens, Greece
[c] University of Belgrade, Faculty of Pharmacy, Department of Physical Chemistry and Instrumental Methods, Vojvode Stepe 450, Belgrade, Serbia

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ABSTRACT

In the current study, three antiepileptic drugs with zwitterionic properties, namely vigabatrin, pregabalin and gabapentin, were chosen as model analytes to undergo derivatization by applying various *n*-alkyl chloroformate/*n*-alcohol combinations, followed by LC–ESI-MS/MS analysis. The employment of 16 combinations per drug using methyl, ethyl, propyl or butyl chloroformate coupled with methanol, ethanol, propanol or butanol, greatly affected a series of parameters of the derivatives, such as retention time on C8 column, signal expressed via areas, limit of detection values, as well as the yields of the main and side reactions. Practically, even slight modification of *n*-alkyl group of either chloroformate or alcohol resulted in significant changes in the chromatographic and mass spectrometric behavior of the novel derivative. It was clearly demonstrated that all the estimated parameters were highly correlated with the length of *n*-alkyl groups of the involved chloroformate and alcohol. The most significant influence was monitored in peak area values, indicating that the length of the *n*-alkyl chain plays an important role in electrospray ionization efficiency. For this parameter, increasing the *n*-alkyl chain from methyl to butyl led to increment up to 2089%, 508.7% and 1075% for area values of derivatized vigabatrin, pregabalin and gabapentin, respectively. These changes affected also the corresponding values of limits of detection, with the estimated improvements up to 1553%, 397.7% and 875.0% for the aforementioned derivatized drugs, respectively. Besides the obvious utilization of these conclusions in the development of bioanalytical methods for these analytes with the current protocol, this study offers valuable data which can be useful in more general approaches, giving insights into the effects of this derivatization reaction and its performances.

1. Introduction

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) equipped, in most cases, with electrospray ionization (ESI) ion source, constitutes a powerful means in modern pharmaceutical analysis. There are two crucial properties which analytes should possess in order to be detected by LC–ESI-MS/MS with appropriate sensitivity: (i) chargeability, existing either through ionic form or created via adduct formation in gas phase, and (ii) relative hydrophobicity, enabling adequate separation from salts and other ion-suppression causing substances [1–3].

Although LC–ESI-MS/MS has an inherent selectivity and sensitivity, which in most cases enables satisfactory direct analysis, the implementation of analyte derivatization sometimes can be essential tool to improve analyte response, especially when ionization efficiencies are extremely low or in case of complex biological matrices [4–6]. Considering that one extra step during sample preparation would extend the total time of analysis, the following criteria should be fulfilled in order to implement a derivatization reaction: (i) addition of just one reagent for derivatization, with simple sample handling; (ii) fast reaction, preferably at room temperature; (iii) formation of derivatives with increased ESI efficiency, either by introducing a chargeable moiety to the compounds of interest (including a group with permanent charge) [7] or by diminishing an opposite charge, and (iv) utilization of small quantity of low-cost reagent [8].

Numerous publications in various fields suggest alkyl chloroformates as favorable reagents for derivatization, due to their characteristics, such as: high reactivity, possibility to react in water medium, negligible cost, high recovery of derivatization reaction and easy extraction of derivatives into organic solvent. Capable to react with different functional groups (like phenol, thiol, amino,

* Corresponding author. Tel.: +381 11 3951 333; fax: +381 11 3972 840.
E-mail address: andja@pharmacy.bg.ac.rs (A. Malenović).

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carboxylic), chloroformates can provide effortless and prompt change of polar compounds, making them suitable, above all, for gas chromatography (GC) analysis. Although this type of derivatization was successfully applied for determination of biogenic amines [9,10], bisfenol A [11], and free fatty acids [12], the most prominent field of chloroformates as derivatization reagents can be found when the subject of analysis are zwitterionic structures, mainly amino acids. Several methods for determination of these structures in blood [13], plasma [14], urine [15,16], meat products [17], supplementation tablets [18] and physiological samples [19] applying GC–MS or in human urine [20], rats brain [21], *Escherichia coli* [22], cyanobacteria [23] applying LC–ESI-MS/MS are described. In the field of metabolic profiling, another considerably attractive area of modern pharmaceutical analysis, alkyl chloroformates were implemented for GC determination of amino acids [24] or amino acids simultaneously with organic acids [25–27] and amines [28,29] as well as for nontargeted metabolite profiling [30,31]. Other specific applications of chloroformates derivatization including determination of amines [32–35], polyamines [36], catecholamines [37,38], linsidomine [39] can also be found in literature. However, an extensive study comparing, via certain criteria, the performance of various alkyl chloroformates in LC–MS^n, has not been reported.

In current study, three antiepileptic drugs—vigabatrin, pregabalin and gabapentin, were selected as model compounds for the derivatization reaction, applying various *n*-alkyl chloroformates. Containing both amino and carboxylic group (zwitterions), they are not suitable for classical reverse-phase chromatographic analysis (RP-HPLC), while coexistence of both positive and negative charge greatly affects ESI efficiency. In such specific case, when it is very hard to enable satisfactory direct analysis, derivatization with alkyl chloroformates seems to be a reasonable solution to overcome both drawbacks, by simultaneously increasing lipophilic character and removing negative moiety. During the derivatization reaction, in presence of pyridine as catalyst, carboxylic and amino group were transformed to ester and carbamate groups, respectively. Wang et al. [40] suggest that the type of ester formed during this derivatization process, directly depends on alcohol, which is present in the reaction mixture. When alkyl chloroformate and alcohol contain the same alkyl group, only one derivative is formed. On the contrary, when reagents with different alkyl chain length are involved in the reaction mixture, two derivatives are created (Fig. 1): one from the combination of chloroformate and alcohol (main reaction) and the other one originated just from utilized chloroformate (side reaction).

The aim of this study was to examine the effects of various *n*-alkyl (from methyl to butyl) chloroformates, combined with different *n*-alcohols (from methanol to butanol) on the chromatographic and tandem mass spectrometric analysis of the derivatives of the selected antiepileptic drugs. Sixteen derivatives per drug were studied in terms of chromatographic retention, signal intensity expressed via areas, limit of detection obtained after MS/MS analysis, as well as the yields of the main and side reaction products. Up to now, only pregabalin was analyzed by GC–MS, following derivatization with ethyl chloroformate [41,42]. Based on the current study, LC–ESI-MS/MS protocols may be developed following the suggested derivatization procedure and covering needs not just for the specific drugs, but for other compounds of similar nature, too.

2. Experimental

2.1. Reagents and chemicals

Vigabatrin, pregabalin and gabapentin reference standards were obtained from British Pharmacopeia Commission Laboratory (Teddington, UK), Pfizer Inc. (Groton, CT, USA) and Kleva Pharmaceutical Company (Athens, Greece), respectively. Acetonitrile (MS grade), methanol, ethanol, propanol, butanol (all alcohols were of HPLC grade), methyl chloroformate, ethyl chloroformate, propyl chloroformate, butyl chloroformate, pyridine, chloroform, ethyl acetate, ammonium formate and formic acid were purchased from Sigma-Aldrich (St. Luis, MO, USA). Di-*iso*-propylether was provided by Riedel-de Häen AG (Seelze, Germany). Hydrochloric acid was obtained from Lach-Ner (Neratovice, Czech Republic), while sodium hydroxide and *n*-hexane were acquired from J.T. Baker (Deventer, Holland) and LGC Promochem GmbH (Wesel, Germany), respectively. Aqueous solutions were prepared with de-ionized and double-distilled water (Resistivity > 18 MΩ) from Simplicity 185 (Millipore, Billerica, MA, USA).

2.2. Instrumentation

Sample preparation was carried out by using Vortex-Genie 2T (Scientific Industries, Inc., Bohemia, NY, USA) and Reacti-Vap III (Thermo Fisher Scientific Inc., San Jose, CA, USA) evaporation unit. The chromatographic analysis was performed using Accela Thermo Scientific system consisted of Accela Pump and Autosampler. A TSQ Quantum Access MAX triple quadrupole spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA), equipped with heated electrospray ionization source (HESI), was utilized for tandem mass spectrometric detection. Data acquisition was performed with Xcalibur 1.2 software.

2.3. Derivatization protocol

Each analyte was dissolved into 0.1 mol L^{-1} hydrochloric acid to obtain the concentration of 100 μg mL^{-1}. 100 μL of this solution was mixed with 150 μL of sodium hydroxide (0.33 mol L^{-1}), 80 μL alcohol (methanol, ethanol, propanol or butanol) in pyridine solution (77: 23 v/v) and 50 μL *n*-alkyl chloroformate reagent solution (mixture of the respective chloroformate, chloroform and *n*-hexane in ratio 17.4: 71.6: 11.0 v/v). After vortexing for 2 min, 500 μL of ethyl acetate was added to perform liquid–liquid extraction. Then, the mixture was vortex-mixed for 2 min and newly formed derivatives were extracted into organic layer. All solutions were left to relax for 10 min and then 350 μL of the upper layer were evaporated to dryness and subsequently reconstituted with 1500 μL of acetonitrile. Each derivative was prepared in triplicate in order to test the reproducibility of the derivatization reaction. Samples were stored in autosampler at 10 °C, before analysis.

2.4. Chromatographic and tandem mass spectrometric conditions

The mobile phase consisted of acetonitrile : 5 mmolL^{-1} ammonium formate (80: 20 v/v). pH of the water phase was adjusted to 2.10 with formic acid. All runs were performed under isocratic conditions, having a flow rate of 600 μL min^{-1}. A Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5 μm particle size) was used and maintained at 30 °C. The injection volume was 3 μL.

Derivatized vigabatrin, pregabalin and gabapentin were detected in positive ESI mode using two Selected Reaction Monitoring (SRM) transitions per analyte, i.e. those that correspond to the most intense fragments (Table 1). Although the most common procedure is to follow just one, the use of two SRM transitions per analyte has stated advantages, as defined by the EU Commission Decision 2002/657/EC [43], presenting a warranty for the selectivity of the method [44]. Vaporizer temperature and capillary temperature were settled at 350 °C and 275 °C, respectively, while spray voltage, sheath gas pressure, ion sweep gas pressure, auxiliary gas pressure, tube lens, skimmer offset and collision

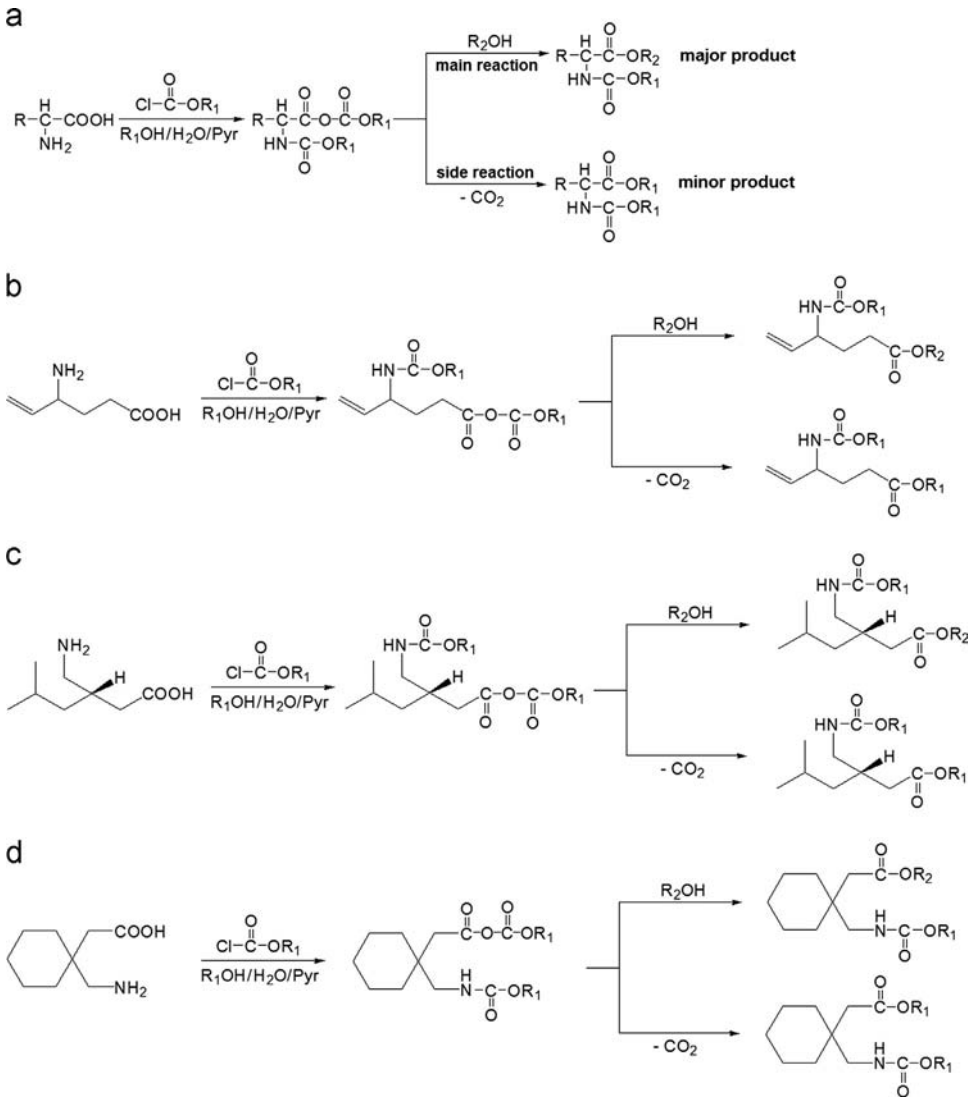

Fig. 1. *n*-alkyl chloroformate/*n*-alcohol derivatization reaction with the main and side reaction for: (a) amino acids [40]; (b) vigabatrin; (c) pregabalin and (d) gabapentin.

energies values were optimized during tuning. Corresponding collision energy (CE) values for the two most intense fragments are presented in Table 1.

For each *n*-alkyl chloroformate a separate method was created, consisting of four scan events, each one configured to monitor two SRM transition, applying suitable CE values, scan time of 0.1 s, scan width of 0.025 *m/z* and peak width 0.70 Q1 (FWHM). With this procedure it was possible to monitor both derivatives, main and side one, as well as to confirm the absence of derivatives whose occurrence was not expected.

3. Results and discussion

Due to a large number of derivatives created using different combinations of *n*-alkyl chloroformate/*n*-alcohol derivatization sets, a coded name for each derivative was given as follows: the first part refers to alkyl chloroformate and it is expressed by the first letter(s) of its alkyl name (methyl=M, Me, Met; ethyl=E, Et, Eth; propyl=P, Pr, Pro and butyl=B, Bu, But), while the same scenario applies for second part that refers to alcohol. Acronyms for vigabatrin derivatives have 2 letters, while for pregabalin and gabapentin 4 and 6 letters, respectively.

3.1. Effect of derivatization reaction on retention times

Derivatization of the three selected molecules and their subsequent LC–ESI-MS/MS analysis led to a series of observations. One of the evident changes was the variation in retention times for the same chromatographic RP system, when the different derivatization sets were applied. As it is shown in Table 1, with increasing chain length (number of added carbons), retention on C8 column is prolonged, leading to the longest retention times when butyl chloroformate/butanol combination is applied. The least lipophilic of all formed derivatives, MM (vigabatrin derivative) was the one with a t_R value of 2.77 min, while the most lipophilic one, ButBut (gabapentin derivative), was eluted at 7.24 min. In addition, it can be also observed that all derivatives of the same number of carbons (for instance, ME and EM derivatives of vigabatrin, eluted at 2.90 and 2.91 min, respectively) had practically the same retention behavior, with limited variations, leading to the conclusion that the number of carbons was the most decisive factor for t_R values in the specific chromatographic system.

In an attempt to correlate chromatographic behavior of the analyzed derivatives to the number of carbons present in their structure, descriptive mathematical equations created by TableCurve2D software v5.01.02 (trial version) were utilized. The equation that succeeded to explain $t_R=f$ (carbon numbers) for all

Table 1
Data for all derivatives obtained after LC–ESI-MS/MS analysis.

Derivative	Carbons Nr	t_R[a] (min)	SRM (*m/z*)[b]	LOD (μg mL^{-1})[c]
Vigabatrin				
MM[d]	9	2.77	201.96→85.15 (17), 127.10 (7)	0.264
ME	10	2.90	215.99→99.12 (15), 141.08 (7)	0.184
MP	11	3.08	229.98→113.09 (13), 155.08 (6)	0.151
MB	12	3.37	244.04→113.09 (14), 169.16 (5)	0.180
EM	10	2.91	215.98→85.14 (17), 127.08 (7)	0.125
EE	11	3.06	229.97→99.11 (16), 141.09 (8)	0.063
EP	12	3.27	244.00→113.10 (15), 155.09 (6)	0.045
EB	13	3.60	258.00→113.10 (14), 169.08 (6)	0.032
PM	11	3.07	230.01→85.15 (19), 127.10 (7)	0.110
PE	12	3.27	244.02→99.15 (16), 141.10 (8)	0.050
PP	13	3.54	258.03→113.10 (14), 155.10 (6)	0.039
PB	14	3.94	272.05→113.11 (14), 169.11 (6)	0.029
BM	12	3.36	244.02→85.14 (20), 127.08 (7)	0.069
BE	13	3.53	258.03→99.11 (17), 141.08 (10)	0.027
BP	14	3.88	272.04→113.09 (15), 155.08 (8)	0.022
BB	15	4.36	286.06→113.09 (15), 169.08 (7)	0.017
Pregabalin				
MeMe	11	3.22	231.95→167.99 (14), 200.00 (7)	0.509
MeEt	12	3.47	245.95→167.98 (15), 199.98 (6)	0.370
MePr	13	3.80	259.96→167.98 (15), 199.99 (5)	0.400
MeBu	14	4.22	273.99→167.99 (16), 199.99 (7)	0.284
EtMe	12	3.49	246.02→142.07 (19), 214.00 (7)	0.284
EtEt	13	3.79	260.02→142.06 (19), 214.00 (7)	0.218
EtPr	14	4.22	274.03→142.07 (20), 213.99 (8)	0.167
EtBu	15	4.76	288.05→142.07 (21), 214.00 (6)	0.147
PrMe	13	3.76	259.98→142.03 (18), 227.97 (5)	0.212
PrEt	14	4.15	273.98→142.03 (20), 227.97 (6)	0.156
PrPr	15	4.69	287.99→142.04 (19), 227.96 (7)	0.125
PrBu	16	5.38	302.00→142.02 (18), 227.95 (6)	0.136
BuMe	14	4.16	274.00→142.03 (18), 242.00 (5)	0.287
BuEt	15	4.68	288.00→142.03 (18), 241.98 (7)	0.181
BuPr	16	5.40	302.01→142.04 (18), 241.98 (6)	0.172
BuBu	17	6.29	316.02→142.03 (19), 241.98 (7)	0.128
Gabapentin				
MetMet	12	3.40	243.96→179.98 (14), 211.98 (6)	0.105
MetEth	13	3.73	257.97→179.98 (15), 211.97 (5)	0.068
MetPro	14	4.14	271.98→179.98 (15), 211.98 (5)	0.056
MetBut	15	4.68	285.99→179.97 (17), 211.98 (7)	0.044
EthMet	13	3.69	257.98→154.06 (20), 225.99 (7)	0.032
EthEth	14	4.12	271.98→154.04 (20), 225.99 (5)	0.021
EthPro	15	4.65	285.99→154.04 (23), 225.98 (6)	0.020
EthBut	16	5.32	300.00→154.03 (23), 225.96 (7)	0.020
ProMet	14	4.06	271.97→154.03 (19), 239.96 (7)	0.027
ProEth	15	4.58	285.97→154.02 (22), 239.95 (7)	0.015
ProPro	16	5.26	299.98→154.02 (21), 239.94 (6)	0.016
ProBut	17	6.15	314.00→154.01 (23), 239.93 (7)	0.013
ButMet	15	4.57	285.96→154.00 (19), 253.92 (6)	0.015
ButEth	16	5.26	299.95→154.00 (18), 253.91 (6)	0.013
ButPro	17	6.17	313.96→153.99 (19), 253.90 (7)	0.012
ButBut	18	7.24	327.96→153.98 (22), 253.88 (7)	0.012

[a] t_R—retention time. Mean values ($n=3$).
[b] SRM—Selected Reaction Monitoring followed by corresponding CE values (eV) given in parentheses.
[c] LOD—Limit of Detection. Mean values ($n=3$).
[d] Code name for each derivative constitutes of 2 parts: The first part refers to alkyl chloroformate and it is expressed by the first letter (s) of its alkyl name (methyl=M, Me, Met; ethyl=E, Et, Eth; propyl=P, Pr, Pro and butyl=B, Bu, But), while the same scenario applies for second part that refers to alcohol. Acronyms for vigabatrin derivatives have 2 letters, while for pregabalin and gabapentin 4 and 6 letters, respectively.

of three derivatized molecules with high coefficient of determination values (R^2), was $\ln y = a + bx^3$ (Table 2). Moreover, the equations obtained for pregabalin and gabapentin had almost the same *a* and *b* coefficients leading to similar retention times of derivatives containing the same carbon number. For example, t_R values for EtEt derivative of pregabalin and MetEth derivative of gabapentin (both having 13 number of carbons) were 3.79 and 3.73 min, respectively. This suggests that the retention behavior was really predominantly affected by the number of carbons atom regardless their initial structure.

Table 2
Mathematical equations which describe chromatographic behavior as a function of the bulk/length of the derivatives and statistical data.

Analyte	Equation[a]	R^2	Adj. R^2	Sig. (*p*-value)[b]
Vigabatrin	$\ln y = 0.898 + 0.000170x^3$	0.9991	0.9986	0.00
Pregabalin	$\ln y = 0.925 + 0.000186x^3$	0.9999	0.9999	0.00
Gabapentin	$\ln y = 0.910 + 0.000184x^3$	0.9998	0.9997	0.00

[a] *x*—number of carbons, *y*—t_R.
[b] Significance level=0.05.

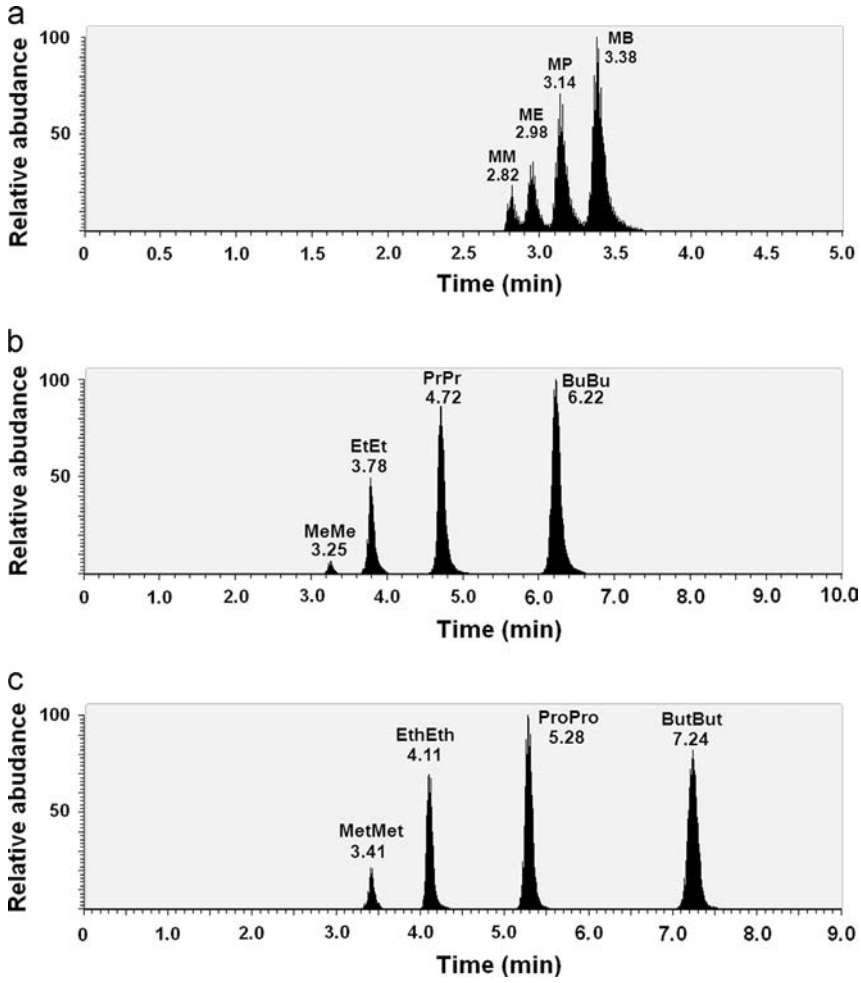

Fig. 2. Total Ion Chromatograms (TIC) obtained from mixtures prepared using equal volumes of various derivatives: (a) mixture of MM, ME, MP and MB derivatives of vigabatrin, (b) mixture of MeMe, EtEt, PrPr and BuBu derivatives of pregabalin and (c) mixture of MetMet, EthEth, ProPro and ButBut derivatives of gabapentin.

3.2. MS/MS signal of the derivatives

Another significant change, due to derivatization via *n*-alkyl chloroformate/*n*-alcohol combinations, was related to signal strength, which was examined by estimating peak area values. The chromatographic peaks were obtained by using two SRM transitions for each derivative, however, these diversities in areas, along with differences in retention times, could be better illustrated in Total Ion Chromatograms (TIC). Therefore, regarding vigabatrin, a TIC originating from a mixture of equal volumes of MM, ME, MP and MB derivatives, clearly indicated that signal intensity was increased with the presence of longer *n*-alkyl chain coming from alcohol (Fig. 2a). Signal increase due to variation of the chain length of *n*-alkyl groups from both chloroformate and alcohol is also depicted in TIC of Fig. 2b (MeMe, EtEt, PrPr and BuBu pregabalin derivatives) and 2c (MetMet, EthEth, ProPro and ButBut gabapentin derivatives). Regarding Fig. 2c, based just on signal intensity, one can assume that peak of ProPro derivative has higher area value than peak for ButBut derivative. However, lengthening of alkyl chain increased not only the signal intensity, but also the lipophilicity of derivatives, causing their longer retention on C8 column and change in the peak shape. The peaks were broadening which resulted in higher area values with smaller signal.

In order to summarize all the results in one figure, all area values were grouped per drug and presented in Fig. 3. Bars clearly demonstrate differences among derivatives for the same chloroformate reagent, which were the result of alcohol change in the reaction mixture. Similar variations were also observed when the length of *n*-alkyl chain of chloroformate reagent was changed. Again, it was clear that lengthening of the *n*-alkyl chain influences the peak areas, with significant increase when longer *n*-alkyl groups were present. The same conclusion has also been drawn by other researchers in the case of amino acids derivatization applying *N*-hydroxysuccinimide ester of *N*-alkylnicotinic acid (C_n-NA-NHS) [45] and was attributed to ESI mechanism. It is believed that lengthening the alkyl chain causes derivatives to have more surfactant-like properties, which increases their concentration at droplet surface, where ionization in ESI is thought to occur. As a result of using various derivatization sets, 16 products per drug were obtained, with remarkable differences in their areas for the same chromatographic system. Direct comparison of the area values between the "shortest" derivative per drug (MM, MeMe, MetMet) and the "longest" one (BB, BuBu, ButBut) for each drug gives a measure of this increase. Therefore, for vigabatrin, the greatest increase was observed (2089%), while for pregabalin and gabapentin the increase was 508.7% and 1075%, respectively. The most evident effect of derivatization with increasing *n*-alkyl length on areas of vigabatrin derivatives can be attributed to its smallest initial size, expressed as number of carbons, among three drugs.

3.3. Effect of extraction solvent on MS/MS signal

In order to investigate whether these area increments are not strictly attributed to ESI mechanism but also to potential influence of organic solvent used for liquid-liquid extraction, three different

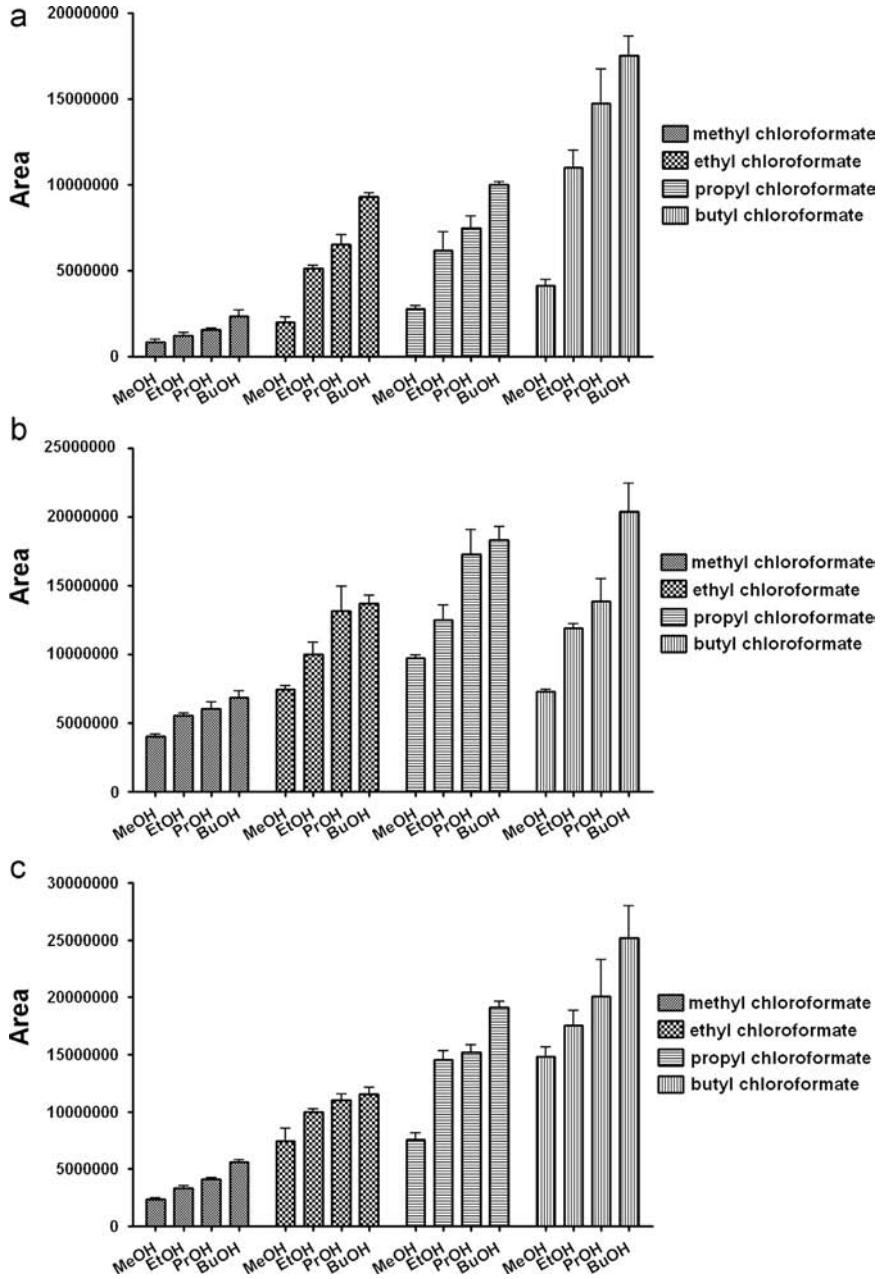

Fig. 3. Differences in areas for all derivatives ($n=3$) using various *n*-alkyl chloroformate/*n*-alcohol derivatization sets: (a) vigabatrin; (b) pregabalin and (c) gabapentin.

solvents (ethyl acetate, di-*iso*-propylether and *n*-hexane) were examined. All these solvents allowed easy sample handling, since they constitute the upper layer. The selection of three water immiscible solvents was based primarily on their physico-chemical properties. Judging from their polarity index values [46] (0.0 for *n*-hexane, 2.2 for di-*iso*-propylether and 4.4 for ethyl acetate) they represent solvents with big variations in polarity and therefore in the affinity with derivatized compounds. The obtained results, presented in Fig. 4 for vigabatrin derivatives, show that all the solvents had the same behavior for derivatives obtained from propyl chloroformate/propanol and butyl chloroformate/butanol combinations, which can be attributed to their increased lipophilicity. For ethyl chloroformate/ethanol combinations, ethyl acetate and di-*iso*-propylether exhibited similar behavior, while less polar *n*-hexane showed decreased extraction efficiency. For methyl chloroformate/methanol derivatives, the less polar solvents (di-*iso*-propylether and *n*-hexane) could not allow sufficient transfer of the derivatives into the organic layer. On the other hand, ethyl acetate, as a more polar solvent, led to approximately 140% signal increase for MM. Based on the aforementioned observations, the following conclusions were reached: (i) ranking order of signal was not affected by solvent selection, (ii) the derivatives bearing lower alkyls require relatively polar (with water immiscible) organic solvent for effective extraction transfer and (iii) from examined extraction solvents, ethyl acetate seems to be the most suitable one for all the derivatives of the selected anti-epileptic zwitterionic molecules.

3.4. Limits of detection and correlation with areas

Limit of detection (LOD) is a characteristic parameter of a specific method, representing a measure of its sensitivity. Estimation of LOD is an integral part of method validation that enables setting of the concentration range of a method intended for a specific application. The latter is of great importance in bioanalysis, since low concentrations of drugs and their metabolites are

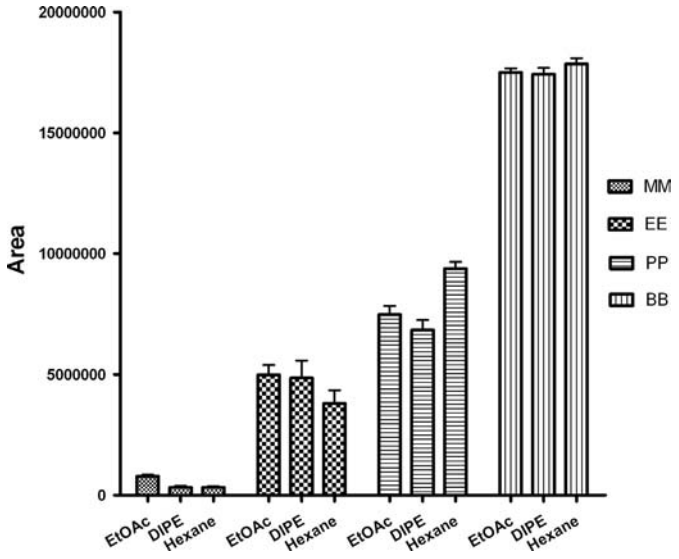

Fig. 4. Effect of extraction solvents (EtOAc: ethyl acetate; DIPE: di-*iso*-propylether; Hexane: *n*-hexane) on ESI signal ($n=3$) of vigabatrin derivatives (MM, EE, PP and BB).

met in complex media. In order to reach the required method sensitivity, derivatization constitutes a tool which can provide improved LOD values for the analytes of interest.

As mentioned above, derivatization with *n*-alkyl chloroformate/*n*-alcohol set led to significant signal increase for the three antiepileptic drugs and therefore, similar improvement for LOD values can be expected. These values for all the derivatives are presented in Table 1 and were obtained from reconstituted samples as described in Section 2.1, diluted with acetonitrile, until a signal-to-noise ratio of 3.3 was reached. It is important to emphasize that absolute LOD values are not of great significance in the current study, since they are highly affected by parameters, such as volumes of reconstitution solution and injection, as well as lower sensitivity of method in the case of real sample analysis. On the contrary, relative differences in LOD values are more important and undoubtedly can serve for general conclusion, when comparison among created derivatives is made. Results presented in Table 1 were in agreement with the expectations and, indeed, LOD values were constantly improving, as lipophilicity of the derivatives increased. For all the derivatives of vigabatrin, pregabalin and gabapentin it was noticed that LOD values decrease was reversely proportional to area increment up to a point when signal increase from *n*-alkyl chain lengthening was prevented by the change in peak shape. In other words, when longer *n*-alkyl groups were attached to one of the analyzed drugs via derivatization reaction, this was translated mainly to peak broadening and not to increase of intensity, which is directly correlated to LOD value. This peak broadening is the result of the increased retention on C8 column, due to lipophilicity increment.

Regarding vigabatrin, the observed area increase of 2089% from MM to BB derivative was translated to a LOD value improvement of 1553%. For pregabalin and gabapentin, the estimated area increase of 508.7% from MeMe to BuBu derivative and 1075% from MetMet to ButBut derivative, respectively, were translated to LOD values improvement of 397.7% and 875.0%, respectively. The common conclusion in all cases was that the initial rapid improvement in LOD values reached, after a specific point, a plateau, where values were slightly improving or remaining practically stable. The most evident effect of derivatization with increasing *n*-alkyl length on LOD values of vigabatrin derivatives, again, can be attributed to its smallest initial size, which is directly correlated to lipophilicity of its derivatives and their subsequent behavior on C8 column.

3.5. Ion suppression study

Qualitative and quantitative determination of possible matrix effect phenomenon gives important information concerning the elution time of matrix elements and constitutes an integral part of bioanalytical method validation protocols. Although the objective of the current study was to obtain general conclusions about selected zwitterionic structures derivatized via chloroformate mediated reaction, matrix effect profiles from blank plasma samples were obtained. To this purpose, the current protocol was applied to 10 μL blank plasma samples and the reconstituted solution was subjected to ion suppression study, in order to assure that interferences causing ion suppression were not coeluted with analytes. In this way, the effects of plasma elements, along with possible interferences of derivatization reagents, (i.e. pyridine) [20], were estimated for the whole chromatographic run, for all SRM transitions, by applying the post-column infusion protocol [47,48]. Blank plasma, treated as it was described in sample preparation section, was injected in the LC–ESI-MS/MS system by the simultaneous post-column infusion of a mixture of the derivatized vigabatrin, pregabalin and gabapentin (each 1000 ng mL^{-1}) in acetonitrile via the Hamilton syringe pump. Syringe pump was connected to the ionization source via T-connection. Flow rate was set at 50 μL min^{-1} for syringe infusion while the mobile phase flow rate was 600 μL min^{-1}. Indicative chromatograms (Fig. 5), presented for derivatized structures using propyl chloroformate/propanol derivatization set, clearly demonstrate that there was no matrix effect at the elution times of the specific analytes. The same situation also occurred in case of other derivatives.

3.6. Estimation of side reaction yield

Fig. 1 illustrates that the main direction of the derivatization reaction with chloroformate reagent, in the presence of alcohol, is the substitution of alkyl group originated from chloroformate with the alkyl moiety of the alcohol. However, this replacement does not occur completely, therefore, the side reaction product is always present to the certain extent. Formation of this product was systematically monitored in all the analyses, and its yield was calculated by comparing the respective area in every reaction (i.e. MM in ME, MP and MB) with the average area that corresponds to the product formed when the combination of chloroformate/alcohol with the same *n*-alkyl group was used (for example only MM exists). The obtained results are presented in Table 3. There is a general agreement with data from literature, since in most cases side reaction yield was less than 10% [40].

Table 3 presents obvious differences in side reaction yields, when various alcohols were utilized. The reason for this phenomenon remains unconfirmed; however, these diversities may be attributed to the size of the alkyl group of alcohol and its consecutive potential to replace the alkyl group of chloroformate. It is indicative that the lowest yield of side reaction was observed in all the cases which included methanol in combination with chloroformates of bulkier group. In other words, small methyl group was not able to provide substitution of longer ethyl, propyl or butyl groups. On the contrary, the yield of the side reaction product increased due to the presence of bulkier group, excluding involvement of ethanol. In the latter case, the yield of the side reaction product was always the highest. The reason for this increase is unclear and one can assume that the selected solvent for extraction (ethyl acetate) may favor transfer of these derivatives in the organic layer. Practically, participation of ethanol in reaction mixture is the main reason for exceeding this "limit" of 10% for side reaction yield, followed by a decrease of this value when a bulkier alcohol (propanol, butanol) is involved.

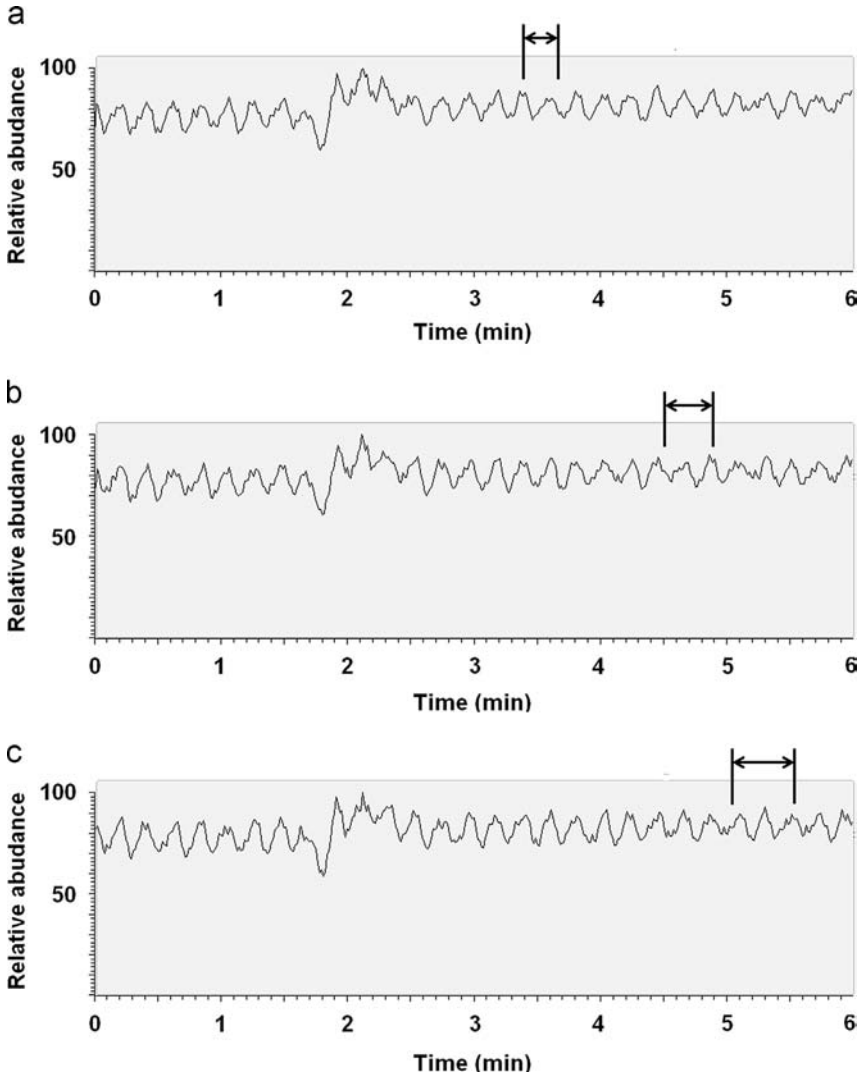

Fig. 5. Study of the matrix effect phenomenon by means of continuous infusion of mixture containing PP (a), PrPr (b) and ProPro (c) and parallel injection of extract of a blank plasma sample.

Table 3
The yields of side reaction products obtained for all *n*-alkyl chloroformate/*n*-alcohol combinations. Average values ($n=3$) are presented for each derivative.

Methyl chloroformate		Ethyl chloroformate		Propyl chloroformate		Butyl chloroformate	
Vigabatrin	% MM		% EE		% PP		% BB
MM		EM	0.80	PM	2.7	BM	4.7
ME	12	EE		PE	11	BE	13
MP	5.7	EP	2.4	PP		BP	5.5
MB	2.7	EB	2.4	PB	6.5	BB	
Pregabalin	% MeMe		% EtEt		% PrPr		% BuBu
MeMe		EtMe	1.6	PrMe	4.1	BuMe	7.1
MeEt	9.5	EtEt		PrEt	11	BuEt	15
MePr	6.9	EtPr	4.3	PrPr		BuPr	7.5
MeBu	8.1	EtBu	7.6	PrBu	8.3	BuBu	
Gabapentin	% MetMet		% EthEth		% ProPro		% ButBut
MetMet		EthMet	1.6	ProMet	3.4	ButMet	7.8
MetEth	9.2	EthEth		ProEth	14	ButEth	21
MetPro	5.1	EthPro	3.2	ProPro		ButPro	8.5
MetBut	7.7	EthBut	3.6	ProBut	8.6	ButBut	

3.7. Perspective

The current study, with three antiepileptic drugs as model analytes to undergo derivatization reaction with *n*-alkyl chloroformate/*n*-alcohol sets, highlights the convenient procedure of this reaction, along with the significant changes in LC–ESI-MS/MS behavior of various derivatives. Starting from this point, present study offers valuable data to the researchers that are planning to develop protocols for the quantitation of these drugs, especially in biological media. These compounds, due to their zwitterionic character, cannot be easily extracted into an organic solvent which is immiscible with water. Therefore, protein precipitation is the basic, if not the only, option for sample preparation, resulting in rather dirty samples with severe matrix effect phenomenon. In such cases, chromatography can be very problematic, while ESI signal is greatly affected by the presence of negatively charged carboxyl group. Derivatization with *n*-alkyl chloroformate/*n*-alcohol set seems to offer solutions to all these problems (liquid–liquid extraction, minimal (if any) matrix effect, utilization of RP columns and signal increase by removing negative charge and increasing chain length). It is possible to choose preferable set of reagents, based on their availability, peak shape, the desirable LOD value, the analytical run time or the need to solve problems, i.e. by increasing lipophilicity character, easier analytes separation from polar elements, that can cause matrix effect, can be achieved. However, results of the current study can be very useful starting point or supportive tool for other applications too, including the very promising field of metabolomics, when a valuable knowledge about a large number of metabolites is to be provided using single derivatization set.

4. Conclusion

The employment of various *n*-alkyl chloroformate/*n*-alcohol combinations for the derivatization of vigabatrin, pregabalin and gabapentin revealed the great importance of *n*-alkyl chain length in a series of parameters of the derivatives, related to their LC–ESI-MS/MS analysis. Even though the effect of chain length on the retention time was expected, the great influence on peak areas clearly demonstrated the differences in ESI efficiency of the derivatives. As a subsequent outcome, a significant improvement in LOD values was observed, enabling the further development of sensitive bioanalytical protocols for these or similar compounds. This great advantage overcomes the existence of an additional step during sample preparation, which constitutes the main drawback of a derivatization procedure. Since chloroformates have been utilized in more general approaches, such as metabolomics, the current study can make a contribution in this field, enabling selection of the most suitable conditions.

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