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Baclofen ester and carbamate prodrug candidates: A simultaneous chromatographic assay, resolution optimized with DryLab®

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ABSTRACT

Baclofen exhibits insufficient CNS-availability when dosed systemically. Hence, prodrug candidates (methyl, ethyl, 1-propyl, 2-propyl and butyl 4-(tert-butoxycarbonyl amino)-3-(4-chlorophenyl) butanoate) were synthesized aiming at CNS-levels appropriate for the treatment of spastic disorders. The characterization of some biopharmaceutically highly relevant physicochemical properties (Log P and aqueous solubility) and the evaluation of biophase levels represent one important component of the project. The overall research aim was to generate an HPLC optimized method using DryLab®, a simulation software for the optimization of a RP-HPLC method, which was optimized using a simulation software (DryLab®), for the simultaneous determination of baclofen and ten synthesized prodrug candidates. The chromatographic resolution predicted and obtained via the simulation is Rs >1.5 for all baclofen derivatives, as well as, with parent baclofen. The method was used to assay the prodrugs and determine their purities, solubility and lipophilicity parameters. The designed analytical method also permits the tracking of the new prodrug candidates' hydrolysis in vitro and in vivo. The determined physicochemical properties indicate for some of the compounds that they might be suitable for CNS-targeting which was exemplified by the detection of significant baclofen levels in rat brain tissues following an i.p. dose of ethyl carbamate (vs. ethyl ester, for which only traces of baclofen were detected).

1. Introduction

Baclofen (BAC), a $GABA_B$ agonist most useful in the symptomatic treatment of spasticity, was experienced to exhibit high therapeutic activity and value in certain – even severe – spastic disorders [1]. The effect appears to directly correlate with the concentrations present at the site of action [1]. Yet, poor availability in the CNS results even when concentrations in the blood are high. Therefore, therapeutic CNS levels are not even approached, unless the drug is directly dosed into the CSF by intrathecal injection [2].

Since, there is a relationship between substance's solubility in lipids and brain penetration [3], it was hypothesized that esters may be suitable, and moreover carbamates were synthesized assuming that they might be cleaved in vivo in the brain.

BAC (Fig. 1) as γ-amino butyric acid analogue has two ionizable groups: carboxylic and amino groups. Brain selectivity of BAC has been improved to some extent by masking the carboxylic moiety by linkage to short-chain alcohols to increase lipophilicity and, hence, BBB penetration [2]. As a necessary requirement of the prodrugs' approach is their capability to revert to the parent drug in the body via enzymatic or chemical mechanisms, quantitatively, reproducible, and at a reasonable rate [4]. Success with carbamate as a latentiating group requires that the prodrug can be hydrolyzed in the body to the parent amine and carbon dioxide [5] through several types of esterases [4]. This approach has been used before for increasing the bioavailability of centrally acting amines to brain succeeding with respect to hydrolysis within the brain tissue and characterized by a pharmacological action with delayed onset and greater duration [4].

Since the formation of carbamates includes a good leaving group, it appears to be an alternative option for more biolabile, yet also sufficiently stable [6] and absorbable [5] BAC derivatives.

Newly synthesized baclofen prodrugs (esters and carbamates) were the analytes, for which a specific, simple, and reliable HPLC method to be used for chemical as well as physicochemical characterization was developed. The development of one robust HPLC method for the simultaneous determination of BAC and the 10 synthesized prodrugs was challenging due to: complexity of the matrix (brain and plasma) where the samples would be assayed, the large number of analytes', cost and time factors required for method development, and the occurrence of by-products, decomposition products, and – possibly – metabolites.

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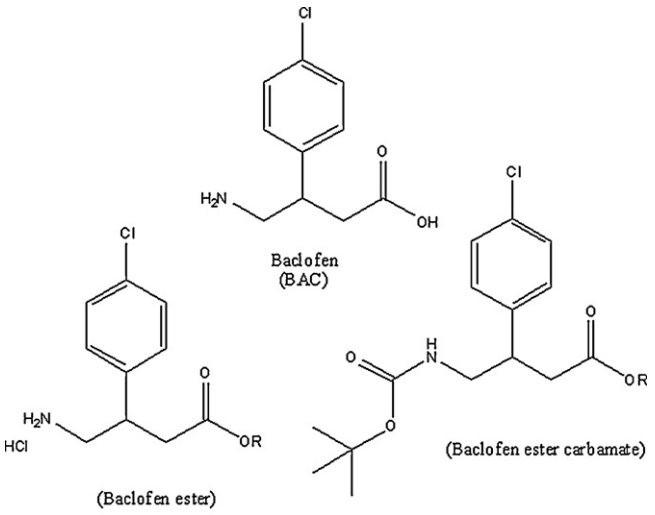

		Esters	Carbamates
R=	CH_3	ME	MC
	CH_2CH_3	EE	EC
	$CH_2CH_2CH_3$	1PE	1PC
	$CHCH_3CH_3$	2PE	2PC
	$CH_2CH_2CH_2CH_3$	BE	BC

Fig. 1. Chemical structures of synthesized baclofen esters and ester carbamates.

Computer-assisted HPLC method development enormously facilitates the optimization of chromatographic selectivity [7], where the *in silico* approach offers easy access to best resolution, least separation time, improvement of peak shape and most economic usage of the organic eluents [7]. It includes the generation of a "critical resolution map" which illustrates the change in resolution (Rs) with one of the critical chromatographic parameters to separation, e.g., percent organic solvent in the mobile phase (%B). Out of the currently available expert systems and simulation software approaches [8–13] DryLab® was selected, which predicts the effect of varying mobile phase composition, temperature, isocratic or gradient separation, and change in column conditions (column dimensions, particle size, flow rate) on the produced chromatogram *in silico*.

The assay development was done for a mixture of baclofen carbamate derivatives and esters along with baclofen and the method was optimized and validated in buffer media used in log P and solubility determinations, and prevalidated for brain tissue. It has proven suitability to be used further as a model for in vivo tracking of prodrugs hydrolysis and regeneration of the pharmacologically active BAC.

2. Experimental

2.1. Chemicals

Baclofen was kindly provided by Novartis Pharma (Cairo, Egypt), pivaloyl chloride by Tübingen University (Germany). All HPLC solvents (acetonitrile, methanol) were obtained from Sigma–Aldrich (Germany) and were used without further purification. All reagents (orthophosphoric acid, perchloric acid, (±)-phenyl alanine and potassium dihydrogen phosphate) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All organic solvents used were obtained from Al-Gomhoria Company (Cairo, Egypt) and were of pure analytical grade. Water used to determine solubility and log P was ultrapure produced by Purelab UHQ water (ELGA, UK).

2.2. Instruments and software

The HPLC equipment was Thermo Finnigan Spectrasystem® (UK) and consisted of: Spectrasystem P2000 pump, UV3000 detector, AS3000 autosampler, and Chromquest 4.2 data system software. A Hypersil C_{18} Gold (250 × 4.6 mm, 5 μm) column and guard column of the same type were used.

The modeling software was DryLab®2000 and PeakMatch®, Molnár Institute for Applied Chromatography (Germany).

2.3. Methods

2.3.1. Synthesis of baclofen esters

In order to protect the carboxylic group of baclofen and to increase its lipophilicity, esters (methyl, ethyl, 1-propyl, 2-propyl and 1-butyl) were synthesized as described previously (e.g., by Leisen et al. [2]) by reaction of baclofen with alcoholic hydrochloric acid for 24 h at ambient temperature. The esters were used as their respective hydrochloride salts.

2.3.2. Synthesis of baclofen ester carbamates

In order to further alter the lipophilicity based on the prepared esters, t-butyloxy carbamates were synthesized from different esters. The method of synthesis has previously been described in Latorre et al. [14] as a new one-pot procedure for the conversion of amines into amides via a carbamate intermediate. The primary amine was treated with an alkoxy carbonyl chloride in the presence of triethyl amine and the corresponding carbamate was obtained in good to high yield (85–95%).

2.3.3. Computer-assisted HPLC method development and optimization for the simultaneous determination of BAC in presence of all prodrug candidates

2.3.3.1. Method development strategy. Stock preparation: Stock solutions containing BAC, the internal standard phenylalanine (PHE) as well as esters and carbamates (E = ester, C = carbamate; M = methyl, E = ethyl, 1P = 1-propyl, 2P = 2-propyl, B = butyl) were prepared by dissolving 5 mg of each compound in 10 ml of 1% perchloric acid for BAC and PHE, ultrapure water for ME, EE, 1PE and 2PE or acetonitrile for MC, EC, 1PC and 2PC. These solutions were further diluted to obtain 50 μg/ml in water (working solutions). The same stock solution in acetonitrile was used throughout the whole study to obtain aqueous working solutions for assay optimization. Long-term instabilities had not been detected for any of the compounds.

A mixed solution containing all compounds (BAC, PHE, ME, EE, 1PE, 2PE, BE, MC, EC, 1PC, 2PC and BC) was prepared as 500 μg/ml of each compound altogether in acetonitrile. This mixed solution was injected to generate the 4 basic runs of the t_G–T study (explanation below).

Gradient time versus temperature study (t_G–T study): The study is planned to determine mainly the effect of gradient time (t_G), temperature (T) on separation of the mixture. Method optimization was designed to start with a gradient elution from the highest to the lowest aqueous composition [15]. Using a mobile phase of 2 components, methanol and 0.01 M phosphate buffer pH 2.6, four basic runs were carried out: 2 linear-gradient runs from 5 to 100% B (B = methanol) with 2 different gradient times: one steep gradient in 20 min and one flat gradient in 60 min, each at 2 different temperatures: a low temperature (40 °C) and a high temperature (70 °C) [16] to explore the effect of temperature on the elution behavior of the analytes. Repetitive injections of the sample mixture were performed for each set-up, until at least 2 consecutive reproducible chromatograms were obtained where neither peak areas nor retention times varied significantly (±10% and ±0.02 min, respectively).

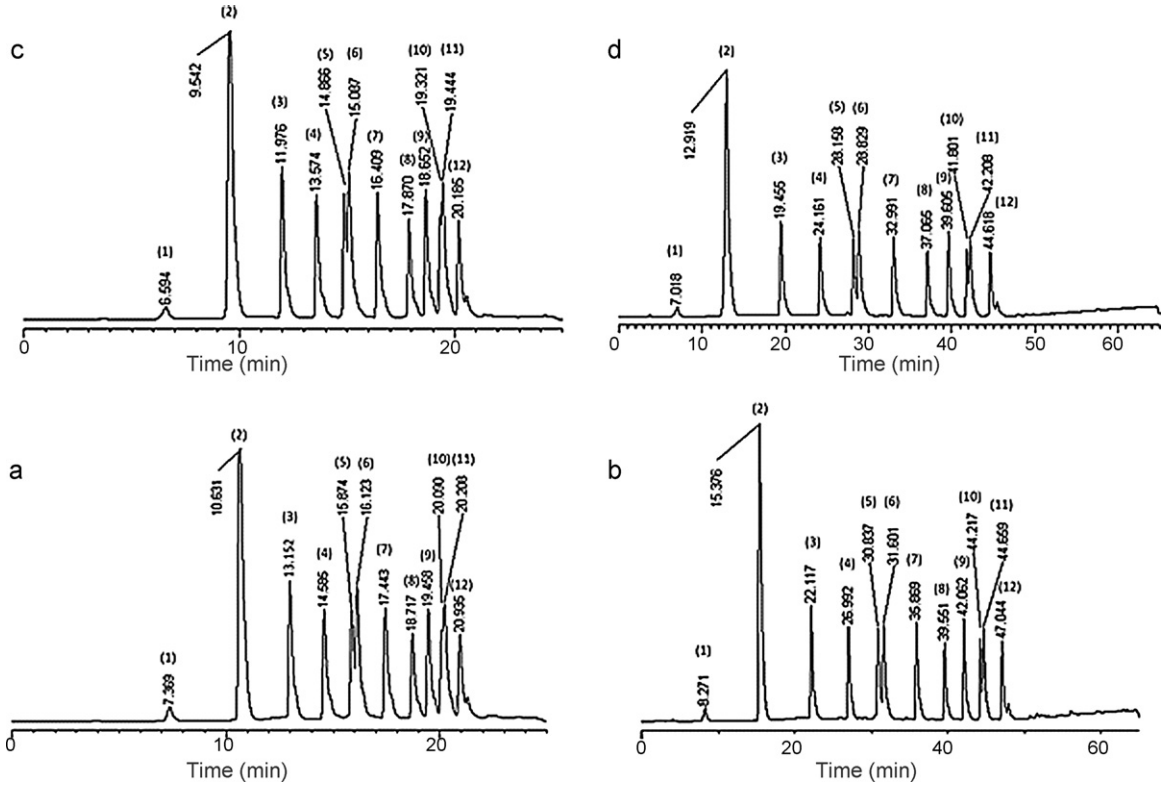

Fig. 2. Chromatograms of the 4 basic runs of the mixture showing their retention times and the peak tracking process: (a) 20 min gradient at 40 °C, (b) 60 min gradient at 40 °C, (c) 20 min gradient at 70 °C, (d) 60 min gradient at 70 °C.

Implementation of the basic runs and peak tracking: The obtained chromatograms were imported to PeakMatch® software in AIA format (*.cdf) for "peak tracking" which refers to the matching of peaks of the same compound between runs where conditions have changed, with the areas of the peaks serving as identifiers for peak tracking since peak areas do not significantly change, in general, as long as the same mixture solution is used. After exporting the chromatograms to PeakMatch® software, areas were compared. Very small peaks of small areas were considered impurities and were excluded. Tracking was completed and accuracy of the extent of match between the same peaks in different chromatograms was calculated by the software, and was expressed as % standard deviation.

The critical resolution map and the critical peak pair: To study the effect of changing the gradient time (t_G) and the column temperature (T) on the resolution of the critical peak pair, the 4 basic runs were exported to DryLab® software in AIA format, after peak tracking was accomplished for subsequent modeling. The "critical resolution map" was automatically computed by the software. The critical resolution in a chromatogram is, by definition, the lowest resolution value between a peak pair among all peaks. This pair is termed the "critical peak pair". DryLab® software then is able to compute the composition of the starting and the terminal %methanol to elute all the compounds within a suitable time.

Implementation of the predicted run and comparison between virtual and experimental results: The HPLC system was conditioned at the computed optimum conditions, temperature and flow rate, then the mixture was injected. The obtained chromatogram was compared to the *in silico* one to show the correlation between the predicted model and the experimental model.

pH study: The effect of small variations in pH on the separation of the mixture components was studied. Two pH values were chosen above and below the previously experimented pH in the t_G–T study, these were 2.0 and 3.2 (i.e. optimum pH 2.6 ± 0.6). This aims to account for the effect of experimental error during the preparation of the buffer aqueous phase. The HPLC system was conditioned for 2 h with each aqueous phase before the injection of the sample mixture and the produced chromatograms of the 3 runs were compared regarding retention time of the peaks, their critical resolution and identity of the critical peak pair.

2.3.3.2. Identification of the peaks under optimized conditions of t_G, T and pH. After chromatographic method optimization was accomplished regarding gradient time, temperature, and pH, the identity of the peaks was finally clarified and specified by injecting all single analytes.

2.3.3.3. Selectivity. Selectivity of the chosen method was demonstrated by comparison between the chromatograms of plain sample matrix (water, blank buffer solutions, blank plasma for future in vivo studies) without the analyzed substance with chromatograms of buffer and plasma containing the analyzed substances, to prove that the response is only due to the compounds of interest. The chromatograms of the blank matrices were compared to chromatograms containing the compounds individually and with the internal standard PHE as well.

2.3.3.4. Optimized method and method validation for aqueous/buffer samples. BAC, ME, EE, 1PE, 2PE, MC, EC, 1PC, and 2PC: Validation was performed with aqueous samples (solubility studies) at high, intermediate, and low concentrations (15, 10 and 1 μg/ml) for all compounds. Calibration standards were evaluated in the range from 1 to 30 μg/ml for each of the compounds.

The method finally applied involved a mobile phase composed of methanol and 0.01 M phosphate buffer pH 2.6 with a solvent gradient from 47% to 74% methanol over 20 min and temperature adjusted to 40 °C. The injection volume was 25 μl, the flow rate was 1 ml/min, and the detection of compounds in eluate was

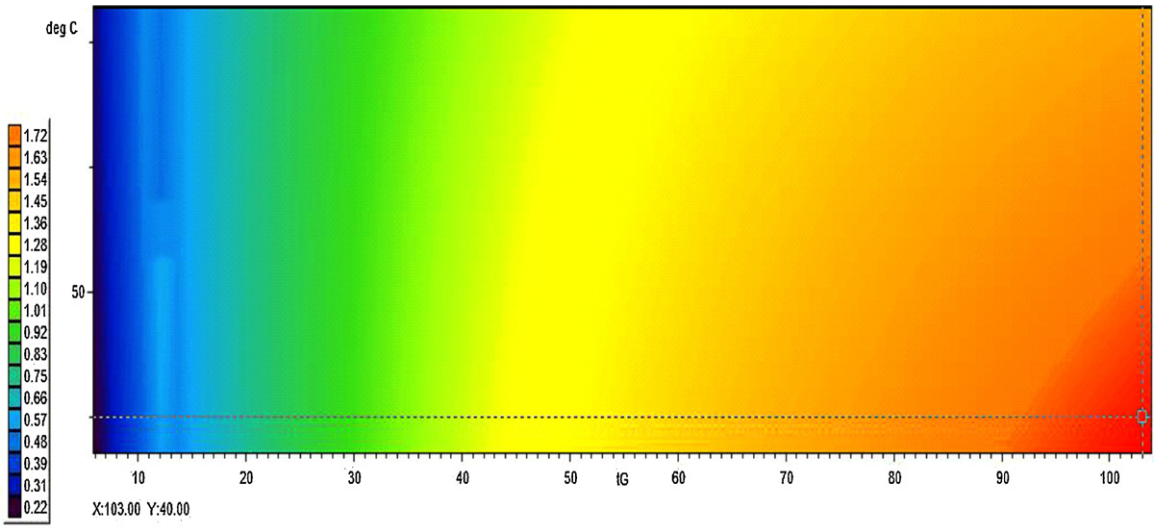

Fig. 3. Two-dimensional resolution map t_G versus T for the studied mixture showing different resolution values for the critical peak pair. Blue areas show the least resolution which increases to the maximum at the red areas. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

done at 220 nm. The concentration of the internal standard was 25 μg/ml and the sample workup was 950 μl sample plus 50 μl internal standard solution (0.5 μg/μl). The retention time for the internal standard (PHE) was 3.9 min, and the retention times for BAC, ME, EE, 1PE, 2PE, MC, EC, 1PC, and 2PC were 4.2, 5.5, 6.8, 8.5, 8.9, 13.8, 15.6, 17.2, and 17.5 min, respectively.

Coefficients of variation were determined by assaying 6 samples of analytes at 3 different concentration ranges.

2.3.3.5. Method for brain tissue samples – sample workup. The rat brain homogenate sample (500 μl aliquot) was vortexed with 50 μl of PHE working stock solution (internal standard) in 2 ml Eppendorf tube for 2 min. After addition of 500 μl of acetonitrile for protein precipitation and after vortexing, the sample was left for at least 20 min to complete precipitation of proteins, followed by 10 min centrifugation at 0 °C at 4000 rpm. The supernatant was filtered using 0.22 μm pore filter. The filtrate was then evaporated at 45 °C under vacuum in the concentrator. Residues were reconstituted in 100 μl acetonitrile plus 100 μl 1% perchloric acid just prior to injection.

2.3.3.6. Method application. Solubility determinations: The model employed for the determination of the water solubility was a saturation model using an amount of the solid sufficient to produce a saturated solution.

Saturated solutions in ultrapure water (pH 6.5) for BAC, BAC esters HCl salts and BAC ester carbamates were prepared. Following 3 days equilibration with occasional shaking on a horizontal shaker, centrifugation took place for 10 min at 4000 rpm. The supernatant was analyzed by HPLC as described below. This test was performed 5 times for each compound.

To validate the method, the study was first performed on BAC, for which solubility is given. Then, it was performed on esters and carbamates.

Log P determinations: As a classical physico-chemical parameter for the evaluation of the lipophilicity [17], the octanol/water distribution coefficients (o/w DC) were determined.

BAC, BAC esters (HCl salts) and BAC ester carbamates were dissolved in ultrapure water. Following partitioning between water and octanol (1:1 v/v) during 30 min on a horizontal shaker and subsequent phase separation by centrifugation (10 min and 4000 rpm) the concentration of different compounds in the aqueous phase was measured by HPLC. Also here, the determination was performed 5 times for each compound.

In vivo studies: Preliminary rat studies on brain distribution of BAC prodrugs were performed and were approved by the University Protection for Animal Care and Use Committee at GUC. The protocol was compliant with the "Principles of Laboratory Animal Care" [NIH Publication # 85-23, revised 1985]. White Wistar rats (n = 15, average body weight, 200 g; fasted overnight; study at 8.00 am) received 1.6 mg/kg BAC-ethyl-ester carbamate (EC) intraperitoneally (dissolved in saline containing 2% (v/v) Tween 80).

After 1, 3, and 5 h of dosage, rats were sacrificed by cervical dislocation. Brains were rapidly excised, weighed and washed with ice-cold tyrode. Each brain was homogenized in 2 ml tyrode containing 16 mM phenyl methyl sulfonyl fluoride (as esterase inhibitor, to prevent post-sampling and pre-analytical hydrolysis) with immediate and consistent cooling. Samples were stored frozen at −80 °C till analysis. Tissue homogenate 500 μl aliquots were analyzed using the developed HPLC assay. Tissue concentrations were calculated in nanograms BAC, EE and EC per gram of brain tissue.

3. Results

3.1. Computer-assisted HPLC method development and optimization for simultaneous determination of BAC in presence of all prodrug candidates

3.1.1. Implementation of the basic runs, peak tracking and design of the resolution map

The 4 basic chromatograms generated at the 2 gradient times t_G = 20 min and 60 min, carried out at the 2 temperatures T = 40 °C and 70 °C are depicted in Fig. 2. The 4 runs showed that long flat gradients at high temperature had generally a positive effect on resolution of critical peak pairs (first pair 5 and 6, second pair 10 and 11).

Tracking was completed and accuracy of the extent of match between the same peaks in different chromatograms was calculated by the software, and was expressed as % standard deviation which did not exceed 10%. Peak tracking showed and confirmed that the elution order was constant for all peaks at all experimental conditions.

In fact, the resolution map stated that optimum separation conditions are: a linear gradient (5–100% methanol) in 100 min at 40 °C and a flow rate of 1 ml/min (Fig. 3). Yet, the elution time of the last

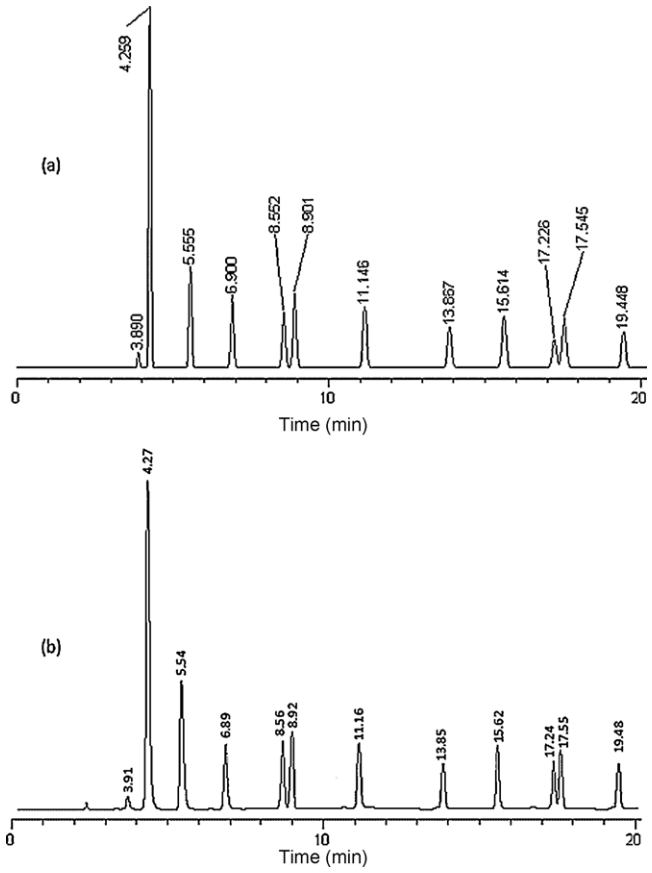

Fig. 4. Chromatograms at optimum conditions, namely: linear gradient 47–74% methanol in 20 min, phosphate buffer pH 2.6 as aqueous phase, flow rate 1 ml/min, and temperature 40 °C: (a) virtual chromatogram at optimized conditions, (b) experimental chromatogram at optimized conditions.

peak is estimated to be 71 min where the critical peak pair was (10) and (11) in the predicted chromatogram under preliminary computed optimum conditions. The long run time was diminished by changing the working conditions from a linear gradient at 5–100% methanol to 5–47%.

3.1.2. Implementation of the predicted run and comparison between virtual and experimental results

The obtained chromatogram significantly matched the *in silico* one upon practical implementation of the computed optimal conditions, the real retention times were found to be in the range of the virtual retention times ±0.1 min, which proved good correlation between the predicted model and the experimental model (Fig. 4).

3.1.3. Determination of the effect of pH under optimized conditions

The retention behavior and selectivity at pH 2.0 and 3.2 were compared with the run at pH 2.6, and it was found that variation in pH in the range of 2.6 ± 0.6 had no effect, neither on the retention times, nor on the resolution of the critical peak pair, nor on the selectivity of the method (Fig. 5).

3.1.4. Identification of the peaks under optimized conditions of t_G, T, and pH

After injecting all single analytes separately, the generated chromatograms clearly revealed the identity of the 12 peaks as being PHE, BAC, ME, EE, 1PE, 2PE, BE, MC, EC, 1PC, 2PC, and BC, respectively, as given in Table 1.

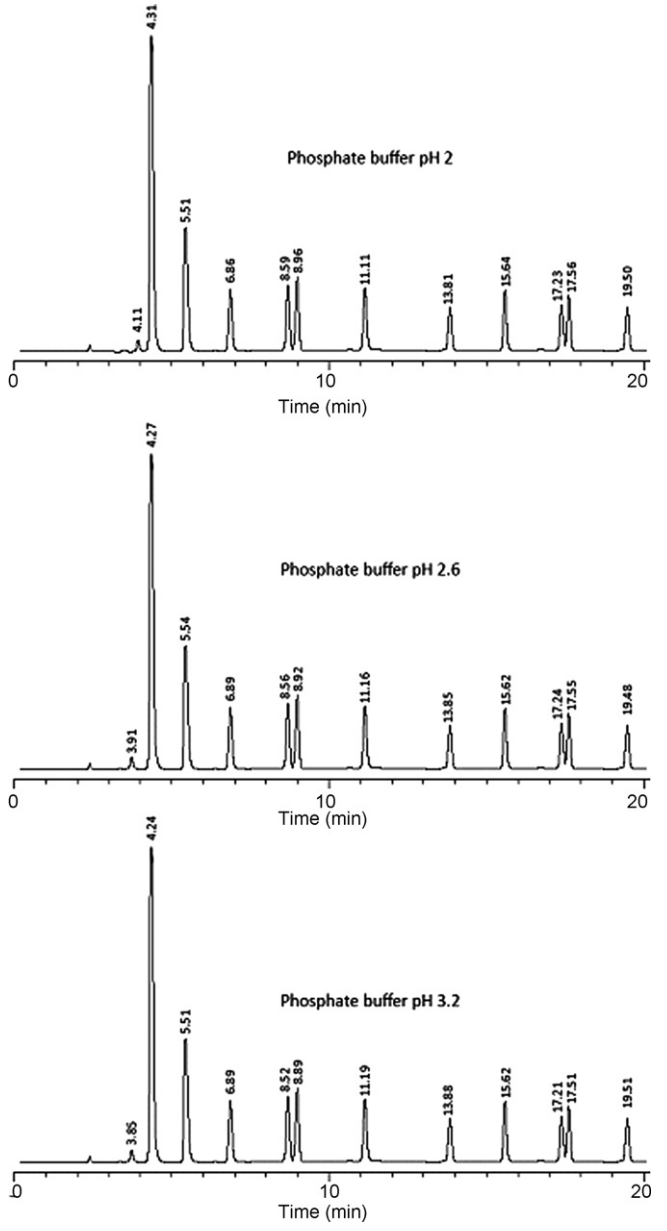

Fig. 5. Effect of small pH variations on the retention times, resolution, and selectivity of the critical peak pair. The same chromatographic conditions were kept constant for the 3 runs except for the pH of phosphate buffer.

Table 1
Identity of the compounds in the mixture as concluded from their individual runs under optimized conditions.

Peak no. in the experimental model	Identity of the peak	t_R of the compound in the mixture (min)
Peak 1	PHE	3.91
Peak 2	BAC	4.27
Peak 3	ME	5.54
Peak 4	EE	6.89
Peak 5	1PE	8.56
Peak 6	2PE	8.92
Peak 7	BE	11.16
Peak 8	MC	13.85
Peak 9	EC	15.62
Peak 10	1PC	17.24
Peak 11	2PC	17.55
Peak 12	BC	19.48

Table 2
Linearity: linear regression of 3 representative calibration curves (1–30 μg/ml), as arithmetic mean ± sdv.

Conc. range (μg/ml)	Slope	Intercept	r^2
BAC	0.1615 ± 0.0166	0.0645 ± 0.0844	0.9997 ± 0.0003
ME	0.1462 ± 0.0018	0.3291 ± 0.3782	1.0000 ± 0.0001
EE	0.1362 ± 0.0090	−0.0436 ± 0.0958	0.9998 ± 0.0001
1PE	0.1459 ± 0.0069	0.2574 ± 0.4031	0.9977 ± 0.0001
2PE	0.1491 ± 0.0140	−1.2720 ± 1.9445	0.9975 ± 0.0012
MC	0.1531 ± 0.0175	−2.3097 ± 2.2072	0.9982 ± 0.0025
EC	0.1315 ± 0.0035	−1.9215 ± 0.6848	0.9970 ± 0.0009
1PC	0.1053 ± 0.0002	−0.6689 ± 0.0261	0.9995 ± 0.0001
2PC	0.1154 ± 0.0064	−2.0608 ± 1.2019	0.9981 ± 0.0027

3.1.5. Selectivity

Neither blank buffer samples nor plasma samples gave any response at the retention times of the studied drug/prodrug candidates. The comparison also showed that the clear separation between the peaks of different compounds and the internal standard (t_R = 3.9 min) is maintained for different matrices.

3.1.6. Method validation

Assay validation for the analytes studied (BAC, ME, EE, 1PE, 2PE, MC, EC, 1PC, and 2PC) in aqueous samples yielded acceptable results and showed that the method is suitable for routine analyses and that is unproblematic for the matrices studied. The calibration standards up to 30 μg/ml were well in the linear range for each of the compounds. Slopes, intercepts and correlation coefficients (r^2) are summarized in Table 2.

LOQs (LODs) were found to be 0.05 (0.025) μg/ml for BAC, 0.07 (0.04) μg/ml for ME, 0.1 (0.05) μg/ml for EE, 0.1 (0.05) μg/ml for 1PE, 0.1 (0.05) μg/ml for 2PE, 0.1 (0.025) μg/ml for MC, 0.3 (0.1) μg/ml for EC, 0.2 (0.1) μg/ml for 1PC and 0.25 (0.1) μg/ml for 2PC.

Precision and accuracy characteristics for all analytes in repetitive assays (n = 6) were determined for the concentration range relevant in solubility studies. Data obtained for high, intermediate, and low concentrations (15, 10, 1 μg/ml) are given in Table 3. The CVs were usually in the range well below 10%.

For the preliminary bioanalytical method with EC in brain tissues, the overall coefficients of variation were determined for EC, EE, and BAC at 2 concentrations (0.2 μg/ml and 1.0 μg/ml for each of the compounds). The respective CVs were between 5 and 10%.

3.2. Aqueous solubilities and lipophilicities of baclofen and its prodrug candidates

BAC, BAC esters as HCl salts (ME, EE, 1PE and 2PE) and BAC ester carbamates (MC, EC, 1PC and 2PC) were tested for their solubility. The water solubilities of esters HCl salts were found to be higher than that of baclofen because they are completely ionized at pH = 6.5–7 (i.e. in ultrapure water used in this experiment). Moreover, it was observed that ester solubilities decreased with increasing the chain length of the ester, along with increasing lipophilicity and molecular weight. The experimental data are summarized in Table 4 (it should be mentioned that testing free esters (not HCl salts) would show different results [2]). The solubility of carbamates in water were much less than that of baclofen because they are completely unionizable, which renders their solubility unaffected by the pH of the dissolution medium. Similarly, carbamate solubilities were found to decrease with increasing chain length of the ester. The water solubilities of 2PE and 2PC were higher than those of 1PE and 1PC, respectively, presumably because of the lipophilicity-influencing effect of the branched hydrocarbon

Table 3
Summary of interday precision and accuracy data for the quantification of different compounds obtained from quality control in vitro samples during routine analysis. The respective values for intraday studies yielded data which were in the same range or better.

Analyte	Concentration (μg/ml)		Found (arith. mean) (μg/ml)	sdv	Precision (C.V. %)	Accuracy (% Bias)
BAC	High	15	15.5367	0.4784	3.0794	3.5778
	Intermediate	10	10.0150	0.1500	7.3126	0.7324
	Low	1	1.0321	0.0668	6.4769	3.2093
ME	High	15	15.1067	0.8427	5.5782	0.7111
	Intermediate	10	10.0900	0.8133	8.0606	0.9000
	Low	1	1.0300	0.0438	4.2542	3.0000
EE	High	15	15.3900	0.3182	2.0679	2.6000
	Intermediate	10	10.0250	0.2178	2.1724	0.2500
	Low	1	1.0433	0.0509	4.8747	4.3333
1PE	High	15	15.0200	0.2382	1.5856	0.1333
	Intermediate	10	10.1050	0.1684	1.6663	1.0500
	Low	1	1.0439	0.0943	9.0327	4.3855
2PE	High	15	15.0083	0.2347	1.5640	0.0556
	Intermediate	10	10.0533	0.1790	1.7801	0.5333
	Low	1	0.9933	0.0894	8.9968	−0.6667
MC	High	15	14.9833	0.1818	1.2136	−0.1111
	Intermediate	10	9.8917	0.1480	1.4960	−1.0833
	Low	1	0.9967	0.0742	7.4455	−0.3333
EC	High	15	15.0317	0.2629	1.7487	0.2111
	Intermediate	10	9.9417	0.2308	2.3213	−0.5833
	Low	1	1.0250	0.0718	7.0013	2.5000
1PC	High	15	15.0733	0.1229	0.8154	0.4889
	Intermediate	10	10.0300	0.1075	1.0720	0.3000
	Low	1	1.0200	0.0687	6.7355	2.0000
2PC	High	15	14.9033	0.2284	1.5323	−0.6444
	Intermediate	10	9.9150	0.3342	3.3704	−0.8500
	Low	1	1.0183	0.0845	8.2958	1.8333

Table 4
Measured solubilities and Log Ps of baclofen and prodrug candidates ($n = 5$).

Compound	Solubility	Log P
B	2.5 mg/ml ± 0.2	−0.56
ME	506.6 mg/ml ± 0.9	−1.49
EE	442.9 mg/ml ± 0.3	−1.06
1PE	93.2 mg/ml ± 0.6	−0.23
2PE	91.6 mg/ml ± 0.5	−0.01
MC	0.41 mg/ml ± 0.01	1.5
EC	0.22 mg/ml ± 0.02	2
1PC	0.13 mg/ml ± 0.005	3
2PC	0.09 mg/ml ± 0.0002	3.4

chain. Neither aqueous solubility for BC nor its log P were estimated as it is practically insoluble, and traces in water – even after saturation – were virtually undetectable.

Although log P (o/w) alone seems to have a limited performance in predicting brain/blood concentration ratios, in combination with other parameters it can still reasonably predict brain–blood partitioning [18]. Specifically, it was found that the optimal log P (o/w) for brain–blood partitioning is approximately 1.5–2.5 or 3 [18]. The hypnotic activity of a number of congeneric series of CNS depressants reached their maximum when log P (o/w) was around 2 [19].

So, out of the tested baclofen ester carbamates, EC (having log P (o/w) of 2 which lies in the optimum range), was chosen for preliminary in vivo studies.

3.3. Brain levels of EC, EE, and BAC in rats

In the preliminary in vivo study, initial information was obtained with respect to the kinetic behavior of the new compounds. As BAC ester carbamates contain two metabolically susceptible moieties (ester group protecting the acidic site and carbamate group protecting the basic site) that may be hydrolyzed in vivo, the net availability of BAC in the target organ was the crucial aspect.

As our HPLC method detects and assays BAC, BAC esters, and BAC carbamates simultaneously, the in vivo metabolic pathways of the prodrugs and the extent of BAC formation can be identified. In the preliminary study for ethyl carbamate (EC) it was proven that (I) the prodrug enters the CNS, (II) ethyl ester (EE) as potential metabolic intermediate is not detectable, and (III) upon prodrug administration BAC is formed and both baclofen and carbamate are detected simultaneously with the BAC-to-prodrug ratio increasing with time (Fig. 6).

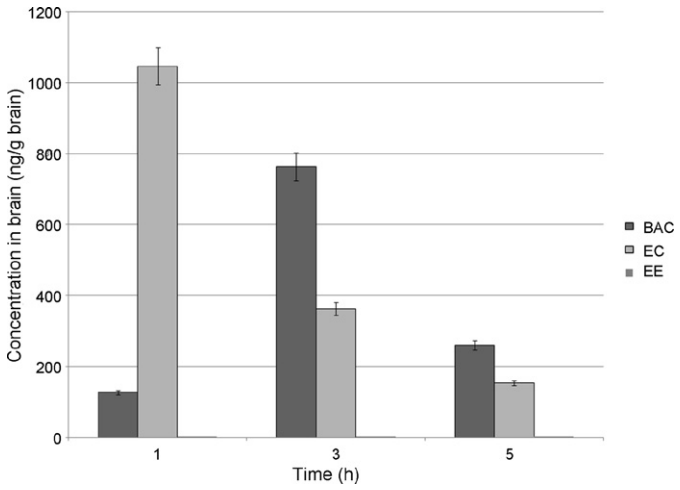

Fig. 6. Baclofen concentration in brain after i.p. injection of BAC, EC and EE ($n = 5$).

4. Conclusions

A newly developed HPLC model for the simultaneous determination of BAC and different prodrugs has been set up and optimized very efficiently. It was applied for the first time for the determination of physicochemical characteristics of new ester and carbamate prodrug candidates of baclofen.

The comprehensive HPLC assay may be used in studies, where analytes may be present simultaneously, or for studies with individual analytes to be assayed separately. Further concept application was performed in in vitro studies as well as animal studies, including plasma and tissue assays. The method(s) turned out to be very robust. Validation data demonstrated excellent reproducibility.

Overall, the current method serves as example for the significant advantages of optimization software in chromatography. This approach appears much more economical, ecologically favorable (solvent-saving), and time-saving than any traditional approach with optimization based on personal experience.

Initial applications of the assay included the evaluation of purity of the compounds as well as lipophilicity and solubility estimations. Chemical purity of the new prodrug candidates was always higher than 99%. None of the analytes was found to be highly soluble. When categorizing the compounds according to the biopharmaceutical classification system (BCS) [20], the initial estimate from the current data would (with no pH dependence studied yet) be "poor solubility" along with high lipid-partitioning.

The (o/w) partitioning of esters HCl salts was found to be related to their water solubility (Table 4). ME and EE HCl salts showed higher water solubility and lower partitioning into the organic layer than BAC. The (o/w) partitioning of carbamates showed very high lipophilicity and preferential partitioning to octanol which appears in accordance with their low water solubilities.

A relationship between substance's solubility in lipids and brain penetration is well-known. More recently it has become usual to relate the logarithm of brain permeation into brain with the logarithm of octanol–water partition coefficient log P (o/w) [3]. Although log P (o/w) alone is known somehow limited in predicting brain/blood concentration ratios, in combination with other parameters it may still reasonably predict brain–blood partitioning [18]. Specifically, it was found that the optimal log P (o/w) for brain–blood partitioning would be between 1.5 and 2.5 [18]. The hypnotic activity of a number of congeners of CNS depressants reached their maximum when log P (o/w) was near to 2 [19]. The log P cut-off for good partitioning into the brain should, hence, be at 2.5. Irrespective of the administration route, parent baclofen, which is characterized by a log P of −0.56, has a small brain-tissue-to-blood ratio, when dosed systemically. So, out of the tested baclofen esters and ester carbamates, MC and EC having log P (o/w) of 1.5 and 2, respectively (which lies in the optimum range), were chosen for further in vivo studies.

And it is hypothesized that *all* synthesized prodrug candidates have a more favorable tissue distribution behavior than parent baclofen. However, the determination of other physicochemical and biopharmaceutical parameters is the subject of additional studies. For these studies as well, the current method served a basis for more specific and problem-oriented (bio-)assay development, which also include metabolism and affinities to absorptive and efflux membrane transporters in different tissues.

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