Pure Appl. Chem., Vol. 79, No. 12, pp. 2339–2349, 2007.
doi:10.1351/pac200779122339

Screening of acetylcholinesterase inhibitors in snake venom by electrospray mass spectrometry*

André Liesener[1], Anna-Maria Perchuc[2], Reto Schöni[2], Nils Helge Schebb[3], Marianne Wilmer[2], and Uwe Karst[1,3,‡]

[1]University of Twente, Chemical Analysis Group and MESA+ Institute for Nanotechnology, P.O. Box 217, 7500 AE Enschede, The Netherlands; [2]Pentapharm Ltd., Engelgasse 109, 4002 Basel, Switzerland; [3]Institute of Inorganic and Analytical Chemistry, University of Münster, Corrensstrasse 30, 48149 Münster, Germany

Abstract: An electrospray ionization/mass spectrometry (ESI/MS)-based assay for the determination of acetylcholinesterase (AChE)-inhibiting activity in snake venom was developed. It allows the direct monitoring of the natural AChE substrate acetylcholine (AC) and the respective product choline. The assay scheme was employed in the screening for neurotoxic activity in fractions of the venom of *Bothrops moojeni*. AChE inhibition was assessed in two fractions. As a positive control, the established AChE inhibitor 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) was used, a dose–response curve for this compound was generated and the IC_{50} value for the inhibitor was determined to be $1.60 \pm 0.09 \times 10^{-9}$ mol L^{-1}. The dose–response curve was used as "calibration function" for the venom inhibition activity, resulting in BW284c51-equivalent concentrations of 1.76×10^{-9} mol L^{-1} and 1.07×10^{-9} mol L^{-1} for the two fractions containing activity. The ESI/MS-based assay scheme was validated using the established Ellman reaction. The data obtained using both methods were found to be in good agreement. The ESI/MS-based assay scheme is therefore an attractive alternative to the standard colorimetric assay.

Keywords: electrospray ionization/mass spectrometry; acetylcholinesterase; inhibitor screening; enzymatic bioassay; acetylcholine.

INTRODUCTION

The catalytic activity of cholinesterases in the degradation of acetylcholine (AC, Fig. 1a) to choline is of central importance in the treatment of Alzheimer's disease (AD). As a general symptom of this disease, reduced cholinergic neurotransmission is observed in the brain of AD patients [1,2]. This deficiency seems to be caused by either reduced activity of choline transferase or by enhanced activity of acetylcholinesterase (AChE), resulting in lower levels of AC [3–5]. Therefore, the predominantly used strategy in the treatment of AD patients is aimed at the potentiation of AC in the brain by lowering its degradation rate. This is achieved by administering potent inhibitors of AChE, such as tacrine, donezepil, galantamine, and rivastigmine. The most significant therapeutic effect is the stabilization or

*Invited contribution to a collection of papers for the IUPAC project 2005-042-1-300 "Chemistry for Biology". Other contributions to the project are published in this issue, pp. 2179–2366.
‡Corresponding author

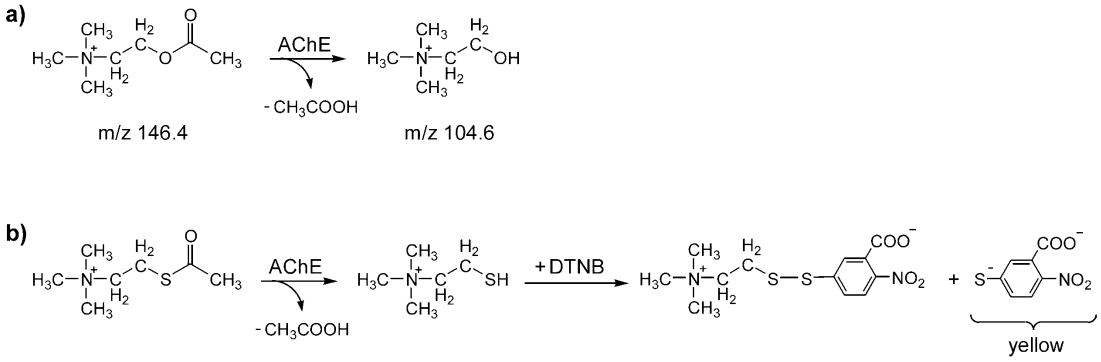

Fig. 1 Reaction schemes underlying the assays for the determination of AChE activity; (a) AChE-catalyzed conversion of AC to choline; detection by means of ESI/MS on the respective signal traces of *m*/*z* 146.4 (AC) and 104.6 (choline); (b) AChE-catalyzed conversion of acetylthiocholine to thiocholine; interception of the thiol by DTNB releases the colored anion, which can be colorimetrically detected.

even improvement of the cognitive functions of the patient for at least one year [6–10]. Since the use of AChE inhibitors is up to now the most effective therapeutic approach in AD treatment, the demand for alternative inhibitors is high.

Identification of inhibiting activity is performed by assessing the enzymatic activity in the presence of the potential inhibitor. The most widely used method for the determination of AChE activity is a colorimetric assay scheme developed by Ellman et al. in 1961 [11]. Since AC and its product choline do not possess any characteristic spectroscopic properties, an alternative substrate has to be used. To allow the detection by means of light absorption, a secondary reaction is coupled to the enzyme-catalyzed conversion. Acetylthiocholine (ATC) serves as substrate for AChE and is hydrolyzed yielding the respective thiol compound. This reactive thiol is intercepted by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) under formation of a disulfide bond with thiocholine and the yellow TNB^{2-} anion is released (Fig. 1b). The formation of this compound can be monitored by measuring the absorption of the reaction mixture at a wavelength of 412 nm [11].

Mass spectrometry (MS) is a possible alternative to this colorimetric assay scheme. As in MS, analytes are detected depending on their mass-to-charge (*m*/*z*) ratio, the spectroscopic properties of the substrate or product compounds are not important. Therefore, MS detection allows the use of natural occurring substrate compounds without any modifications to the molecular structure or the need of coupling a secondary reaction for detection. In the case of the determination of AChE activity, the naturally substrate AC and its product choline are detected at their respective *m*/*z* ratios.

The concept of employing electrospray ionization/MS (ESI/MS) as a means of detection in enzymatic bioassays was introduced by the group of Henion [12,13]. A first approach to identify potential inhibitors and determine their IC_{50} values by means of ESI/MS was presented by Wu et al. in 1997 [14]. Thomas et al. and Lewis et al. developed a MS assay scheme based on matrix-assisted laser desorption/ionization (MALDI) from porous silicon surfaces (DIOS) for the monitoring of the AChE-catalyzed hydrolysis of AC. Using the DIOS/MS assay scheme, they also investigated the inhibition of AChE by several known inhibitors [15,16]. Wall and coworkers employed both a DIOS/MS and an ESI/MS assay scheme for the determination of kinetic parameters of the AC hydrolysis mediated by AChE and studied also the inhibition of this reaction by tacrine. The values obtained from both methods were found to be in good agreement [17]. Shen and colleagues presented a high-throughput approach based on the DIOS/MS system for the screening of potential AChE inhibitors from a compound library reporting a throughput of 4000 samples in a 5-h measurement period [18]. An alternative high-throughput screening approach based on MS was presented by Ozbal et al. [19]. All of these investigations on potential AChE inhibitors by means of MS were only conducted with pure compounds and not

with natural extracts. Recently, Irth and coworkers described the high-performance liquid chromatography (HPLC)/MS-based online assay for screening of AChE inhibitors in a crude extract of Narcissus c.v. "Bridal Crown". After separation of potential inhibitors on a reversed-phase liquid chromatograpy (RPLC) system, first the enzyme AChE and subsequently, after a reaction time of 3 min, the substrate AC was added. After a reaction time of 1 min, substrate and product were detected by ESI/MS. Despite the complicated set-up with two post column reaction coils, active inhibitors were successfully detected in the plant extracts [20].

One of the most promising sources for potent AChE inhibitors are snake venoms. Besides the effects on the hemostatical system, blood coagulation and fibrinolysis, also strong neurotoxic effects, resulting in the immobilization and finally death of the prey, have been reported [21–23]. One possible mode of venom action is the selective inhibition of mammalian AChE by, for instance, fasciculins [24].

Recently, an ESI/MS-based method for proteolytic activity screening was developed in our laboratory [25,26]. As this method is robust and easy to use with any common ESI/MS instrumentation, we applied this concept to screening of snake venom fractions for the presence of AChE inhibitors. The assay scheme was employed in the inhibition screening of the venom of *Bothrops moojeni*. The results from the ESI/MS assay were validated with a colorimetric assay based on the Ellman scheme.

EXPERIMENTAL

Chemicals

The snake venom used in this study was provided by Pentapharm (Basel/CH) and was fractionated by size exclusion chromatography (SEC) as described by Perchuc et al. [27]. AChE (235 U/mg) from electric eel, AC bromide, acetylthiocholine iodide (ATC), 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) and DTNB were purchased in the highest purity available from Sigma-Aldrich Chemie (Zwijndrecht/NL). The p.a. grade ammonium acetate was obtained from Merck (Darmstadt/D). All solvents were purchased in LC/MS grade quality from Biosolve (Valkenswaard/NL).

Sample preparation

All solutions were prepared in buffer (15 mM ammonium acetate/ammonia, pH 7.5 in water) and stored at –18 °C until use. The AC bromide and ATC iodide were dissolved in buffer yielding substrate solutions of 1×10^{-4} mol L^{-1} each. The DTNB was dissolved in buffer to give a 1×10^{-3} mol L^{-1} solution. The inhibitor BW284c51 was dissolved to give a stock solution with a concentration of 1×10^{-4} mol L^{-1}, which was diluted to yield solutions of 3×10^{-5} mol L^{-1}, 1×10^{-5} mol L^{-1}, 3×10^{-6} mol L^{-1}, 1×10^{-6} mol L^{-1}, 3×10^{-7} mol L^{-1}, 1×10^{-7} mol L^{-1}, 3×10^{-8} mol L^{-1}, 1×10^{-8} mol L, and 1×10^{-9} mol L^{-1}, respectively. AChE was dissolved in buffer to yield a solution with a content of 5 mg L^{-1}, unless stated otherwise.

The venom of *B. moojeni* was fractionated by preparative gel permeation chromatography, yielding 19 fractions with venom components separated by their molecular masses in solutions. The gel fractions (F28–F46) were frozen and kept at –18 °C until use.

Instrumental set-up of the FIA/ESI/MS system

For the flow-injection analysis (FIA) experiments, a flow-injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn/D) was connected to the mass spectrometric detector. The carrier stream was set to 0.3 mL min^{-1} of 75/25 (volume per volume) mixture of methanol and water.

Mass spectrometric detection

For detection, an Esquire 3000$^+$ ion trap mass spectrometer (Bruker Daltonik, Bremen/D) equipped with an ESI source was used. All measurements were performed using the positive ion MS mode. Mass spectra were recorded over a range from *m*/*z* 100 to *m*/*z* 1000 in full scan mode. Ionization of the analytes was achieved in the ESI interface with 40 psi nebulizer gas, 10 L dry gas/min of 365 °C and –5000 V on the capillary inlet. The resulting data was analyzed using DataAnalysis software version 3.1 (Bruker Daltonik, Bremen/D).

ESI/MS assay procedure

All ESI/MS-based inhibition assays were performed according to the following general procedure. AChE (50 μL) and 50 μL of the respective venom fraction solution were combined with 300 μL buffer and pre-incubated at room temperature (RT) for 10 min. The reaction was started by adding 400 μL of the substrate AC solution to the mixture. Aliquots of 50 μL were sampled from the reaction mixture every 2 min for a complete time span of 20 min. The aliquots were quenched in 950 μL methanol to stop effectively the conversion by rapid denaturing of the catalytic enzyme. The quenched samples were subsequently injected into the FIA/ESI/MS system and analyzed in triplicate. The injection volume was set to 5 μL.

For the reference assays without inhibition, 50 μL of buffer were added to the enzyme instead of the venom fraction solution. In the case of the BW284c51 assays, 50 μL of the inhibitor solution were pre-incubated with the enzyme. Due to the dilution steps in the assay scheme, the effective concentration of BW284c51 ranged from 6.25×10^{-7} mol L^{-1} to 6.25×10^{-11} mol L^{-1}.

UV/vis assay procedure

In order to allow the direct comparison between ESI/MS assays and UV/vis assays, the reaction conditions and ratios of reagents had to be similar. Therefore, the colorimetric inhibition assays were carried out according to the following scheme. 300 μL of the AchE, 300 μL of venom fraction solution, and 160 μL of the DTNB solution were mixed, diluted with 1640 μL buffer, and pre-incubated at RT for 10 min in order to allow the DTNB to react with possibly present thiol functions in the protein content of this mixture. 100 μL of this mixture was transferred to the wells of a 96-well microtitation plate. The reaction was started by adding 100 μL of the substrate ATC solution to each well. The reaction progress was monitored by measuring the increase of absorption at 412 nm on a SpectraMax 250 plate reader (Molecular Devices, Munich/D).

For the reference assays without inhibition, 50 μL of buffer were added to the enzyme instead of the venom fraction solution. In the case of the BW284c51 assays, 50 μL of the inhibitor solution were pre-incubated with the enzyme. Due to the dilution steps in the assay scheme, the effective concentration of BW284c51 ranged from 6.25×10^{-6} mol L^{-1} to 6.25×10^{-11} mol L^{-1}.

RESULTS AND DISCUSSION

For the investigation of inhibitor activity toward AChE in the venom of *B. moojeni*, the venom fractions are assayed individually against AChE and the natural substrate AC. In the course of the reaction, AC is hydrolyzed by AChE to choline and acetic acid (Fig. 1a). This is monitored by ESI/MS. Typical mass spectra obtained from quenched samples at the beginning of the reaction and at the end are shown in Fig. 2.

After 2 min reaction time, signals for both substrate and product are observed. After a reaction time of 10 min, the signal for AC is not longer perceptible and the product signal increased significantly.

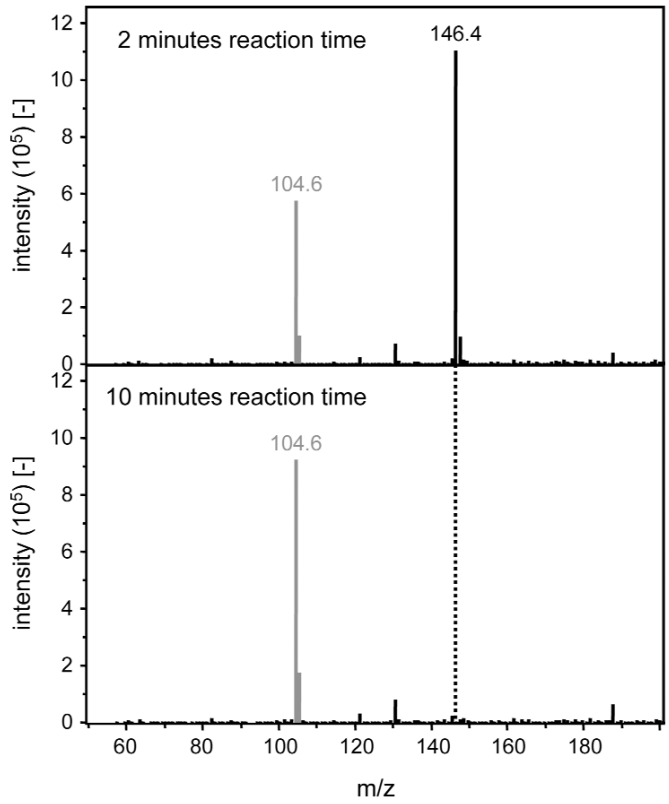

Fig. 2 Mass spectra obtained from samples of the reaction mixture after 2 min and after 10 min reaction time. The mass spectrum after 2 min reaction time shows signals for both, the substrate compound AC (*m*/*z* 146.4) and the product choline (*m*/*z* 104.6). After 10 min reaction time, only the signal for choline is observed, while the substrate signal has completely disappeared (position of AC signal indicated by the dashed line).

No additional signals appear in the mass spectra, not even at longer reaction times (monitored until 30 min). Therefore, it can be concluded that the conversion of the substrate is completed.

Subsequent injections of the quenched samples allow monitoring of the progress of the enzyme-catalyzed hydrolysis (Fig. 3). Figure 3 shows the signal traces for the substrate and the product. The consumption of the AC and the formation of choline is directly observed by the decrease and increase of the peaks on the respective *m*/*z* traces. Integration of the peak areas gives a measure of the concentration of the respective compounds present at a given time. Quantification of the analytes in MS is usually achieved by addition of an internal standard. The concept of using the substrate as internal standard for the relative quantification of the product compound was discussed by several authors for the use in MALDI/MS assay schemes [28–30]. Since absolute quantification of the concentrations of the analytes is not necessary to judge the assay performance, the relative reaction progress (RRP) at any sampling time was determined on the basis of this approach. The RRP is calculated from the ratio of product and substrate in the sample. The RRP in percent is expressed as the product peak area (S_P) as a fraction of product plus the remaining substrate peak areas (S_S).

$$\mathrm{RRP}(\%) = \frac{S_p}{S_s + S_p} \times 100 \tag{1}$$

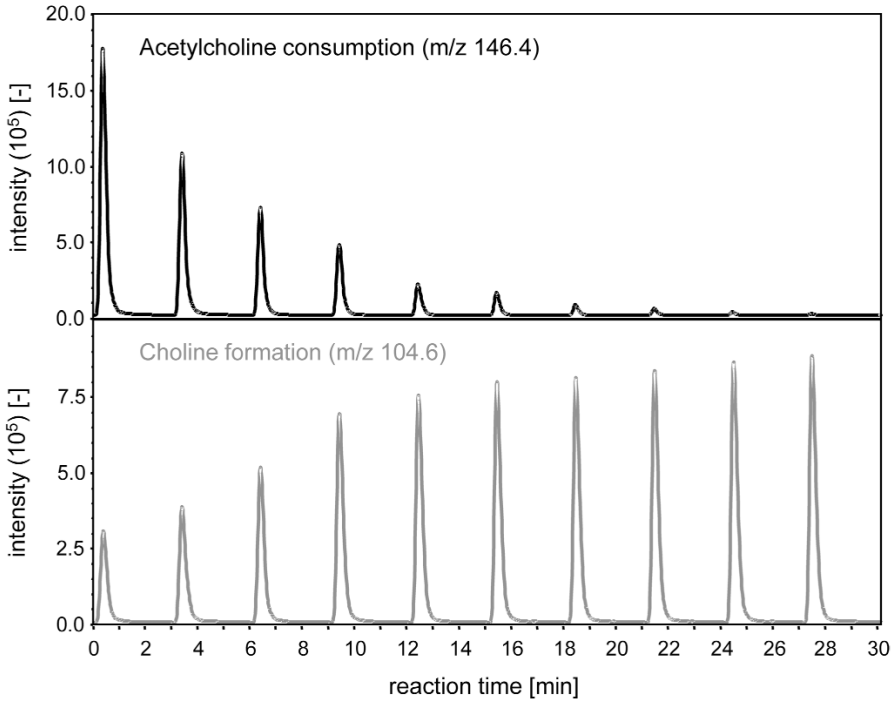

Fig. 3 Injection profile of enzymatic conversion by AChE (2 mg L^{-1}) recorded on the signal traces of substrate and product showing clearly the decrease in AC concentration and the increase of choline concentration in the reaction mixture over the course of time.

The value for the RRP ranges from 0 % (no product formed) to 100 % (complete conversion of the substrate). Plotting the RRP values vs. the reaction time for each assay results in a time-resolved reaction profile (Fig. 4).

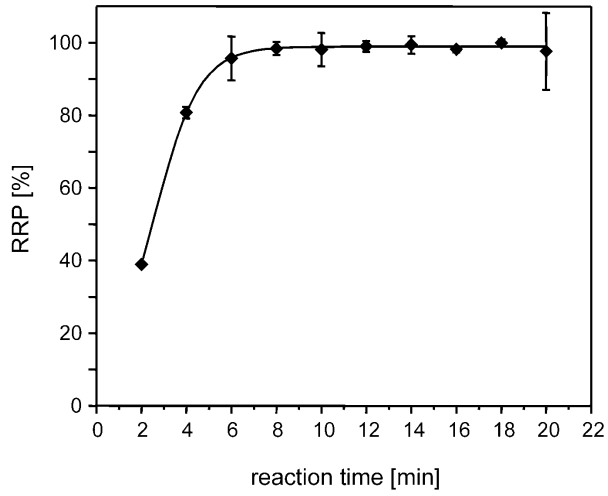

Fig. 4 Complete reaction profile showing the RRP at different time points; derived from the ESI/MS assay scheme of the reference assay without any inhibitor present. The relative standard deviation ranges from 1.3 to 10.9 % (n = 3).

The profile depicted was derived from the reference assay without any inhibitor present. The profile shows a typical course of an enzyme-catalyzed reaction with a high initial reaction rate. The conversion of the substrate seems to be completed after 8 min reaction time.

In order to assess inhibitor activity in the snake venom, the RRP profiles derived from the assays in the presence of each venom fraction were compared to the reference profile. This can be investigated closer using a correlation plot (Fig. 5a). In this figure, the RRP values of the reactions in presence of the venom fraction F33 and F34 are plotted vs. the RRP values of the reference assay. Close correlation (no change in catalytic activity of AChE) is indicated by points close to the dotted line. The correlation plots of the assays in the presence of F33 and F34 show significant divergences from the line, thus indicating a loss in enzymatic activity in the respective assays. This leads to the conclusion that in those fractions an inhibitor for AChE must be present. The correlation plots of the assays in the presence of the other venom fractions (shown as example for F29) did not exhibit any significant divergences. Therefore, no inhibitory activity is assigned to be present in those fractions.

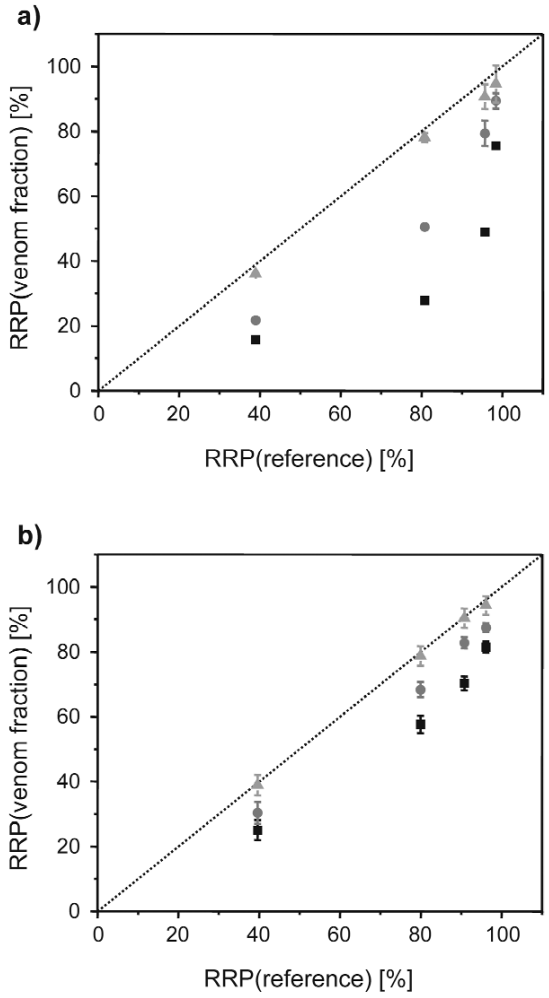

Fig. 5 Correlation plots derived from (a) the ESI/MS assays and (b) from the colorimetric assays. The RRP at the first 8 min reaction time in the presence of the venom fractions F34 (■), F33 (●), and F29 (▲) is plotted vs. the RRP of the reference assays. The dashed line indicates the position for complete correlation of reference and screening assays.

To verify this finding, control assays following a modified Ellman procedure were performed. The RRP in percent at a given time point in the absorption assay was calculated from the ratio of absorbance to maximal absorbance (after completion of the conversion). Plotting the RRP values of the individual assays vs. the values obtained from the reference assay results in a correlation plot similar to the one derived from the MS assays (Fig. 5b).

As assessed from the ESI/MS-based assays, a significant divergence from the reference assay is observed indicating the slower reaction progress in the presence of F33 and F34. Also, no significant divergences could be observed in the correlation of the other fraction assays. This confirms the conclusion drawn from the ESI/MS assays that only in fractions F33 and F34, an AChE-inhibiting compound is present.

In order to further characterize the inhibition potential, the inhibitory activity found in the fractions was related to that of the known inhibitor BW284C51. Therefore, dose–response profiles for the inhibition of AChE by BW284C51 were generated employing both, the ESI/MS- and the UV/vis-absorption-based assay schemes. Assays were performed in the presence of varying concentrations of inhibitor, and the respective RRP values were calculated as described above. The RRP values of the inhibition assays after 6 min reaction time were related to the RRP value found in the reference assay at the same time point (100 % assay performance) and plotted vs. the concentration of BW284C51 (Fig. 6).

From these plots, the IC_{50} values of BW284C51 toward AChE were determined to be $1.60 \pm 0.09 \times 10^{-9}$ mol L^{-1} (ESI/MS assay) and $2.48 \pm 0.24 \times 10^{-9}$ mol L^{-1} (UV/vis-absorbance assay), respectively.

The dose–response profiles from both assay schemes were used to relate the inhibitory activity in the venom fractions F33 and F34 to the inhibitory activity of BW284C51. Therefore, the assay performance of the respective reactions after 6 min reaction time was determined and the corresponding theoretical concentrations of BW284C51 were calculated (Table 1).

Table 1 Equivalent concentrations of BW284C51 calculated for the inhibition activities found in F33 and F34.

Venom fraction	Equivalent concentration of BW284C51	
	ESI/MS	UV/vis absorption
F 34	1.76×10^{-9} mol L^{-1}	1.42×10^{-9} mol L^{-1}
F 33	1.07×10^{-9} mol L^{-1}	0.89×10^{-9} mol L^{-1}

Considering the fact that two different assay schemes were employed, one based on the direct detection of product and substrate and the other employing a secondary reaction for the generation of a detectable product, the values calculated for the IC_{50} of BW284C51 and for the equivalent concentrations for the venom fractions correlate well.

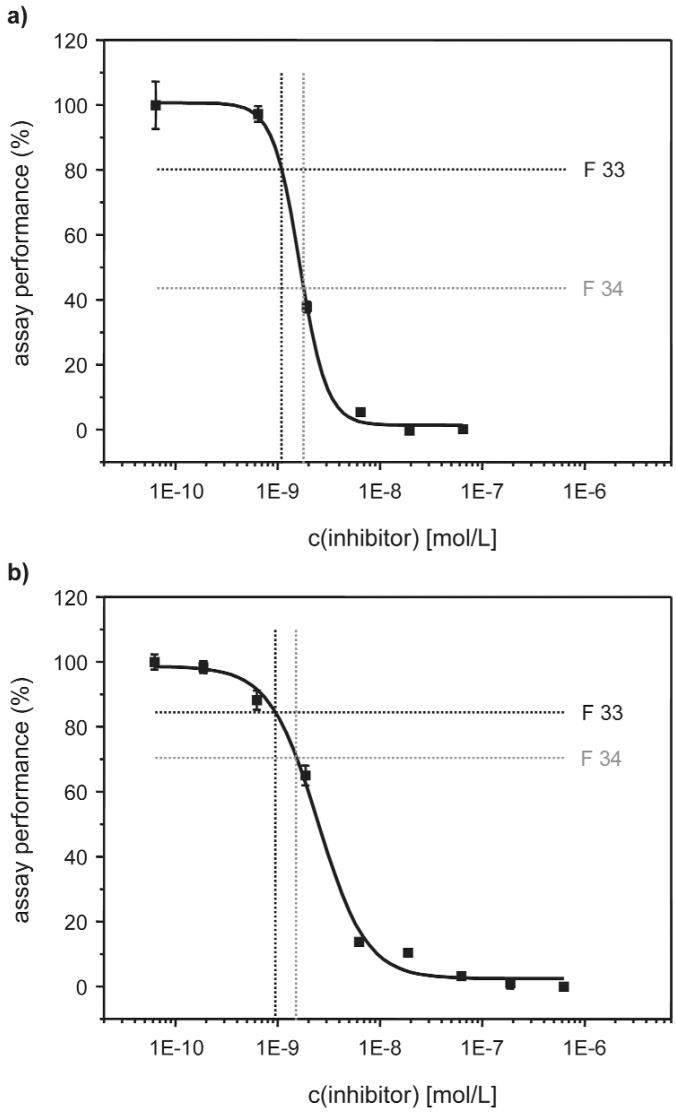

Fig. 6 Dose–response plots for the AChE inhibitor BW284C51 derived from (a) ESI/MS assays and (b) colorimetric assays; the respective assay performance in the presence of F33 and F34 are indicated by the horizontal dashed lines; the equivalent concentrations of BW284C51 are given by the intersections with the response curve.

CONCLUSION

A robust ESI/MS-based assay was developed for the rapid screening for AChE-inhibitory activities in complex biological samples. The assay was applied successfully to the inhibition screening of snake venom. The inhibitory activity found in the venom fractions was quantified in relation to the known AChE inhibitor BW284C51 using its dose–response plots as calibration curve, thus giving an independent measure for comparison of inhibition activity in different fractions. All findings resulting from the ESI/MS assay scheme were controlled by colorimetric assays, thus validating the ESI/MS methodology. The results were found to be in good agreement with each other. ESI/MS is therefore an excellent alternative detection method for the monitoring of enzyme-catalyzed assays. The major advantage

over the optical method is the possibility to monitor the naturally occurring substrate AC and its product choline, which are not detectable by any optical means. The ESI/MS assay scheme proved to be generally applicable not only to the investigation of pure substances, but also for highly complex biological samples. Since ESI/MS assays offer the opportunity to monitor several enzymatic conversions in parallel, further work will be directed to the development of a multiplexing inhibition assay scheme.

ACKNOWLEDGEMENT

We thank the Fonds der Chemischen Industrie (Frankfurt/Main), the Nederlandse Organisatie voor Wetenschappelijk Onderzoek NWO (Den Haag) and the Studienstiftung des Deutschen Volkes (Bonn) for financial support of the work.

REFERENCES

1. D. L. Price. *Annu. Rev. Neurosci.* **9**, 489 (1986).
2. P. Kasa, Z. Rakonczay, K. Gulya. *Prog. Neurobiol.* **52**, 511 (1997).
3. P. Davies, A. J. Maloney. *Lancet* **2**, 1403 (1976).
4. N. R. Sims, D. M. Bowen, S. J. Allen, C. C. T. Smith, D. Neary, D. J. Thomas, A. N. Davison. *J. Neurochem.* **40**, 503 (1983).
5. S. T. Dekosky, R. E. Harbaugh, F. A. Schmitt, R. A. Bakay, H. C. Chui, D. S. Knopman, T. M. Reeder, A. G. Shetter, H. J. Senter, W. R. Markesbery. *Ann. Neurol.* **32**, 625 (1992).
6. J. J. Sramek, V. Zarotsky, N. R. Cuttler. *Drug Develop. Res.* **56**, 347 (2002).
7. M. Farlow. *Int. Psychogeriatr.* **14**, 93 (2002).
8. B. Ibach, E. Haen. *Curr. Pharm. Des.* **10**, 231 (2004).
9. D. G. Wilkinson, P. T. Francis, E. Schwam, J. Payne-Parrish. *Drugs Aging* **21**, 453 (2004).
10. D. B. Hogan, B. Goldlist, G. Naglie, C. Patterson. *Lancet Neurology* **3**, 622 (2004).
11. G. L. Ellman, K. D. Courtney, V. Andres, R. M. Featherstone. *Biochem. Pharmacol.* **7**, 788 (1961).
12. E. D. Lee, W. Mück, J. D. Henion, T. R. Covey. *J. Am. Chem. Soc.* **111**, 4600 (1989).
13. F. Y. L. Hsieh, X. Tong, T. Wachs, B. Ganem, J. Henion. *Anal. Biochem.* **229**, 20 (1995).
14. J. Wu, S. Takayama, C.-H. Wong, G. Siuzdak. *Chem. Biol.* **4**, 653 (1997).
15. J. J. Thomas, Z. Shen, J. E. Crowell, M. G. Finn, G. Siuzdak. *Proc. Natl. Acad. Sci. USA* **98**, 4932 (2001).
16. W. G. Lewis, Z. Shen, M. G. Finn, G. Siuzdak. *Int. J. Mass Spectrom.* **226**, 107 (2003).
17. D. B. Wall, J. W. Finch, S. A. Cohen. *Rapid Commun. Mass Spectrom.* **18**, 1482 (2004).
18. Z. Shen, E. P. Go, A. Gamez, J. V. Apon, V. Fokin, M. Greig, M. Ventura, J. E. Crowell, O. Blixt, J. C. Paulson, R. C. Stevens, M. G. Finn, G. Siuzdak. *ChemBioChem* **5**, 921 (2004).
19. C. C. Ozbal, W. A. LaMarr, J. R. Linton, D. F. Green, A. Katz, T. B. Morrison, C. J. H. Brenan. *Assay Drug Dev. Technol.* **2**, 373 (2004).
20. C. F. de Jong, R. J. E. Derks, B. Bruyneel, W. Niessen, H. Irth. *J. Chromatogr., A* **1112**, 303 (2006).
21. T. Matsui, Y. Fujimura, K. Titani. *Biochim. Biophys. Acta* **1477**, 146 (2000).
22. F. S. Markland. *Toxicon* **36**, 1749 (1998).
23. K. F. Stocker. *Medical Use of Snake Ven*om, Chaps. 2 and 3, CRC Press, Boca Raton (1990).
24. C. Cervenansky, F. Dajas, A. L. Harvey, E. Karlsson. "Fasciculins, anticholinesterase toxins from mamba venoms: Biochemistry and pharmacology", in *Snake Toxins*, A. L. Harvey (Ed.), pp. 303–321, Pergamon Press, New York (1991).
25. A. Liesener, U. Karst. *Analyst* **130**, 850 (2005).
26. A. Liesener, A.-M. Perchuc, R. Schöni, M. Wilmer, U. Karst. *Rapid Commun. Mass Spectrom.* **19**, 2923 (2005).

27. A.-M. Perchuc, L. Menin, R. Stöcklin, B. Bühler, R. Schöni. *Pathophysiol. Haemost. Thromb*. **34**, 241 (2005).
28. A. G. Craig, C. A. Hoeger, C. L. Miller, T. Goedken, J. E. Rivier, W. H. Fischer. *Biol. Mass Spectrom.* **23**, 519 (1994).
29. R. P. Pauly, F. Rosche, M. Wermann, C. H. S. McIntosh, R. A. Pederson, H.-U. Demuth. *J. Biol. Chem.* **271**, 23222 (1996).
30. M.-J. Kang, A. Tholey, E. Heinzle. *Rapid Commun. Mass Spectrom.* **14**, 1972 (2000).