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Short communication

Using short columns to speed up LC–MS quantification in MS binding assays☆,☆☆

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ABSTRACT

The present study describes the use of short columns to speed up LC–MS quantification in MS binding assays. The concept of MS binding assays follows closely the principle of traditional radioligand binding but uses MS for the quantification of bound marker thus eliminating the need for a radiolabelled ligand. The general strategy of increasing the throughput of this type of binding assay by the use of short columns is exemplified for NO 711 binding addressing GAT1, the most prevalent GABA transporter in the CNS. Employing short RP-18 columns with the dimension of 20 mm × 2 mm and 10 mm × 2 mm at flow rates up to 1000 μL/min in an isocratic mode retention times of 8–9 s and chromatographic cycle times of 18 s could be achieved. Based on the internal standard [$^2H_{10}$]NO 711 fast chromatography methods were developed for four different columns that enabled quantification of NO 711 in a range from 50 pM up to 5 nM directly out of reconstituted matrix samples without further sample preparation. A validation of the established methods with respect to linearity, intra- and inter-batch accuracy and precision showed that the requirements according to the FDA guideline for bioanalytical methods are met. Furthermore the established short column methods were applied to the quantification of NO 711 in saturation experiments. The results obtained (i.e., K_d- and B_{max}-values) were almost identical as compared to those determined employing standard column dimension (55 mm × 2 mm).

1. Introduction

Techniques to characterize the affinity of test compounds towards a target are part of the fundamental screening tools in the drug discovery process [1,2]. As the sensitivity required to record binding interactions is exceptionally high, most of the currently available methods use labels such as radioisotopes or fluorophores [3]. During the last decade, however, an increasing number of approaches not demanding a label have been developed [4,5]. Among the latter are a variety of methods based on mass spectrometry, some of which have been successfully implemented in the drug screening process [2,6–8].

MS binding assays, recently introduced by us, to characterize binding to membrane bound targets belong to this category. They follow the concept of conventional radioligand binding and are however, designed to measure binding of a native (i.e., not labelled) marker by means of LC–ESI-MS/MS. In contrast to radioligand binding assays MS binding assays avoid all the drawbacks (e.g., legal requirements, generation of radioactive waste, etc.) associated with radioisotopes and represent a universal and at the same time easily applicable tool for the characterization of nearly any ligand's binding to a defined target provided that its affinity is high enough [2].

As for MS binding assays, LC–ESI-MS/MS analysis is performed directly out of the matrix resulting from the binding assays (without any sample preparation step). The time devoted to MS quantification of the marker is essentially the period required for chromatography. This can be kept quite short (i.e., 2–3 min) – even with standard HPLC equipment – providing considerable throughput capacity. As modern screening techniques demand highest efficiency there is, however, still a need to shorten the time period for MS quantification. The most straightforward conception solving this problem is to forgo chromatographic separation before MS quantification. Very recently we were able to demonstrate the feasibility of this analytical strategy employing a new MALDI–MS/MS system (FlashQuant) [9]. The measurement of a binding sample spotted onto a 96 well format MALDI plate took only 1.7 s, however, 14 s were required in total as the mean duration per sample. This discrepancy is a result of the fact that moving the individual spots under the laser beam takes distinctly longer than the measurement of the spot itself. Certainly, with an increased throughput to be expected, MALDI–MS/MS will be the method of choice for marker quantification in MS binding assays in the future. At present, however, LC–ESI-MS/MS, comprising a fast HPLC enabling total run times with less than 1 min, could also be a promising analytical

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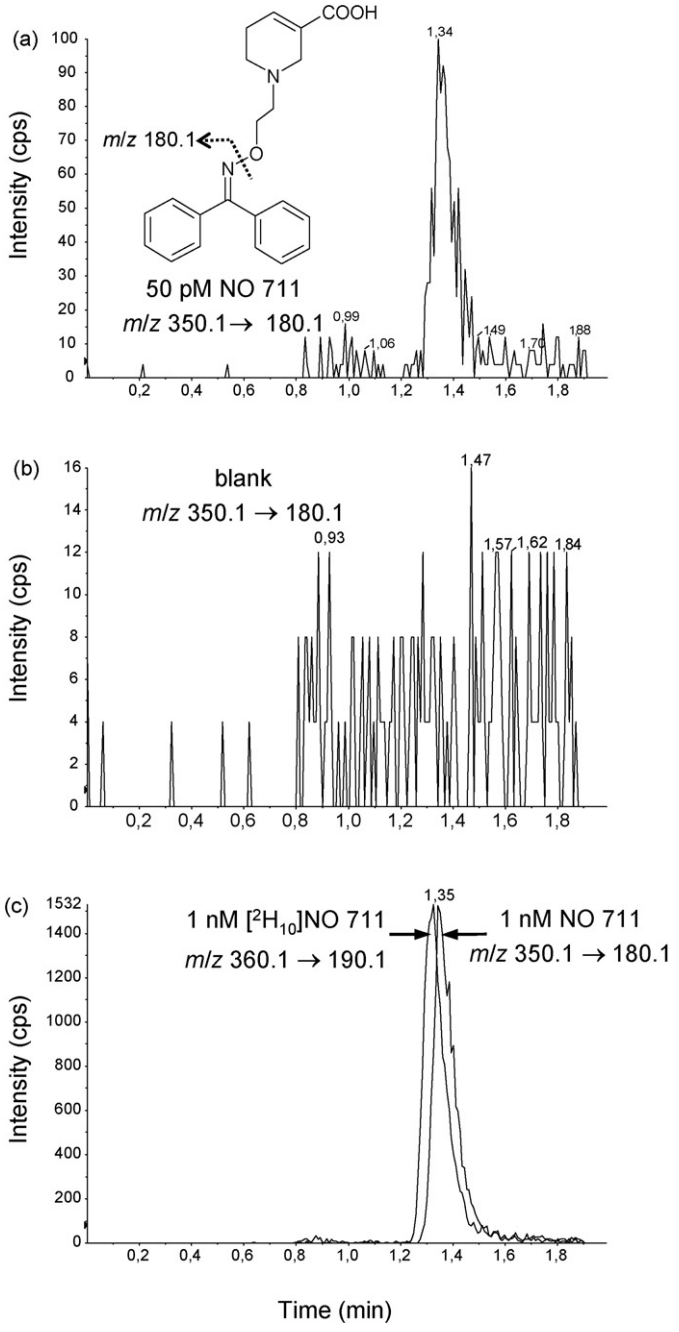

Fig. 1. SRM LC–ESI-MS/MS chromatograms of NO 711 and [$^2H_{10}$]NO 711 in matrix samples obtained with an API 3200 employing a Purospher STAR RP18 column (55 mm × 2 mm, isocratic flow 350 μL/min, mobile phase: 10 mM ammonium formate buffer pH 7.0 (A), methanol (B), acetonitrile (C) (A:B:C, 50:20:30, v/v/v), injection volume 30 μL); (a) matrix sample containing 50 pM NO 711 and 1 nM [$^2H_{10}$]NO 711, (b) matrix blank, (c) matrix sample containing 1 nM NO 711 and 1 nM [$^2H_{10}$]NO 711.

approach to increase throughput. There are a number of strategies to speed up HPLC coupled to ESI-MS/MS, e.g., employing UPLC, monolithic columns, column switching, fast gradients, etc. [10–12]. Our intention was to achieve this goal by reducing the column length while increasing the flow rate at the same time. We selected our formerly established and extensively employed MS binding assay for mGAT1 (murine GABA transporter subtype 1) using NO 711 (see Fig. 1), as a marker, as an example for the realization of this strategy [13]. GAT1 is the most abundant GABA transporter subtype in the CNS and represents a relevant drug target for several therapeutic indications such as epilepsy, anxiety and pain [14,15].

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water was obtained by distillation of demineralised water (obtained by reverse osmosis) in house. Ammonium formate p.a. for mass spectrometry was from Fluka (Taufkirchen, Germany). NO 711 (MW 350.4) and [$^2H_{10}$]NO 711 (360.5) were synthesized as described [13].

2.2. Preparation of standards and quality controls

660 μg NO 711 and 512 μg [$^2H_{10}$]NO 711, respectively, were dissolved in 5 mL water (volumetric flasks) resulting in a concentration of 264.7 μM NO 711 and 332.8 μM [$^2H_{10}$]NO 711, respectively. Both stock solutions were further diluted to 1 μM with water and aliquots were frozen at −20 °C. On the day of the assay 1 μM solutions were thawed and serially diluted in methanol to yield 1 nM [$^2H_{10}$]NO 711 (internal standard, IS) and the desired standard concentrations. Following this procedure no signs of degradation of NO 711 or [$^2H_{10}$]NO 711 could be detected over several years. Matrix blanks (see below) were supplemented with 200 μL of the respective analyte and IS solutions (in triplicate), dried over night at 50 °C and finally reconstituted in 200 μL 10 mM ammonium formate buffer pH 7.0 (A) and methanol (B) (A:B, 95:5, v/v) to obtain calibration standards. In the same way, quality control samples (QC, six-fold per concentration) were prepared to assess precision and accuracy. In the same way samples containing 50 pM or 1 nM NO 711, respectively, and 1 nM [$^2H_{10}$]NO 711 were prepared for method development.

2.3. MS Binding assays

All experiments were performed with identically constituted triplicates, as previously described in detail [13]. In short, 9 concentrations of NO 711 (2.5–240 nM) were used in saturation experiments. Non-specific binding was defined as binding remaining in the presence of 100 mM GABA. Incubation was terminated by transfer of 200 μL per well onto a 96-well filter plate (Acroprep, glass fibre, 1.0 μm, 350 μL, Pall, Dreieich, Germany) with a 12 channel pipette. After rapid vacuum-filtration the filters were washed with ice cold 0.9% NaCl (5 × 150 μL). Subsequently the filter plate was dried (60 min, 50 °C), allowed to cool down to RT and finally eluted with 3 × 100 μL methanol into a 1.2 mL polypropylene deep well plate (Brand, Wertheim, Germany). The eluates generated in the binding experiment were supplemented with 200 μL 1 nM [$^2H_{10}$]NO 711 (in methanol) as internal standard before the plate was dried over night (50 °C). Matrix blanks were prepared analogously by incubation of the mGAT1 membrane preparation without NO 711. Finally dried samples were reconstituted in 200 μL 10 mM ammonium formate buffer pH 7.0 (A) and methanol (B) (A:B, 95:5, v/v).

2.4. LC–ESI-MS/MS

LC–ESI-MS/MS was performed using a API 3200 or a API 5000 triple quadrupole mass spectrometer (as indicated in the following) with a TurboV-ion source (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent HPLC system (Agilent 1200 vacuum degasser, binary pump and oven, Agilent, Waldbronn, Germany) and a SIL-HT(A) autosampler (Shimadzu, Duisburg, Germany) or a HTS-PAL autosampler (CTC-Analytics, Zwingen, Switzerland). For