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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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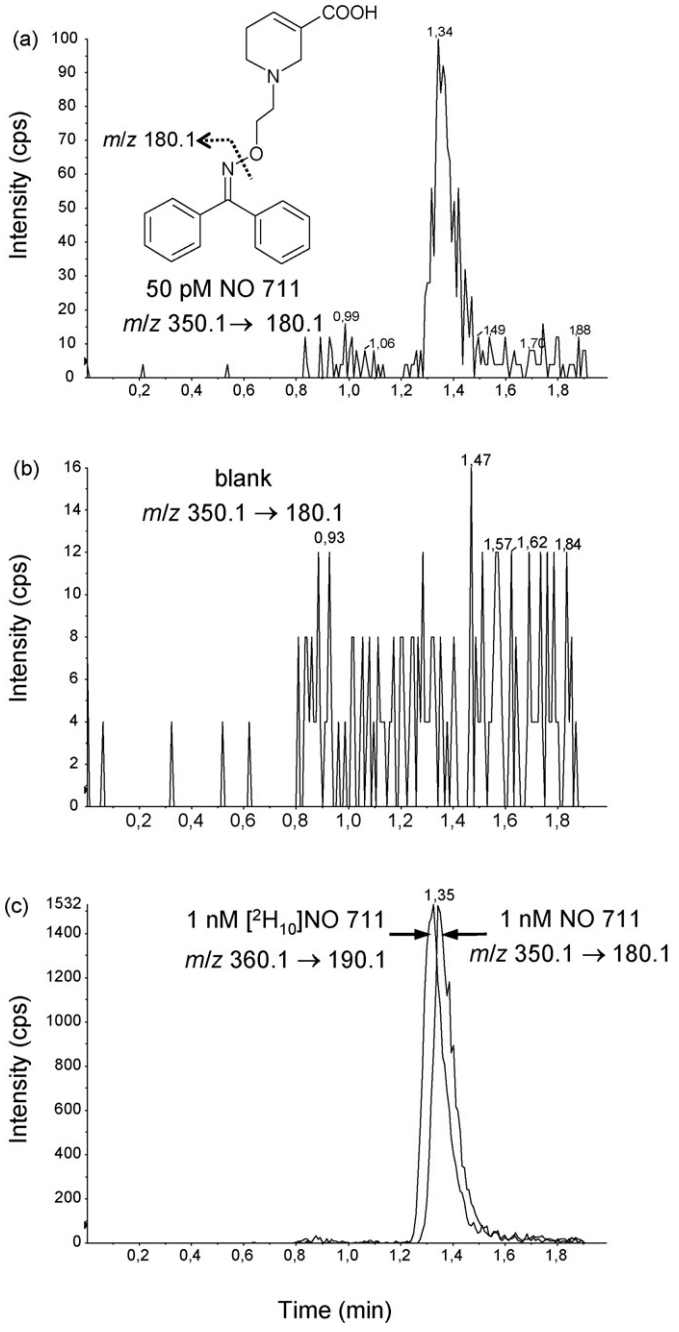

Fig. 1. SRM LC–ESI-MS/MS chromatograms of NO 711 and [$^2H_{10}$]NO 711 in matrix samples obtained with an API 3200 employing a Purospher STAR RP18 column (55 mm × 2 mm, isocratic flow 350 μL/min, mobile phase: 10 mM ammonium formate buffer pH 7.0 (A), methanol (B), acetonitrile (C) (A:B:C, 50:20:30,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

HPLC the following cartridges in the respective cartridge holders were used: Purospher STAR RP18 (endcapped, 55 mm × 2 mm, 3 μm; Merck, Darmstadt, Germany), Luna C18(2) (20 mm × 2 mm and 10 mm × 2 mm, 3 μm; Phenomenex Torrance, CA, USA) and SynergiFusion-RP (20 mm × 2 mm and 10 mm × 2 mm, 2.5 μm; Phenomenex). All columns were protected with the respective 4 mm × 2 mm SecurityGuard cartridges (Phenomenex). The column temperature was set at 20 °C in all cases. Employing the Purospher STAR RP18 cartridge (55 mm × 2 mm) 10 mM ammonium formate buffer pH 7.0 (A), methanol (B), acetonitrile (C) (A:B:C, 50:20:30, v/v/v) was used as mobile phase with a flow rate of 350 μL/min and the injection volume was 30 μL. The effluent up to 0.8 min and after 1.9 min (total run time of 2.0 min) was diverted to waste by a Valco valve in order to protect the mass spectrometer. For the 20 mm × 2 mm cartridges 10 mM ammonium formate buffer pH 7.0 (A) and acetonitrile (B) were used in ratios A:B (v/v) of 5:95 (Luna C18(2)) and 20:80 (SynergiFusion-RP), respectively, at 1000 μL/min. For the 10 mm × 2 mm cartridges 10 mM ammonium formate buffer pH 7.0 (A) and acetonitrile (B) was used in ratios A:B (v/v) of 5:95 (Luna C18(2)) and 10:90 (SynergiFusion-RP), respectively, at 800 μL/min. Aliquots of 5 μL were injected onto the 20 mm × 2 mm and the 10 mm × 2 mm cartridges. The effluent up to 0.1 min and after the total run time of 0.3 min was diverted to waste. The autosampler was rinsed with water (A) and acetonitrile (B) (A:B, 20:80, v/v) after aspiration.

Temperature (T), ion spray voltage (IS), collision energy (CE), curtain (CUR), nebulizing (GS1), auxiliary (GS2) and collision (CAD) gas of the API 3200 were set as determined by FIA optimization using matrix samples containing 1 nM NO 711 and 1 nM [$^2H_{10}$]NO 711 (injection volume 5 μL). Purospher STAR RP18 55 mm × 2 mm: T: 625 °C, IS: +1750 V, CE: 28 eV, CUR: 20 psi (138 kPa), GS1: 32 psi (221 kPa), GS2: 65 psi (448 kPa) and CAD: 3 psi (21 kPa); SynergiFusion-RP 10 mm × 2 mm and 20 mm × 2 mm as well as Luna C18(2) 10 mm × 2 mm and 20 mm × 2 mm: T: 700 °C, IS: +1500 V, CE: 28 eV, CUR: 22 psi (152 kPa), GS1: 65 psi (448 kPa), GS2: 65 psi (448 kPa) and CAD: 5 psi (34 kPa). For NO 711 and [$^2H_{10}$]NO 711 the transitions (*m*/*z*) 350.1–180.1 and 361.1–190.1, respectively, were analysed in the positive ion selected reaction monitoring mode operating Q1 and Q3 under low mass resolution conditions with dwell times of 250 ms (Purospher STAR RP18, 55 mm × 2 mm) or 100 ms (20 mm × 2 mm and 10 mm × 2 mm cartridges). The potentials at the quadrupoles were set as determined by means of the quantitative optimization protocol. Data were collected and quantified (without further manipulation like smoothing, etc.) using Analyst 1.4.2 (Applied Biosystems, Darmstadt, Germany).

2.5. Method validation

Linearity, precision and accuracy were controlled according to the FDA guidance for bioanalytical method validation [16]. Linearity for each matrix batch (i.e., all samples prepared at a single day with identical target material) was determined individually by plotting the peak area ratio (*y*) of NO 711 relative to that of the IS *vs*. the concentration of NO 711 (*x*). Calibration functions based on 6 or 7 calibration standards were generated by least squares linear regression employing Prism 4.02 (GraphPad Software, San Diego, CA, USA). Calibration curves for the Purospher STAR RP18, 55 mm × 2 mm column were calculated without weighting. All calibration curves for short columns were achieved with a weighting factor of $1/x^2$ (as this procedure yielded better results as compared to non-weighting). The LLOQ was defined as the lowest concentration of NO 711 yielding a response of at least 5 times the response as compared to the blank response, acceptable accuracy (80–120%), and sufficient precision (within 20%). Intra- and inter-batch accuracy and precision were determined for QC samples at three concentration levels with six replicates each based on individual calibrations for five different matrix batches. No signs of degradation of NO 711 or [$^2H_{10}$]NO 711 could be detected under the described conditions during extensive practical experience over several years.

3. Results and discussion

3.1. Sensitivity requirements

Sensitivity requirements for MS binding assays are clearly defined by the analyte concentrations, reflecting total as well as non-specific marker binding in saturation experiments at nominal marker concentrations (M_{tot}) of about $0.1K_d \leq M_{tot} \leq 10K_d$ and target concentrations (T_{tot}) of $\leq 0.1K_d$. In the case of NO 711 binding to mGAT1 previous experiments had revealed that total binding under these conditions is roughly in the range of 100 pM to 3 nM NO 711 in the binding samples whereas non-specific binding accounts for concentrations of maximally 500 pM down to below 10 pM NO 711. As preliminary investigations showed that NO 711 concentrations representing the lowest range of non-specific marker binding could hardly be analyzed with the mass spectrometer designated for this purpose (API 3200), we decided to aim at a concentration range for the quantification of NO711 in matrix samples from 50 pM up to low nM. To forgo MS quantification of few samples representing the non-specifically bound marker, in saturation experiments, does not result in appreciable problems because non-specific binding is known to increase linearly with the nominal marker concentration [13,17]. Therefore these "missing datapoints" (i.e., concentrations of non-specifically bound NO 711) can easily be obtained by extrapolation using linear regression once established on the basis of experimental data resulting from non-specific marker binding at higher concentrations (an example for analysis of a representative saturation experiment is shown in Fig. 4).

3.2. Standard method based on 55 mm × 2 mm column

As the current project utilizing LC columns of 10 mm and 20 mm length for an increased throughput should be realized employing an API 3200 triple quadrupole mass spectrometer we first transferred our LC–ESI-MS/MS method formerly established for an API 2000 based on the internal standard [$^2H_{10}$]NO 711 and a Purospher STAR RP18 column (55 mm × 2 mm, 3 μm, 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) to this MS instrument. Besides optimization of compound specific and source dependent parameter settings of the mass spectrometer only minor modifications as compared to the original method were made. The only step worth mentioning is a slightly changed procedure to obtain the samples containing the analyte to be analyzed. Whereas the liberation of the bound marker from the filter residue by elution with methanol remained unchanged in contrast to the original procedure the internal standard (as well as the analyte for preparation of calibration standards) dissolved in methanol was added before drying and final reconstitution of the samples in 10 mM ammonium formate buffer pH 7.0 (A) and methanol (B) (A:B, 95:5, v/v). Since extensive practical experience indicated that slight variations of the calibration function can be hardly avoided for different batches we decided to establish individual calibrations for each batch of binding assay.

Lastly a validation with respect to linearity, intra- and inter-batch accuracy and precision according to the FDA guideline [16] based on five different batches was performed (Table 1). The demanded limits were met from 50 pM as the LLOQ up to 5 nM with RSDs being below 10% and accuracies in the range from 90

Table 1
Validation of NO 711 quantification by LC–ESI-MS/MS with API 3200 and Purospher STAR RP18 (55 mm × 2 mm).

Sample (n)[a]	Intra-series												Inter-series		
	Series 1[b]			Series 2[c]			Series 3[d]			Series 4[e]			Series 5[f]			M	Acc	Pre
	M[g]	Acc[h]	Pre[i]	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre			
50 pM Cal (6)	49.5	98.9	8.1	53.6	107.2	5.9	46.5	93.9	2.6	57.5	114.9	8.6	57.4	114.8	7.5			
75 pM Cal (3)	77.5	103.3	2.7	75.7	100.9	5.4	78.9	105.2	4.3	74.9	99.9	7.2	83.4	111.2	9.0			
100 pM Cal (3)	101.8	101.8	5.1	97.3	97.3	4.1	105.7	105.7	4.4	93.6	93.6	4.9	107.8	107.8	4.9			
200 pM Cal (3)	190	95.0	8.3	175	87.5	1.7	197	98.7	1.8	171	85.5	3.4	200	100.3	5.2			
500 pM Cal (3)	517	103.3	1.0	517	103.3	1.9	475	94.9	5.1	506	101.2	4.4	494	98.8	1.6			
1000 pM Cal (3)	1002	100.2	0.9	1004	100.4	1.5	1029	102.9	1.7	1017	101.7	4.9	968	96.8	4.3			
5000 pM Cal (3)	4930	98.6	1.3	5000	100.0	1.1	5000	99.9	1.1	5000	99.9	1.9	5010	100.1	3.5			
100 pM QC (6)	99.4	99.4	7.8	89.8	89.8	1.3	96.2	96.2	5.4	93.7	93.7	7.4	107.8	107.8	6.0	97.4	97.4	7.1
500 pM QC (6)	539	107.9	3.3	483	96.5	3.5	498	99.6	2.2	534	106.8	5.7	503	100.6	3.9	511	102.3	4.6
2500 pM QC (6)	2490	99.8	4.0	2470	98.7	1.4	2350	94.0	2.2	2500	100.0	4.0	2470	99.0	3.7	2460	98.4	1.3

[a] Calibration standard (Cal) or quality control (QC), in brackets number of replicates; [b–f] resulting calibration function: series 1: $y = 1.109x + 0.01872$, $r^2 = 0.9998$; series 2: $y = 1.051x - 0.008593$, $r^2 = 0.9998$; series 3: $y = 1.116x + 0.02161$, $r^2 = 0.9998$; series 4: $y = 0.8420x - 0.01070$, $r^2 = 0.9995$; series 5: $y = 0.9662x + 0.0005911$, $r^2 = 0.9989$; [g] mean of calculated concentration (pM); [h] accuracy in %; [i] precision as RSD in %.

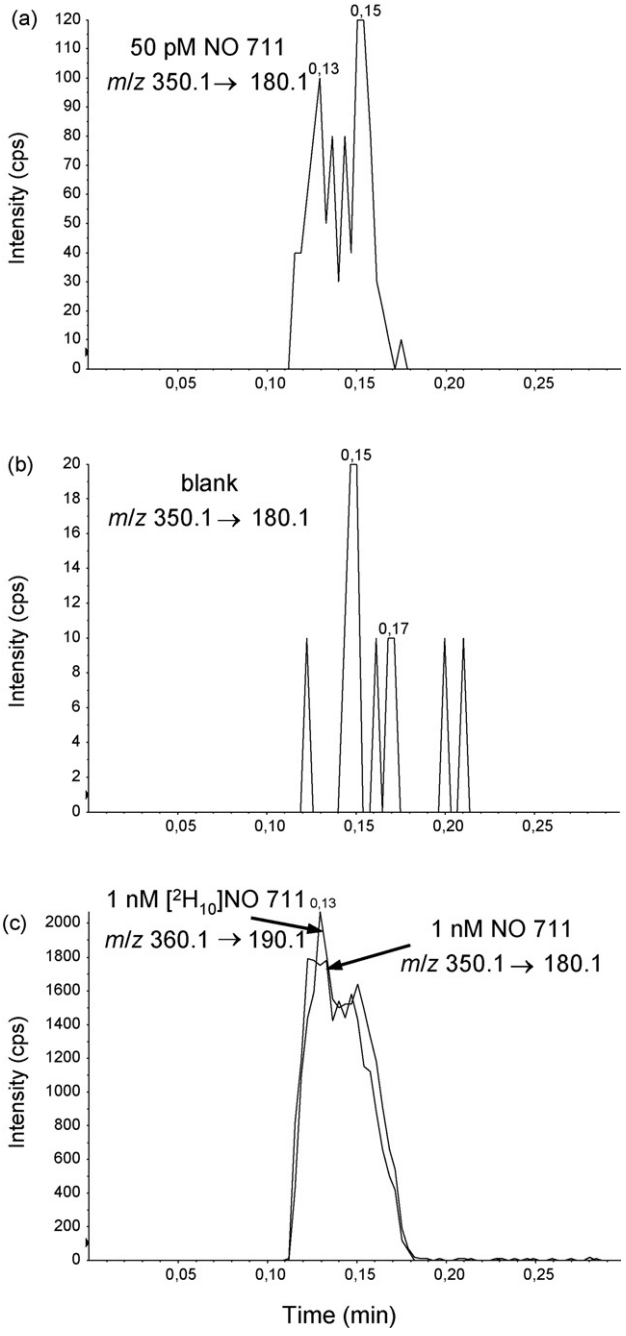

Fig. 2. SRM LC–ESI-MS/MS chromatograms of NO 711 and [$^2H_{10}$]NO 711 in matrix samples obtained with an API 3200 employing a Luna C18(2) (10 mm × 2 mm, isocratic flow 800 μL/min, mobile phase: 10 mM ammonium formate buffer pH 7.0 (A) and acetonitrile (B) (A:B, 5:95 (v/v), injection volume 5 μL); (a) 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.

to 110%. Representative SRM chromatograms for 50 pM and 1 nM calibration standards as well as a matrix blank are shown in Fig. 1.

3.3. Development and validation of methods based on short columns

As the Purospher STAR RP18 was not available in a column length smaller than 30 mm, we selected another standard C18 (LunaC18) stationary phase with the dimension of 20 mm × 2 mm and 10 mm × 2 mm for the intended reduction of the time required for HPLC. Furthermore a polar embedded C18 stationary phase

Table 2
Optimized chromatographic conditions for quantification of NO 711 by LC–ESI-MS/MS employing short columns.

Column	Mobile phase (A/B)[a]	Flow rate (μL/min)	Pressure (bar)	Retention time (min)[b]	k[c]
Luna C18(2) (20 mm × 2 mm	5:95	1000	62	0.13	0.43
Luna C18(2) (10 mm × 2 mm)	5:95	800	33	0.13	0.49
SynergiFusion-RP (20 mm × 2 mm)	20:80	1000	97	0.15	0.65
SynergiFusion-RP (10 mm × 2 mm)	10:90	800	43	0.14	0.61

[a] % (v/v), A: 10 mM ammonium formate pH 7.0, B: acetonitrile.
[b] Retention time for NO 711 in matrix samples.
[c] System void volume was determined experimentally by FIA–MS (without column), void volume of column and precolumn was calculated according to $v = 0.7r^2\pi L$.

(SynergiFusion-RP) with identical dimension (20 mm × 2 mm and 10 mm × 2 mm) was chosen for this purpose. Employing a 1 nM NO 711 matrix sample we varied the isocratic mobile phase composed of 10 mM ammonium formate buffer pH 7.0 (A) and acetonitrile (B) from A:B, 50:50 to 5:95 (v/v) and investigated flow rates from 500 to 1200 μL. The desired short retention times ranging, at best, from 5 to 10 s as well as satisfactory signal intensities were obtained when eluents with rather high acetonitrile proportions (80–95%) were used. In the case of the analytical standard procedure based on the 55 mm × 2 mm column, matrix samples directly resulting from the binding assays had been used for quantification without any prior purification. Therefore, to protect the LC–MS/MS system, the LC column had been provided with a guard column and the switching valve integrated in the API 3200 system had been used to direct the effluent containing the analyte more or less exclusively to the MS instrument. For the established methods based on short columns, we decided to keep these settings unchanged, as we still attempted to analyse unpurified matrix samples. As the integrated valve is operated in time steps of only 0.1 min we optimized the chromatographic conditions for all four short columns in a way to obtain the peaks for NO 711 and [$^2H_{10}$]NO 711 between 0.1 and 0.2 min so that data acquisition could be completed within the time period of 0.1 and 0.3 min when the HPLC effluent was switched to the ESI source. The optimal conditions found using 5 μL injections volumes of spiked matrix samples are specified in Table 2. Finally, the source dependent parameters for the respective chromatographic conditions were optimized by flow injection analysis employing the quantitative optimization option of the API 3200. It should be noted furthermore that the dwell time was reduced from 250 ms (55 mm × 2 mm column) to 100 ms for the short column method. Representative SRM chromatograms are shown in Fig. 2.

Although the Shimadzu SIL HT(A) autosampler works comparably fast the "overhead" time to the chromatographic cycle time of 18 s produced by the autosampler (including a rinsing step) could not be reduced below 39 s. It should be emphasized, however, that this "overhead" time can be further reduced employing an even faster autosampler or by the use of two (or more) autosamplers [18–20]. Alternatively, omitting the rinsing step of the autosampler, at the expense of an enhanced carryover, may be considered to this end as well. But, so far, no effort has been made to determine the extent this would have on the results of the MS binding assays.

With the aim of quantifying NO 711 concentrations down to 50 pM also the effect of increased injection volumes (i.e., >5 μL) on the signal intensity was studied. Unfortunately injection volumes higher than 5 μL did not result in enhanced peak heights but rather tended to peak distortion. It should be mentioned that this is a clear difference to the method developed for the standard column dimension (55 mm × 2 mm) which shows an almost linear relation between injection volume and signal intensity up to at least 50 μL (data not shown). Therefore we investigated whether the sensitivity employing the established fast chromatography could be further enhanced by a more powerful mass spectrometer. Preliminary experiments with an API 5000 instead of the

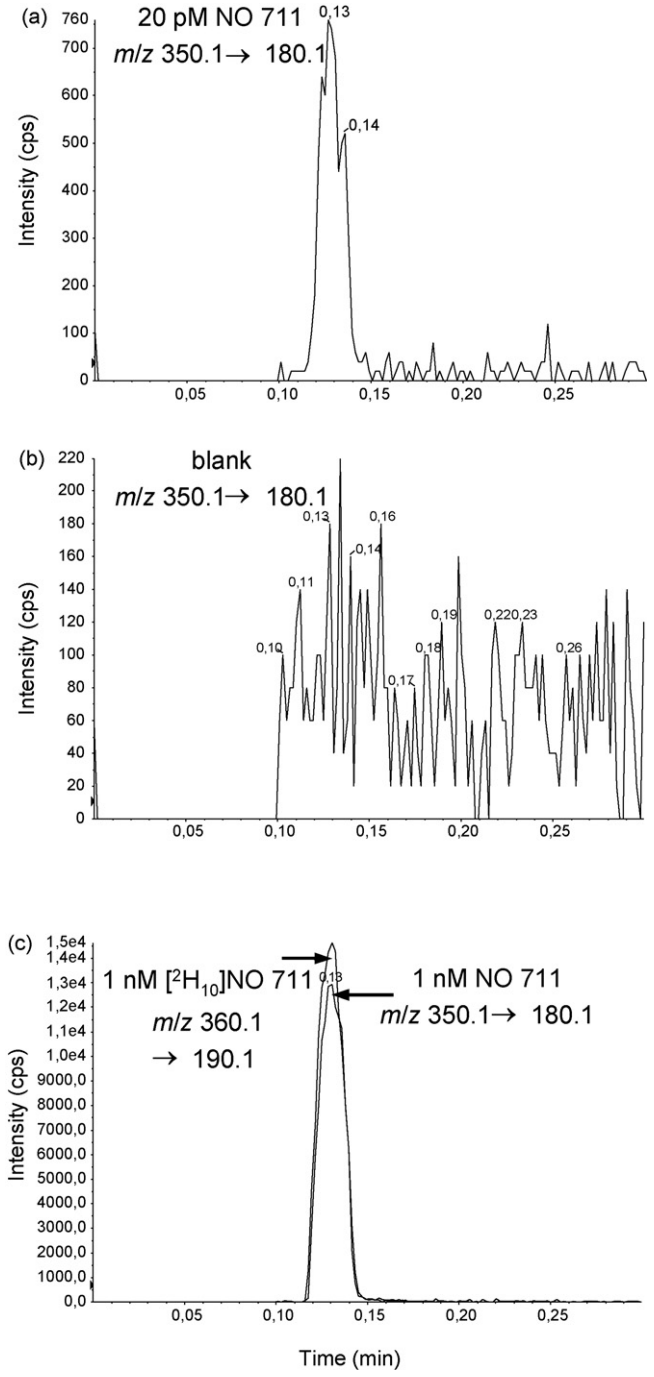

Fig. 3. SRM LC–ESI-MS/MS chromatograms of NO 711 and [$^2H_{10}$]NO 711 in matrix samples obtained with an API 5000 employing a Luna C18(2) (10 mm × 2 mm, isocratic flow 800 μL/min, mobile phase: 10 mM ammonium formate buffer pH 7.0 (A) and acetonitrile (B) (A:B, 5:95 (v/v)), injection volume 5 μL); (a) matrix sample containing 20 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.

Table 3
Validation of NO 711 quantification by LC–ESI-MS/MS with API 3200 and Luna C18(2) (10 mm × 2 mm).

Sample (n)[a]	Intra-series															Inter-series		
	Series 1[b]			Series 2[c]			Series 3[d]			Series 4[e]			Series 5[f]					
	M[g]	Acc[h]	Pre[i]	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre
50 pM Cal (6)	53.3	106.5	5.8	–	–	–	[j]	49.2	98.5	15.5	51.1	102.2	14.9	48.4	96.8	7.3		
75 pM Cal (3)	70.4	93.9	7.5	81.3	108.4	6.9	79.8	106.4	14.7	73.8	98.4	8.3	80.1	108.1	6.1			
200 pM Cal (3)	181	90.5	3.7	180	89.8	3.1	193	96.5	7.6	176	87.9	8.1	208	103.8	2.9			
500 pM Cal (3)	501	100.2	7.8	443	88.7	3.6	482	96.5	6.7	463	92.7	5.6	499	99.9	0.4			
1000 pM Cal (3)	945	94.5	1.9	946	94.6	5.0	983	98.3	2.3	1049	104.9	15.0	1009	100.9	4.1			
3000 pM Cal (3)	3330	111.0	2.4	3240	108.1	3.2	3100	103.3	5.3	3190	106.3	6.2	2890	96.3	3.2			
7000 pM Cal (3)	7240	103.4	2.9	7770	111.0	3.9	7140	102.1	2.3	7370	105.3	5.2	6810	97.3	4.4			
100 pM QC (6)	95.3	95.3	2.8	105.2	105.2	8.5	96.8	96.8	12.2	111.3	11135	13.3	100.6	100.6	3.2	101.8	101.8	6.4
1000 pM QC (6)	1004	104.1	2.0	1121	112.1	5.3	1097	109.7	8.0	980	98.0	6.4	904	90.4	6.5	1029	102.9	8.6
5000 pM QC (6)	4880	97.7	2.2	5180	103.6	2.1	5580	111.6	4.4	5210	104.1	8.2	4710	94.2	2.8	5110	102.2	6.5

[a]Calibration standard (Cal) or quality control (QC), in brackets number of replicates; [b–f]resulting calibration function: series 1: $y = 0.8898 + 0.004920$, $r^2 = 0.9953$; series 2: $y = 0.8309x - 0.0008320$, $r^2 = 0.9805$; series 3: $y = 0.9276x - 0.0007101$, $r^2 = 0.9984$; series 4: $y = 1.1088x - 0.01025$, $r^2 = 0.9932$; series 5: $y = 1.096x + 0.001565$, $r^2 = 0.9959$; [g]mean of calculated concentration (pM); [h]accuracy in %; [i]precision as RSD in %; [j]excluded for calibration as criteria for LLOQ of the CDER guideline were not met.

Table 4
Validation of NO 711 quantification by LC–ESI-MS/MS with API 3200 and Luna C18(2) (20 mm × 2 mm).

Sample (n)[a]	Intra-series															Inter-series		
	Series 1[b]			Series 2[c]			Series 3[d]			Series 4[e]			Series 5[f]					
	M[g]	Acc[h]	Pre[i]	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre
50 pM Cal (6)	51.7	103.5	6.8	55.1	110.3	3.7	47.6	95.2	15.0	51.3	102.7	11.3	48.1	96.2	6.7			
75 pM Cal (3)	73.5	99.0	8.3	67.1	89.4	3.9	84.9	113.2	12.8	72.7	97.0	11.9	83.4	111.3	6.2			
200 pM Cal (3)	180	90.2	2.9	181	90.4	7.7	214	106.8	9.5	179	89.6	14.1	203	101.5	8.5			
500 pM Cal (3)	517	103.4	2.4	452	90.5	4.2	477	95.3	7.8	454	90.7	8.7	477	95.4	8.1			
1000 pM Cal (3)	943	94.3	1.7	983	98.3	6.5	888	88.8	5.8	1018	101.8	14.1	1009	100.9	2.3			
3000 pM Cal (3)	3230	107.7	1.8	3240	108.0	2.9	3210	107.2	4.0	3092	103.1	4.7	2990	99.6	4.3			
7000 pM Cal (3)	7200	102.8	0.2	7930	113.2	3.4	6880	98.2	7.4	7870	112.5	1.4	6820	97.4	3.1			
100 pM QC (6)	96.0	96.0	4.2	99.1	99.1	9.2	89.0	89.0	14.0	108.7	108.7	9.7	99.7	99.7	6.5	98.6	98.6	7.3
1000 pM QC (6)	1016	101.6	1.9	1103	110.3	4.6	1005	100.5	7.0	991	99.1	7.8	902	90.2	4.9	1006	100.6	7.1
5000 pM QC (6)	4980	99.6	3.4	5370	107.3	4.9	4870	97.3	2.8	4852	97.0	14.1	4790	95.9	5.0	4970	99.4	4.6

[a]Calibration standard (Cal) or quality control (QC), in brackets number of replicates, [b–f]resulting calibration function: series 1: $y = 0.8886x + 0.009661$, $r^2 = 0.9977$; series 2: $y = 0.7907x + 0.01241$, $r^2 = 0.9808$; series 3: $y = 0.9913x + 0.003214$, $r^2 = 0.9936$; series 4: $y = 1.093x - 0.006590$, $r^2 = 0.9845$; series 5: $y = 1.088x + 0.005150$, $r^2 = 0.9980$; [g]mean of calculated concentration (pM); [h]accuracy in %; [i]precision as RSD in %.

Table 5
Validation of NO 711 quantification by LC–ESI-MS/MS with API 3200 and employing SynergiFusion-RP (10 mm × 2 mm).

Sample (n)[a]	Intra-series															Inter-series		
	Series 1[b]			Series 2[c]			Series 3[d]			Series 4[e]			Series 5[f]					
	M[g]	Acc[h]	Pre[i]	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre
50 pM Cal (6)	50.7	101.5	6.1	–	–	–	[j]	49.1	98.2	13.6	50.9	101.7	18.4	48.7	97.4	10.0		
75 pM Cal (3)	76.5	102.0	1.6	77.7	103.6	5.1	79.4	105.8	1.2	–	–	–	[k]	79.8	106.5	3.7		
200 pM Cal (3)	176	87.8	4.6	191	95.7	4.8	199	99.5	6.5	175	87.6	12.0	207	103.4	6.1			
500 pM Cal (3)	517	103.3	4.7	430	86.1	3.8	511	102.0	9.2	463	92.7	7.9	517	103.4	1.7			
1000 pM Cal (3)	938	93.8	1.9	964	96.4	4.4	885	88.5	9.2	1057	105.7	7.5	1001	100.1	1.5			
3000 pM Cal (3)	3270	109.2	1.6	3330	111.0	3.7	3020	100.8	14.5	3110	103.7	1.1	2930	97.6	1.0			
7000 pM Cal (3)	7160	102.3	1.0	7500	107.2	8.0	7210	103.0	12.0	7490	107.0	1.3	6680	95.5	4.0			
100 pM QC (6)	95.8	95.8	3.3	102.4	102.4	10.0	96.6	96.6	10.0	104.9	104.9	12.9	104.8	104.8	13.4	100.9	100.9	4.4
1000 pM QC (6)	1012	101.2	2.8	1105	110.5	7.4	1026	102.6	4.2	996	99.6	6.7	961	96.1	8.5	1020	102.0	5.3
5000 pM QC (6)	4780	95.6	4.3	5070	101.4	3.0	5210	104.3	3.8	4900	98.1	6.3	4940	98.8	3.7	4980	99.6	3.3

[a]Calibration standard (Cal) or quality control (QC), in brackets number of replicates, [b–f]resulting calibration function: series 1: $y = 1.029x + 0.01025$, $r^2 = 0.9973$; series 2: $y = 0.9339x + 0.001503$, $r^2 = 0.9855$; series 3: $y = 0.9560x - 3.375 \times 10^{-6}$, $r^2 = 0.9848$; series 4: $y = 1.101x - 0.003138$, $r^2 = 0.9941$; series 5: $y = 1.040x + 0.002662$, $r^2 = 0.9959$; [g]mean of calculated concentration (pM); [h]accuracy in %; [i]precision as RSD in %; [j]excluded for calibration as criteria for LLOQ of the CDER guideline were not met; [k]excluded for calibration as RSD exceeded 15%.

Table 6
Validation of NO 711 quantification by LC–ESI-MS/MS employing with API 3200 and SynergiFusion-RP (20 mm × 2 mm).

Sample (n)[a]	Intra-series															Inter-series		
	Series 1[b]			Series 2[c]			Series 3[d]			Series 4[e]			Series 5[f]					
	M[g]	Acc[h]	Pre[i]	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre	M	Acc	Pre
50 pM Cal (6)	53.9	107.8	8.2	–	–	–	[j]	50.7	101.5	15.7	51.0	102.0	14.1	49.6	99.2	9.8		
75 pM Cal (3)	69.6	92.8	9.4	79.4	105.8	3.5	75.4	100.5	4.8	73.6	98.1	2.5	–	–	–	[k]		
200 pM Cal (3)	175	87.3	0.9	177	88.3	6.7	174	87.2	1.5	181.3	90.7	9.4	213.5	106.8	4.0			
500 pM Cal (3)	507	101.4	4.7	452	90.4	6.8	503	100.6	3.2	458	91.6	6.8	550	110.0	11.2			
1000 pM Cal (3)	967	96.7	3.7	961	96.1	3.9	918	91.8	7.6	1058	105.8	9.3	988	98.8	3.9			
3000 pM Cal (3)	3303	110.1	1.2	3120	105.6	1.8	3315	110.5	3.1	3280	109.2	3.6	2950	98.3	2.4			
7000 pM Cal (3)	7270	103.8	2.2	7970	113.8	5.3	7450	106.4	2.6	7040	100.6	4.6	6870	98.1	3.9			
100 pM QC (6)	91.8	91.8	4.4	99.6	99.6	10.6	107.3	96.6	9.1	111.0	111.0	9.1	100.7	100.7	13.3	102.1	102.1	7.3
1000 pM QC (6)	1041	104.1	2.7	1129	112.9	3.9	981	98.1	4.0	945	94.5	4.0	907	90.7	6.0	1001	100.1	8.7
5000 pM QC (6)	4830	96.5	1.7	5250	104.9	2.7	5022	100.4	6.3	5050	101.0	6.3	4730	94.6	2.2	4980	99.5	4.1

[a]Calibration standard (Cal) or quality control (QC), in brackets number of replicates; [b–f]resulting calibration function: series 1: $y = 0.8662x + 0.02206$, $r^2 = 0.9957$; series 2: $y = 0.8042x + 0.009357$, $r^2 = 0.9770$; series 3: $y = 0.9820x - 0.008601$, $r^2 = 0.9847$; series 4: $y = 1.098x - 0.005112$, $r^2 = 0.9960$; series 5: $y = 1.013x + 0.001379$, $r^2 = 0.9981$; [g]mean in pM; [h]accuracy in %; [i]precision as RSD in %; [j]excluded for calibration as criteria for LLOQ of the CDER guideline were not met; [k]excluded for calibration as RSD exceeded 15%.

Table 7
Comparison of K_d- and B_{max}-values obtained for a single mGAT1 saturation experiment as determined with different LC–ESI-MS/MS quantification methods (see also Fig. 2).

	K_d (nM)	B_{max} (pmol/mgP)
Purospher STAR RP 18 (55 mm × 2 mm)	23.1	35.0
Luna C18(2) (20 mm × 2 mm)	23.2	36.0
Luna C18(2) (10 mm × 2 mm)	21.1	35.0
SynergiFusion-RP (20 mm × 2 mm)	24.1	36.5
SynergiFusion-RP (10 mm × 2 mm)	23.1	37.0

API 3200 showed indeed that NO 711 can then be quantified down to 20 pM in matrix samples (see Fig. 3 and supplementary material).

In order to characterize the performance of the developed short column methods (employing the API 3200) we performed again a validation with respect to linearity, intra- and inter-batch accuracy and precision based on five different batches. The results for the four established short column methods are documented in Tables 3–6. Inter-series accuracies were again between 90–110% and inter-series precisions expressed as RSDs were again below 10% in each case. Intra-assay accuracies and precisions, however, were distinctly lower as compared to the method for the standard column dimension (55 mm × 2 mm). The same was true for the standards defining individual calibration functions. In single cases, the 50 pM standard did not meet the FDA guideline requirements (accuracy beyond 80–120% or RSD beyond 20%) so that the LLOQ was defined as the lowest calibration standard fulfilling these requirements (i.e., 75 pM). Despite these restrictions all four short column methods could be shown to meet the acceptable specification for bioanalysis according to the FDA guideline. Taken together, the obtained results indicate that chromatography can be reduced to a minimum almost without compromising on sensitivity and reliability of ESI-MS/MS.

3.4. Application of LC–ESI-MS/MS methods based on short columns to MS binding assays

When saturation experiments for mGAT1 were analyzed by LC–ESI-MS/MS employing the short columns mentioned above (using the API 3200 for MS-MS detection) sound results were achieved. With respect to the reliability of the underlying quantification method it would be not very meaningful to give means for K_d- and B_{max}-values calculated from all performed binding assays as the deviations observed could be the result of a poor LC–ESI-MS/MS method but also of differences between the employed target material. Therefore we show only a single saturation experiment performed together with the preparation of the matrix samples of "series 5", representing a single batch (series 5 Tables 3–6), that was analysed using the four short columns as well as the 55 mm × 2 mm standard column and the respective calibration functions. As can be seen from Fig. 4a the NO 711 concentrations determined for total and non-specific binding display no essential differences with regard to the quantification method employed. Non-specific binding below 50 pM NO 711 (at nominal marker concentrations below 50 or 80 nM, respectively) was calculated by extrapolation from the straight line obtained by linear regression for the concentrations of non-specifically bound NO 711 at nominal concentrations ≥50 nM NO 711 as outlined above. In Fig. 4b the saturation isotherms based on the five quantification methods are depicted. The differences between the K_d- and B_{max}-values calculated from these saturation isotherms (see Table 7) are negligibly small, indicating again that even the short column methods (showing moderate validation characteristics, see series 5 Tables 3–6) lead to reliable results.

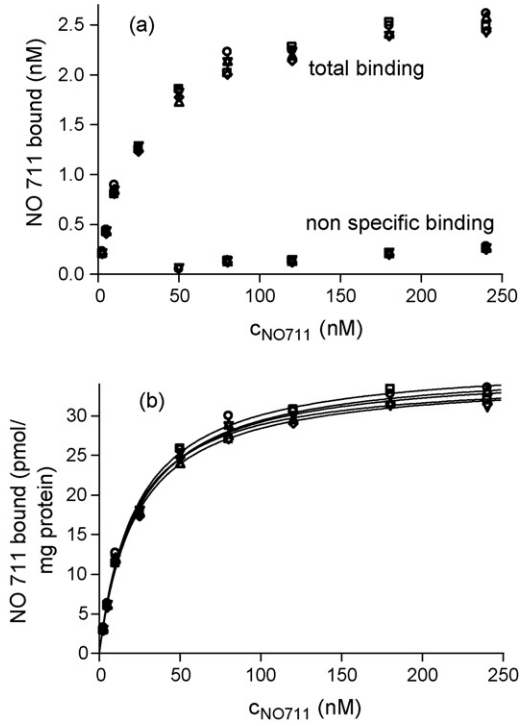

Fig. 4. (a) Means (calculated from triplicates) for total and non-specific binding of NO 711 (given as concentration in the samples) as well as (b) specific NO 711 binding (given as pmol/mg protein) together with the calculated saturation isotherm obtained with an API 3200 for a single mGAT1 saturation experiment (performed as described in Section 2) using different HPLC columns (as indicated by the following symbols: (◊) Purospher STAR RP18 55 mm × 2 mm; (Δ) Luna C18(2) 20 mm × 2 mm; (▽) Luna C18(2) 10 mm × 2 mm; (□) SynergiFusion-RP 20 mm × 2 mm; (○) SynergiFusion-RP 10 mm × 2 mm). Non-specific binding below 50 pM NO 711 was calculated by extrapolation from the straight lines obtained by linear regression for the concentrations of non-specifically bound NO 711 at nominal concentrations ≥50 nM NO 711.

4. Conclusions

The present study documents that NO 711 as a marker for mGAT1 binding can be quantified reliably by ESI-MS/MS after fast chromatography employing short analytical columns (20 mm × 2 mm and 10 mm × 2 mm) in a concentration range suitable to analyze MS binding assays without additional requirements as compared to a standard method based on a 55 mm × 2 mm column. The established short column methods led to retention times of 8–9 s and reduced the chromatographic cycle time to 18 s. Furthermore quantification of NO 711 was possible directly out of the matrix samples obtained by elution of the target material, subsequent drying and reconstitution without any sample preparation step. Accordingly, the present study indicates the feasibility of highly efficient marker quantification in MS binding assays in a high throughput manner even when only standard LC–ESI-MS/MS equipment is employed. The established short column methods identified the autosampler as the bottleneck limiting throughput capacity. The use of extremely fast autosamplers or the combination of two autosamplers should allow to make use of the full potential of the established short column methods. In the context of other strategies applied in the bioanalytical field to speed up LC–MS such as MUX sources [21], the RapidFire technology [22] or parallel staggered LC separations in combination with multiple ESI emitters [23] and concepts omitting any chromatographic separation step before introduction of the sample into the MS such as chip based nanoESI approaches [24], the FlashQuant system [25] or nanoextractive electrospray ionization sources [26], the estab-

lished short column technique impresses by its simplicity as it is based only on standard LC–MS/MS equipment.

Finally, the general significance of this strategy of rapid marker quantification exemplarily developed for NO 711 as a marker of mGAT1 binding should be mentioned. As the matrices of analogous MS binding assays for further membrane proteins can be expected to be rather similar and the choice of the marker in this approach is rather flexible (e.g., with respect to matrix effects) – independent of the nature of the expressed target – the procedure established for mGAT1 should be a promising starting point for further applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.12.006.

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