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Sensitive determination of G-protein-coupled receptor binding ligands by solid phase extraction–electrospray ionization–mass spectrometry

Thomas Letzel [a,b,*], Rico J.E. Derks [a], Cornelius T. Martha [a], André van Marle [c], Hubertus Irth [a]

[a] *Leiden/Amsterdam Center for Drug Research, Department of Analytical Chemistry and Applied Spectroscopy, Faculty of Exact Science, Vrije Universite it Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*
[b] *Department for Basic Life Sciences, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Weihenstephan, Germany*
[c] *Department of Medicinal Chemistry, Faculty of Exact Science, Vrije Universite it Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

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Abstract

High affinity Histamine H2-receptor binding ligands were assayed by automated solid phase extraction (SPE) coupled via electrospray ionization with a Quadrupole-Time-of-Flight mass spectrometer (Q-ToF-MS). The mass spectrometric behavior of these analytes was tested in aqueous solutions with several (nine) volatile salts, in different pH, and with various methanol contents. Out of the high amount of available ligands, three fluorescent-labeled molecules (5706, 5707, and 5708) were studied in detail. The limits of detection (LODs) for all three compounds obtained in mass spectrometric detection was 1 fmol (absolute) in continuous flow and FIA (flow injection analysis) measurements. The results obtained with FIA-fluorescence detection gave LODs a factor 10–100 times higher.

A systematic investigation of sample solving conditions, loading flow conditions, and elution flow conditions made the automated SPE–MS coupling efficient. Ideally, the ligands were dissolved in MeOH–25 mM phosphate buffer (30:70 v/v; pH 11), the SPE loading flow comprised MeOH–25 mM phosphate buffer (30:70 v/v; pH 11) and the SPE elution flow contained MeOH–100 mM ammonium formate solution (90:10 v/v; pH 3). Using this method on a C_{18}-modified silica cartridge (C18, 5 μm, 100 A, 300 μm i.d. × 5 mm, LC Packings) assures high recovery and achieved LODs for all three compounds of 5 fmol (absolute). As an absolute amount of ligands specifically bound on H2-receptors in biochemical experiments is, as will be published elsewhere, between 10 and 100 fmol, the SPE–MS method for the basic compounds can be directly applied for these Histamine H2-receptors.

Keywords: GPCR; Histamine H2-receptor; SPE–MS; Mass spectrometry

1. Introduction

Molecules that are involved in intercellular communication are successful proven targets in drug discovery [1,2]. However, in screening processes to identify new compounds that affect the activity of the targets can include many negative aspects of targets or screened compounds that can cause wrong results. In the case of G-protein-coupled receptors (GPCRs)—being the target for drug development [3]—many lead compounds prove to be problematic because they stick to plastics, are poorly soluble, too hydrophobic, unstable, and rapidly degraded. A probable cause for at least a few of these adverse chemical properties may lay in the fact that GPCRs are membrane spanning cell surface receptors that upon agonist binding, activate an G-protein mediated intracellular signaling cascade. In fact, the human genome possesses several thousands of protein-coding genes (30–40 thousands [4,5] versus 20–25 thousands [6]), many of which can be expected to be associated to vital life functions and disease. Therefore, many GPCRs are the subject of screening programs, which are aimed to find novel and easy feasibilities to synthesize molecules with a good solubility and toxicological profile altering the activity of the GPCRs. In general, the selection of the compounds is based on ligand binding and or functional properties of the receptors. Knowing the degradation speed and the metabolism profile of the potential drugs is also important. In the attempts to come up with

* Corresponding author. Fax: +49 8161 71 44 04.
E-mail address: T.Letzel@lrz.tum.de (T. Letzel).

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new compounds pharmacological and toxicological profiles of compounds and targets assays are miniaturized and novel biochemical approaches to find new leads can be anticipated.

In order to increase automating possibilities and sensitivity of this screenings, it is important to know which analytical properties are needed. One of the most sensitive and promising technique in analytical chemistry today is the mass spectrometry; therewith ligands can be obtained in complex with the proteins [7–10], the inhibition efficiency of ligands can be monitored [11,12] and ligand screening as well as identifications can be performed very sensitive [13,14].

Here, we use the Histamine H2-receptor [15] as a model GPCR developing a ligand screening technique. Close to a thousand inverse agonists [16] for the Histamine H2-receptor have been described including a number of high affinity but highly hydrophobic fluorescent compounds [17].

However, in our days hydrophilic antagonists (like famotidine (Fig. 1A), ranitidine (Fig. 1B), cimetidine (Fig. 1C), and nizatidine) are used as pharmaceutical drugs [18,19], to treat and prevent ulcers in the stomach and intestines, to treat conditions in which the stomach produces too much acid and to treat conditions in which acid comes up into the esophagus and causes heartburn, such as gastroesophageal reflux disease caused by that receptor [20].

Several studies have been accomplished to monitor this compounds in both, human plasma and urine [21–38]. In all cited references, high-performance liquid chromatography (HPLC) was used in combination with ultraviolet (UV) or mass spectrometric (MS) detection [21,32]. In almost all of these analytical investigations, pretreatment steps were chosen extracting the analytes from urine and plasma (like liquid–liquid extraction or solid phase extraction (SPE)). However, only a few research groups automated the sample pretreatment [22,32,36]. Detection limits for Histamine H2-receptor inhibitors like famotidine, ranitidine and cimetidine in human plasma and urine were in the values of 0.2–10 ng mL^{-1} [21–28], between 1 and 10 ng mL^{-1} [29–34], and between 15 and 100 ng mL^{-1} [35–38], respectively. With a typical medication for Histamine H2-receptor-inhibitors like famotidine [21,25] and cimetidine [36] of 40 to 1600 mg per day, respectively, they can be found unmetabolized in urine (22–31% famotidine [25] and 56–85% cimetidine [36]) and in plasma also a few hours after administration (20–150 ng mL^{-1} famotidine [25] and low mg mL^{-1} cimetidine [35–37]). Even though the cited methods [21–38] might be sensitive enough to detect non-metabolized ligands; however, the detection of reactive metabolic products or ligands from binding experiments (with an absolute amount of 10–100 fmol bound molecules), UV detection with LODs in the 1 pmol range (absolute) is not sensitive enough. The detection of these hydrophilic compounds (with non problematic characteristics in pharmacology and analytical chemistry) could be optimized to limits of 3 fmol (absolute) for famotidine with the LC/MS setup of Campanero et al. [21] and thus showed the potential of MS to be a suitable and versatile detection method.

In this study, we present the optimization of an automated SPE-method directly coupled with mass spectrometry to detect basic and hydrophobic GPCR ligands using H2-receptor antagonists with high affinity [17]. The described techniques and buffer systems might serve as a lead for the development for sensitive detection of small molecular as lead compounds obtained from biochemical or pharmacological assays in general.

2. Experimental

2.1. Chemicals and materials

Famotidine (M_r 337.4; Fig. 1A) was received from Merck Sharp & Dohme (Haarlem, The Netherlands), Ranitidine (M_r

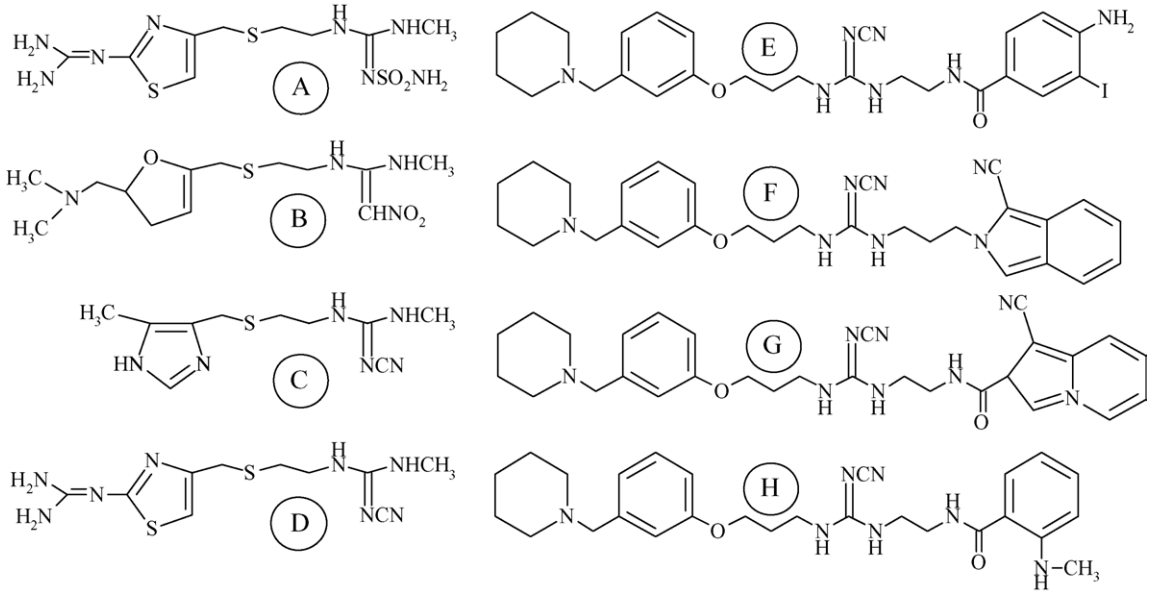

Fig. 1. H2-receptor ligands studied to obtain best mass spectrometric conditions and further analytical parameter: (A) famotidine (pK_i: 7.74 ± 0.13); (B) ranitidine (pK_i: 6.89 ± 0.13); (C) cimetidine (pK_i: 5.64 ± 0.07); (D) tiotidine (pK_i: 7.40 ± 0.30); (E) iodoaminopotentidine (pK_i: 9.04 ± 0.14); (F) 5706 (pK_i: 8.31 ± 0.33); (G) 5707 (pK_i: 8.80 ± 0.24); (H) 5708 (pK_i: 8.90 ± 0.19).

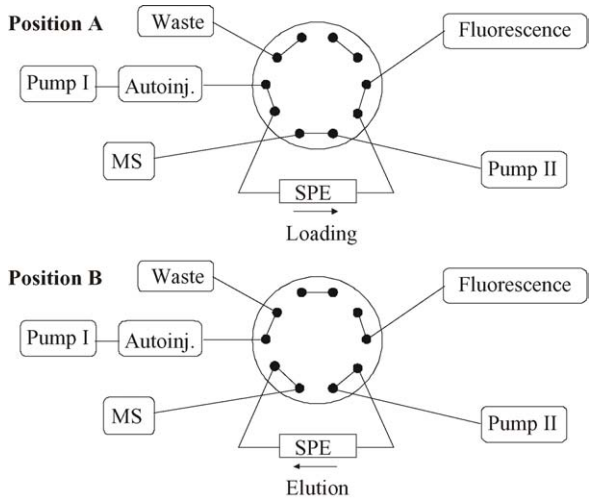

Fig. 2. Block diagram of the SPE–MS system with automated column switching in position A at the loading step and in position B at the elution step.

314.4; Fig. 1B) from GlaxoSmithCline (Middlesex, UK), Cimetidine (M_r 352.3; Fig. 1C) from Sigma (St. Louis, MO, USA), and Tiotidine (M_r 312.4; Fig. 1D) from Imperial Chemistries (UK). Iodoaminopotentidine (M_r 603.4; IAPT; Fig. 1E) and the fluorescent-labeled receptor ligands 5706 (M_r 497.0; Fig. 1F), 5707 (M_r 526.0; Fig. 1G), and 5708 (M_r 491.0; Fig. 1H) were synthesized in house [17]. Ammonium bicarbonate (>99%), dimethyl sulfoxide (p.a.), methanol (99.8%; MeOH), sodium acetate, and sodium carbonate (p.a.) were purchased from J.-T. Baker (Deventer, The Netherlands). Acetic acid (>99.8%, AAc), ammonium acetate (>98%), and formic acid (>99.8%; FAc) were obtained from Riedel-de Haën (Seelze, Germany), potassium carbonate (p.a.), potassium dihydrogenphosphate (p.a.), tri-potassium phosphate-7-hydrate (p.a.), and tri-sodium citrate dihydrate (p.a.) from Merck (Darmstadt, Germany), ammonium formate (>97%) from Aldrich (Steinheim, Germany) and triethylammonium bicarbonate from Sigma (St. Louis, MO, USA). High purity water was taken from a Milli-Q water system (Millipore, Eschborn, Germany).

2.2. Instrumentation

The solid phase extraction unit coupled to the mass spectrometric and the fluorescent detection (the latter used for breakthrough experiments) is shown in Fig. 2. Core of the setup was a 10-port valve on a Switchos II device (an advanced microcolumn switching unit from LC Packings, Amsterdam, The Netherlands). The loading pump (pump I in Fig. 2), integrated in the Switchos II, was pumping the loading solvent via an auto sampler (234, Gilson, Villiers-le-Bel, France) to the 10-port valve. After analyte injection and in valve position A, the solvent was flushed through the extraction column. In breakthrough measurements, the outlet was connected to a fluorescence detector (Model FP-1520, Jasco, Tokyo, Japan) and in other experiments to a collect vessel. The elution pump (pump II in Fig. 2) from Shicoh Engineering (Yamato, Japan) was in this valve position directly bypassed to the mass spectrometer. Switching the 10-port valve into position B connected the elution flow with the extraction column and bypassed the loading flow to the fluorescence detector (or to waste). A μ-precolumn cartridge (C18, 5 μm, 100 A, 300 μm i.d. × 5 mm) in a precolumn holder (both LC Packings, Amsterdam, The Netherlands) was used as SPE column.

The technical setup was controlled by the Ultichrom software (LC Packings, Amsterdam, The Netherlands) for automation. The internal instruments could be controlled directly and the external instruments via the analog outputs with starting and stopping signals.

The mass spectrometric detection was performed with electrospray ionization Q-TOF 2 (Micromass, Manchester, UK) mass spectrometer. The measurements were performed in positive ionization mode with 353 K source temperature, 423 K desolvation temperature, 250 L h^{-1} desolvation gas flow, 50 L h^{-1} cone gas flow, 17 psi gas cell pressure (i.e. 20 V collision voltage for optimum transfer through collision cell), 2500 V capillary voltage, and cone voltage as optimized for each ligand. The detected mass-range was set to a range of 5 Da beginning with the molecule mass as lowest mass. The data acquisition parameters were 1.0 s $scan^{-1}$, 0.1 s dwell time, full TOF MS continuous scan mode using the option 'MS profile' for each substance in the according mass range. Nitrogen (purity 5.0; Praxair, Oevel, Belgium) and argon (purity 5.0; Praxair) were used as desolvation/cone gas and collision gas, respectively.

A syringe (Hamilton, Reno, NV, USA) in a syringe pump (Harvard Apparatus 22, Harvard, MA, USA) was used for continuous infusion measurements.

2.3. Stock solutions and calibration

Stock solutions of tested ligands were prepared by dissolving each compound in 1 mL dimethyl sulfoxide, resulting in l0 mmol L^{-1} solution. Diluting the stock solution in several solvents as described for each experiment gives the working solutions. Solutions with concentrations in the range of l00 pmol L^{-1} to 100 nmol L^{-1} were obtained via a second stock solution (10 μmol L^{-1} out of the 10 mmol L^{-1} solution).

For quantification studies of the ligand 5708 without SPE step, eight solutions were prepared in the range of 100 pmol L^{-1} to 1 μmol L^{-1} in MeOH–ammonium formate solution (50 mM; pH 3) (80:20 v/v). These calibration solutions (0.1/0.5/1/10/50/100/500/1000 nmol L^{-1}) were subjected to continuous flow measurements with a flow rate of 10 μL min^{-1} or by 10 μL injections into a MeOH–ammonium formate (50 mM; pH 3) flow (80:20 v/v; flow rate 10 μL min^{-1}) connected with MS. In continuous flow experiments, the signal intensity was obtained by accumulating the counts for 1 min measurements and in flow injection analysis–mass spectrometry (FIA–MS) measurements the signal intensity was obtained by peak area integration.

For quantification studies of ligand 5708 in FIA-fluorescent detection, eight solutions were prepared in the range of l0 nmol L^{-1} to 1 μmol L^{-1} in MeOH–ammonium formate solution (50 mM; pH 7) (50:50 v/v). These calibration solutions (10/20/30/40/50/100/500/1000 nmol L^{-1}) were measured by

5 μL injections into a MeOH–ammonium formate (50 mM; pH 7) flow (50:50 v/v; flow rate 10 μL min^{-1}) connected with fluorescent detection. For comparative studies, three solutions (100, 500, and l000 nmol L^{-1}) were prepared in phosphate buffer (25 mM; pH 7). These calibration solutions were measured by 5 μL injections into a phosphate buffer (25 mM; pH 7) flow with 10 μL min^{-1} flow rate and subsequent fluorescent detection.

The excitation/emission wavelength pair was set to 360/440 nm in both fluorescence studies and the signal intensities were obtained by peak area integration.

Identical solutions were made for the ligands 5706 and 5707 and detected with mass spectrometric or fluorescence detection. The excitation/emission wavelength pair was set to 320/400 nm for ligand 5707 and to 330/455 nm for ligand 5706.

For quantification studies of the ligands with SPE, four solutions of each ligand were prepared in the range of 100 pmol L^{-1} to 10 nmol L^{-1} in MeOH–phosphate buffer (10 mM; pH 11) (30:70 v/v). These calibration solutions (0.1/0.5/1/10 nmol L^{-1}) were applied by 10 μL injections into the MeOH–phosphate buffer (10 mM; pH 11) loading flow (30:70 v/v; flow rate 50 μL min^{-1}). The elution was realized with a MeOH–ammonium formate (100 mM; pH 3) flow (80:20 v/v; flow rate 20 μL min^{-1}) connected with MS.

2.4. Mass spectrometric assays

2.4.1. Salt systems

To test signal intensities in different salt containing aqueous solutions, working solutions of each ligand (see Fig. 1A–E) were prepared with salts and pH as listed in Table 1. The 10 μmol L^{-1} samples were obtained from the 10 mmol L^{-1} stock solutions, the pH was controlled by a pH meter (691, Metrohm, Herisau, Switzerland). The samples were measured in continuous flow (flow rate 10 μL min^{-1}) and the signal intensities were obtained by 1 min integration.

2.4.2. Organic modifier and pH

To determine organic modifier and pH dependency, working solutions of four ligands (Fig. 1E–H) were prepared in 10 mM ammonium acetate, 20 mM ammonium bicarbonate and 10 mM ammonium formate, respectively, in concentrations of 10 μmol L^{-1} (out of the 10 mmol L^{-1} stock solution). The solutions contained 10, 25, or 50% MeOH, respectively and were adjusted to pH 3.0, 4.0, or 5.0, respectively. The samples were measured in continuous flow (10 μL min^{-1} flow rate) and calculated by 1 min integration.

2.5. SPE elution assay

To test elution properties, 100 nmol L^{-1} working solutions of three ligands (Fig. 1F–H) were prepared in ammonium bicarbonate (20 mM; pH 11.0). The 10 μL of each sample was injected into a loading flow of MeOH–phosphate buffer (50 mM; pH 11) (30:70 v/v; flow rate 20 μL min^{-1}) in valve position A (Fig. 2). After flushing the column 10 min with loading buffer, the valve was switched into position B. The elution flow was varied in a set of measurements with different MeOH contents. The elution flow with 20 μL min^{-1} flow rate contained X% MeOH and $(100 - X)$% ammonium formate solution (final concentration was 10 mM; pH 3) with $X = 50, 60, 70, 80, 90$. The elution flow was connected with the mass spectrometer and both peak size and intensity were determined.

2.6. SPE pre-concentration assay

A working solution of ligand 5708 (1 μmol L^{-1} in ammonium formate (10 mM; pH 7.0)) was prepared to determine pre-concentration properties, and 10 μL samples were injected into the loading flow of which the MeOH contents were varied. The loading flow contained in a set of measurements X% MeOH and $(100 - X)$% phosphate buffer (final concentration was 10 mM, pH 11) with $X = 0$, 10, 20, 30, respectively. The flow rate was 100 μL min^{-1} and the valve was set to position A (Fig. 2). After flushing the column 10 min with loading solvent, the valve was switched into position B. The elution flow at a 20 μL min^{-1} flow rate contained MeOH–ammonium formate solution (100 mM; pH 3) (90:10 v/v). The elution flow was connected to the mass spectrometer and both peak size and intensity were determined.

2.7. Sample solution assay

To test the solution efficiency of the hydrophobic substances in the sample vials, working solutions of 5708 (1 μmol L^{-1})

Table 1
Applicability and estimation of different salt systems for the mass spectrometric detection of the H2-receptor ligands shown in Fig. 1

Salt system	pH	MS efficiency	Comments
Ammonium acetate (10 mM)	6.9	Very useful	High MS response, low contamination potential
Ammonium bicarbonate (20 mM)	6.7 (+AAc), 7.5 (+FAc)	Very useful	High MS response, low contamination potential
Ammonium formate (10 mM)	6.4	Very useful	High MS response, low contamination potential
Dest. H_2O, no salt	6.8	Very useful	High MS response, not usable in biochemical reactions
Potassium carbonate (2 mM)	7.9	Restricted useful	Good MS response, but factor 10 lower than above, low contamination potential
Sodium acetate (2 mM)	6.9	Not useful	Low MS response, high contamination potential
Sodium carbonate (2 mM)	7.4	Restricted useful	Good MS response, but factor 10 lower than above, low contamination potential
Sodium citrate (l mM)	7.9	Restricted useful	Good MS response, problematic if stainless steel is used
Triethylammonium bicarbonate (20 mM)	7.5	Not useful	Low MS response, high contamination potential

were prepared in X% MeOH and (100 − X)% phosphate buffer (final concentration was 10 mM, pH 11) with X = 0, 10, 20, 30, respectively. The 10 μL samples were injected into the loading flow of MeOH/phosphate buffer (10 mM; pH 11) (30:70 v/v; flow rate 100 μL min^{-1}) in valve position A (Fig. 2). After 10 min flushing the column with loading solvent, the valve was switched into position B. The elution flow at a 10 μL min^{-1} flow rate contained 90% MeOH and 10% ammonium formate solution (100 mM; pH 3). The elution flow was connected to the mass spectrometer and both peak size and intensity were determined.

3. Results and discussion

3.1. Mass spectrometric optimization studies

3.1.1. Volatile salt solutions

Solvents and additives that are needed in biomedical and biochemical samples (such as BSA, EDTA, Tris or HEPES) are often incompatible with mass spectrometric detection. These samples mostly contain non-volatile salts, ion pairing agents or cluster building compounds which need to be avoided as much as possible in the used samples. Avoiding these interferences is an important issue and, therefore, it is important to find optimal buffer/salt systems combining biological integrity with detection sensitivity.

To develop the sensitive detection properties, several volatile salts were tested for their utility in mass spectrometric detection. Compounds A–E in Fig. 1 were dissolved in the salt systems shown in Table 1, and in case of ammonium bicarbonate adjusted to neutral pH with acetic or formic acid. Due to their basic structure, the molecules are protonated at neutral pH and, therefore, detectable with electrospray ionization in the positive ion mode.

The mass spectrometric responses for the ligands in ammonia ion-containing solutions (ammonium bicarbonate, ammonium acetate and ammonium formate) were in all three sets significantly higher as compared to the other salts. Dissolved in Milli-Q water the molecules gave also high MS response, however, pure water was not used further on, due to low compatibility with bioassays. Sodium and potassium carbonate can be used if the alternative ammonia salts cannot be taken (e.g., due to incompatibility with molecules or bioassay). Due to the presence of high amounts of sodium or potassium ions, the resulting sodium or potassium adducts have to be taken into account in MS detection. However, the mass spectrometric response of protonated analytes in these salts was up to a factor 10 lower than in ammonia ions-containing solutions. Molecules in sodium citrate solutions had a mass spectrometric response similar to sodium carbonate samples. Since citrate is known to build metal-complexes [39] and give problems in combination with stainless steel (often used in chromatography). However, it can be used in alternative systems where metal surfaces are exchanged by other materials. Sodium acetate and triethyl ammonium bicarbonate could not be used due to very low mass spectrometric response of the analytes and high contamination of the mass spectrometric cone.

3.1.2. Modifier content and pH

To obtain best mass spectrometric conditions in the positive ion mode, the content of organic modifier and the pH was varied. Therefore, four analytes (Fig. 1E–H) were dissolved in ammonium bicarbonate, ammonium acetate, and ammonium formate solutions adjusted to different pH and containing different methanol contents.

Fig. 3 shows the mass spectrometric response of 5708 obtained by integration of 1 min continuous-flow measure-

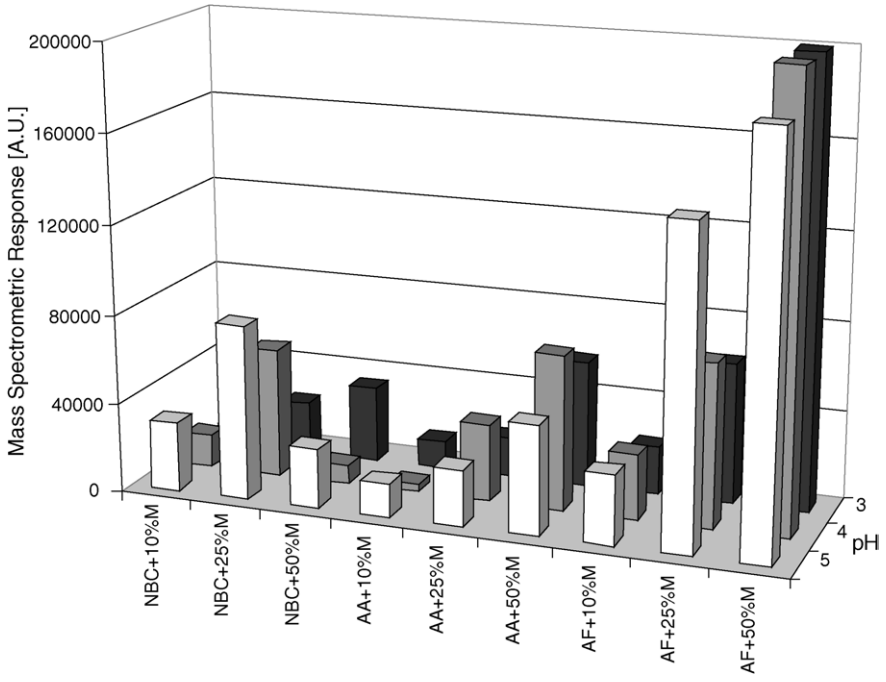

Fig. 3. Mass spectrometric responses of ligand 5708 in different salt systems (ammonium bicarbonate (NBC); ammonium acetate (AA); ammonium formate (AF)) with different MeOH (M) contents (10, 25 and 50%) at different pH values (3–5).

ments. Analytes dissolved in ammonium acetate and ammonium formate solution gave higher MS response with increasing methanol content, whereby results with ammonium bicarbonate showed no trend. The effect (of increased signal intensity with increasing methanol contents) is explained by a decreased surface tension of aqueous droplets caused by organic modifier [40]. Leading to a more efficient droplet desolvation, it results in more gaseous ions that can be detected in the mass spectrometer.

The influence of pH (Fig. 3) showed no general trend. The protonation of ligands seemed not to be influenced in acidic solutions leading to a similar response in all conditions. This result confirms the observation that the basic molecules are effectively charged at both neutral and lower pH. However, 5708 dissolved in ammonium formate and methanol (50:50 v/v) adjusted to pH 3 or 4 gave the highest mass spectrometric response.

The other ligands (data not shown) behave the same as described for ligand 5708. Dissolved in ammonium bicarbonate the ligands gave no trend, dissolved in ammonium formate as well as in ammonium formate the responses increased by raising methanol contents. The pH did not influence the response significantly, except for the ammonium formate solution with 50% MeOH. The mass spectrometric response in these solvents adjusted to pH 3 and 4 were significant higher (up to a factor 100) compared to the other solutions.

Therefore, in forthcoming samples, ammonium formate solutions adjusted to pH 3 with high methanol contents were used as spraying solvent. The corresponding results show best mass spectrometric response for all compounds.

3.2. Mass spectrometric and fluorescent limits of detection

The LODs of ligands 5706, 5707, and 5708 (Fig. 1F–H), were obtained in mass spectrometric and in fluorescence detection. Due to the fluorescent labels on these compounds, they are ideal for comparison measurements. The mass spectrometric quantification was performed in FIA and in direct-infusion measurements. The first technique was performed by sample injections into a carrier flow coupled to MS and the latter by continuous introduction of sample into the mass spectrometer. Several ligands were dissolved in methanol and ammonium formate solution (80:20 v/v; pH 3) that was also used as carrier flow. Fluorescent detection was performed in methanol and ammonium formate solution (50:50 v/v; pH 7) as well as in phosphate buffer (pH 7.0).

The LODs for ligand 5708 tested under several conditions are listed in Table 2. In contrast to pharmaceutical publications dealing with urine and plasma so far, the limits of detection in this study are stated in absolute amounts rather than concentrations of detectable compounds. In receptor binding assays, specific bound ligands are eluted after binding to the receptors and pre-concentrated on SPE material, therefore, the absolute amount of ligands bound to receptors is of interest. The LODs were 1 fmol for both mass spectrometric detection methods and 75 fmol in the fluorescence detection dissolved in MS compatible and 250 fmol in pharmaceutical compatible conditions. In both methods, the mass spectrometric LODs for 5706 and 5707 were for both ligands 1 fmol absolute (data not shown). The LODs for 5706 and 5707 in fluorescent detection were in the middle to higher fmol range similar to compound 5708 (data not shown).

3.3. Solid phase extraction optimization studies

The solid phase extraction pre-treatment of the H2-receptor antagonists' famotidine, ranitidine, and cimetidine has been investigated in several previous studies [32,36,38]. These methods could not be directly transferred to the more hydrophobic fluorescent-labeled drugs used in these study. The recoveries of basic molecules are often poor due to the high potential of non-specific surface binding. Different stationary phases and elution properties for these kinds of compounds were studied for basic drugs earlier [41–45]. However, not all of them are MS compatible.

3.3.1. Column elution

As shown before, the analytes could be detected in ammonium formate solutions with high amount of methanol adjusted to pH 3. The SPE cartridge was loaded reproducibly with an amount of analytes. The subsequent elution was performed in different sets with methanol contents up to 90%. Best elution efficiency and peak shape were obtained with an elution flow of MeOH–ammonium formate (90:10 v/v). If the elution flow contained more than 70% MeOH the ligand could be eluted, however, by lowering the concentration of methanol peak tailing increased progressively. Methanol contents below 70% allowed no quantitative analysis due to insufficient elution. The experiments demonstrated for ligand 5708 gave same results for the ligands 5706 and 5707. In all forthcoming experiments, the elution flow was MeOH–ammonium formate solution (l00 mM; pH 3) (90:10 v/v) with a flow rate of 20 μL min^{-1}.

3.3.2. Column loading

In a set of different experiments, methanol contents and pH of the loading flow were varied to determine the most efficient way to load the basic analytes. The amount of analyte breakthrough was detected by fluorescence detection and recoveries

Table 2
Mass spectrometric and fluorescent quantification of the H2-receptor ligand 5708 with method description, limits of detection (S/N; 3/1), R^2-value, calibration range

Quantification method/condition	LOD (fmol)	R^2-value; (calibration range)	Detection conditions
MS quantification (MeOH:H_2O; pH 3.0)	1	0.9725 (1 fmol to 200 pmol)	*m/z* 492.0 FIA (10 μL injection)
MS quantification (MeOH:H_2O; pH 3.0)	1	0.9998 (1 fmol to 200 pmol)	*m/z* 492.0 (cont. flow; 10 μL min^{-1})
Fluorescence quantification (MeOH:H_2O; pH 7.0)	75	0.9984 (50 fmol to 5 pmol)	Ex./em. 360/440 FIA (10 μL injection)
Fluorescence quantification (phosphate buffer; pH 7.0)	250	0.9651 (50 fmol to 5 pmol)	Ex./em. 360/440 FIA (10 μL injection)

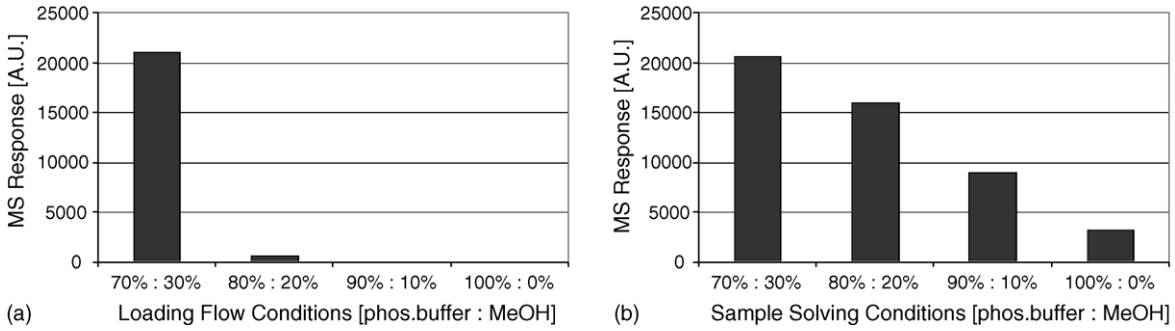

Fig. 4. Mass spectrometric responses of ligand 5708: (a) in different SPE loading conditions (0–30% MeOH) and (b) in different sample solving conditions (0–30% MeOH). For (a) the samples were solved in MeOH–phosphate buffer (30:70 v/v; pH 11) and for (b) the loading flow was MeOH–phosphate buffer (30:70 v/v; pH 11). The elution flow was in both sets MeOH–ammonium formate solution (90:10 v/v; pH 3; flow rate 4 μL min^{-1}).

were determined by MS (data not shown). Analytes loaded with MeOH–ammonium formate solution at pH 7 showed breakthrough and no extraction occurred. No breakthrough occurred when analytes were loaded with MeOH–ammonium formate solution, pH 11, even after flushing the column for more than 30 min. The molecules could not be retarded on a C18 material at pH 7 due to the carrying charges described above, whereby the uncharged molecules at pH 11 were detected in good recoveries without breakthrough.

A set of measurements with several contents of MeOH in the loading flow was tested to determine best anti-sticking properties. Sample solution conditions as well as elution conditions were kept constant during all measurements. As shown in Fig. 4(a), no ligand was detected if the MeOH content was below 30%. At 30% MeOH, the ligands were effectively trapped on the column whereas a specific binding was suppressed. Higher amounts of MeOH did not increase the amount of trapped analytes.

In all forthcoming experiments, the loading flow was MeOH–phosphate buffer (25 mM; pH 11) (30:70 v/v) with a flow rate of 50 μLmin^{-1}.

3.3.3. Sample dilution

Several contents of MeOH in the sample solving solution were tested due to expected ligand sticking to the sample vials. Loading conditions as well as elution conditions were kept constant in all measurements. As shown in Fig. 4(b), the amount ligand detected was increasing if the MeOH content was raised from 0 to 30%. With 30% MeOH, the non-specific binding of this ligands was suppressed and the transfer into the loading flow was quantitatively.

In all forthcoming experiments, the sample was dissolved in MeOH–phosphate buffer (25 mM; pH 11) solution (30:70 v/v).

3.4. SPE–MS limits of detection

LODs of 5 fmol for ligands 5706, 5707, and 5708 were obtained in SPE–MS as shown in Table 3. All ligands were dissolved in MeOH–phosphate buffer (25 mM; pH 11) solution (30:70 v/v) and loaded also with this solution at a flow rate of 50 μL min^{-1}. The subsequent elution was realized with MeOH–ammonium formate solution (100 mM; pH 3) (90:10 v/v) at a flow rate of 20 μL min^{-1}.

Table 3
SPE–MS limits of detection (S/N; 3/1) of ligands 5706, 5707, and 5708 (Fig. 1F–H) with method description, R^2-value and calibration range

Compound	LOD (fmol)	R^2-value ($n = 4$); (calibration range)	Detection conditions
5706	5	0.9865; (1–l00 fmol)	*m/z* 492.0 SPE–MS (10 μL injection)
5707	5	0.9998; (1–l00 fmol)	*m/z* 527.0 SPE–MS (10 μL injection)
5708	5	0.9763; (1–l00 fmol)	*m/z* 498.0 SPE–MS (10 μL injection)

4. Conclusions

The systematically developed SPE–ESI–MS method clearly achieves the sensitive analysis of high affine, hydrophobic, and basic H2-receptor ligands. This analytical method breaks the way to detect ligands in further receptor experiments resulting in maximal 100 fmol amount. Results from ligand adsorption experiments with several H2-receptor bound molecules will be published (with assay details) in a further publication [46].

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