Advances in mass spectrometry-based post-column bioaffinity profiling of mixtures

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Received: 30 August 2010 / Revised: 29 October 2010 / Accepted: 31 October 2010 / Published online: 24 November 2010
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Abstract In the screening of complex mixtures, for example combinatorial libraries, natural extracts, and metabolic incubations, different approaches are used for integrated bioaffinity screening. Four major strategies can be used for screening of bioactive mixtures for protein targets—pre-column and post-column on-line, at-line, and off-line strategies. The focus of this review is on recent developments in post-column on-line screening, and the role of mass spectrometry (MS) in these systems. On-line screening systems integrate separation sciences, mass spectrometry, and biochemical methodology, enabling screening for active compounds in complex mixtures. There are three main variants of on-line MS based bioassays: the mass spectrometer is used for ligand identification only; the mass spectrometer is used for both ligand identification and bioassay readout; or MS detection is conducted in parallel with at-line microfractionation with off-line bioaffinity analysis. On the basis of the different fields of application of on-line screening, the principles are explained and their usefulness in the different fields of drug research is critically evaluated. Furthermore, off-line screening is discussed briefly with the on-line and at-line approaches.

Keywords Mass spectrometry · On-line based bioassays · Post-column bioaffinity screening · Bioactive mixture analysis

Abbreviations

Mant- 2′-O-(N-methylantraniloyl)guanosine-3′,5′-cGMP cyclic monophosphate
AMC 7-Amido-4-methylcoumarin
ACH E Acetylcholinesterase
ACHBP Acetylcholine binding protein
ATP Adenosine triphosphate
APCI Atmospheric pressure chemical ionization
Z-FR CBZ–Phe–Arg
CYPs Cytochromes p450
cAMP Cyclic adenosine monophosphate
cGMP Cyclic guanosine monophosphate
ESI Electrospray ionization
ER Estrogen receptor
EIC Extracted-ion chromatogram
IT TOF Ion-trap–time-of-flight hybrid MS
GST Glutathione-S-transferase
HPLC High-performance liquid chromatography
HRS High-resolution screening
HTS High-throughput screening
MALDI Matrix-assisted laser-desorption ionization
MS Mass spectrometry
NMR Nuclear magnetic resonance spectroscopy
PKA Protein kinase A
SIM Selected-ion monitoring
SRM Selected-reaction monitoring
SPE Solid-phase extraction

Introduction

Within the pharmaceutical industry, microplate-based high-throughput screening (HTS) methodology is used for most bioaffinity screening applications from the early hit stage...
up to the phase of lead development. These approaches suffer from a number of limitations, especially the purity of the compounds screened. When dealing with mixtures, identifying the individual bioactive compounds can be a cumbersome endeavor. This is also true when combinatorial chemistry and parallel synthetic libraries are used as starting points for drug discovery.

Conventionally, when dealing with bioaffinity assessment of mixtures, they are first fractionated [1], the collected fractions are evaporated, and microplate bioassays are used to detect the bioactive fractions, eventually in parallel with compound identification by mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) spectroscopy [2–7]. The general workflow of such a process is depicted in Fig. 1. The challenge in these procedures lies in the difficulty of correctly matching the dereplicated bioactive compounds in particular fractions with their chemical structure [8, 9]. Typical examples include fractionation of intact proteins before matrix-assisted laser-desorption ionization (MALDI) MS analysis [10], and many types of biochemical assay formats that are conducted after fractionation of bioactive mixtures, such as natural extracts [11, 12], metabolic mixtures [13], and environmental mixtures [14–17]. The main drawback is that the high resolution obtained from the separation step (chromatographic peaks of seconds to tens of seconds) is often lost in the low-resolution fractionation process (fractions in the minute range are common).

Recent advances in separation science and detection technology have fuelled research into adjusted or newly designed bioassay formats [18, 19]. Our research interest is in bioassays hyphenated to analytical separation techniques. Pre and on-column bioaffinity screening formats will be reviewed by us in the near future [20]; this paper focuses on MS based post-column bioassay formats.

The direct on-line post-column coupling of a bioassay to a chromatographic separation is an effective alternative to HTS. This so-called high-resolution screening (HRS) demands rapid and straightforward on-line bioassays in order to achieve high resolution and sensitivity [21–23]. HRS was pioneered by the research groups of Przyjazny [24, 25] and Irth [26], using HRS systems based on UV or fluorescence detection. In this review, the focus is on HRS systems in which MS detection is an integrated part of the analytical approach. There are currently three general ways to implement MS detection in HRS:

1. MS detection replaces UV or fluorescence detection, thus providing MS-based readout of the bioassay (Fig. 2a);
2. MS detection is performed in parallel with the (fluorescence-based) on-line bioassay (Fig. 2b); and
3. MS detection is performed in parallel with micro-fractionation before off-line bioassay (Fig. 2c).

In all three approaches, chemical information derived from MS detection can be directly correlated with the bioaffinity information [27–29]. Available examples of MS-based HRS systems are reviewed and the merits and limitations of the various approaches to MS-based HRS bioassays are discussed.

On-line bioaffinity analysis with direct MS readout

History

Initial proof-of-principle of HRS approaches was performed with colorimetric (UV–visible) and fluorescence readout. In these studies, high-affinity binding partners, for example biotin – (strept)avidin and/or digoxin – anti-digoxin antibodies were used. The HPLC eluent with biotin and biocytin as analytes was mixed with a continuous flow of an avidin solution, in which a dye ligand with increased absorption properties upon avidin binding provided the detection principle [24]. Subsequently, UV detection was replaced by fluorescence detection in a system in which biotin, upon binding, results in fluorescence enhancement of the fluorescently labeled avidin [25]. Although the fluorescence-based bioassay was more sensitive, the technique was intrinsically compromised by the need for unknown ligands to cause fluorescence enhancement upon binding. A ligand displacement approach with a fluorescent antibody tracer enabled analysis of unknown ligands by the use of an immobilized ligand affinity column before detection [26]. Various other applications were developed along these lines. Alternatives to the affinity trapping columns were also...
The first example of an on-line bioassay with electrospray ionization (ESI) MS rather than fluorescence readout was described by Hogenboom et al. [35]. A typical bioassay setup with direct MS readout is depicted in Fig. 2a. The competitive bioassay specifically used the high-affinity streptavidin and fluorescein-biotin interaction. Because the affinity interaction between biotin and streptavidin is one of the strongest currently known interactions ($K_a = 0.6 \times 10^{15} \text{ L mol}^{-1}$), this affinity complex is easily preserved during the ESI MS process. The use of fluorescein-biotin enabled straightforward comparison with the fluorescence readout demonstrated previously. A lower affinity interaction between anti-digoxin and digoxin ($K_a = \text{approx. } 10^9 \text{ L mol}^{-1}$) was also tested and gave comparable results.

MS for on-line bioassay readout

The first example of an on-line bioassay with electrospray ionization (ESI) MS rather than fluorescence readout was described by Hogenboom et al. [35]. A typical bioassay setup with direct MS readout is depicted in Fig. 2a. The competitive bioassay specifically used the high-affinity streptavidin and fluorescein-biotin interaction. Because the affinity interaction between biotin and streptavidin is one of the strongest currently known interactions ($K_a = 0.6 \times 10^{15} \text{ L mol}^{-1}$), this affinity complex is easily preserved during the ESI MS process. The use of fluorescein-biotin enabled straightforward comparison with the fluorescence readout demonstrated previously. A lower affinity interaction between anti-digoxin and digoxin ($K_a = \text{approx. } 10^9 \text{ L mol}^{-1}$) was also tested and gave comparable results. An important consideration here is that in-solution dissociation constants cannot simply be correlated with the degree of conservation of non-covalent complexes in the gas phase. Typically, complexes involving electrostatic interactions are easily preserved or even strengthened because of the lack of solvent shielding in a vacuum. In contrast, complexes involving hydrophobic interaction tend to dissociate readily in the gas phase. This implies that for every target–ligand interaction, the optimum bioassay conditions for on-line MS based bioassays have to be determined.

Derks et al. demonstrated one of the main advantages of MS as readout, namely the ease of measuring different ligand–target interactions simultaneously in one bioassay.
They measured streptavidin–biotin and anti-digoxin antibodies–digoxin interactions in one bioassay format. The small ligand molecules (biotin and digoxin) were chromatographically separated before on-line bioaffinity analysis with direct MS readout.

When coupling the effluent of an LC separation on-line to a bioassay, one has to be aware of specific compatibility problems. First, the effluent has to be diluted to bioassay-compatible conditions. Therefore, the organic modifier concentration has to be lowered to a maximum of 5 to 10% for most (soluble) enzyme and receptor targets. This means that dilution by a factor of 10 to 20 has to be established, assuming that very high organic modifier concentrations are often used at the end of reversed-phase gradients. Furthermore, as many LC separations use acid modifiers, dilution (with bioassay buffer, target enzyme, and tracer ligand or substrate) must also result in a change in pH to a bioassay compatible pH. For this, the buffer capacity should be sufficient; but for MS compatibility, the other hand, the buffer concentration should not be too high. For the process of mixing in the post-column bioassay, a standard inverted Y-piece suffices. The actual on-line incubation can occur in normal PEEK tubing, but when slightly longer reaction times are required (usually for enzymes), a knitted reaction coil can be used instead to reduce post-column band broadening. A typical advantage of this technique is that the very short post-column incubation times enable use of slightly higher concentrations of organic modifier than with traditional bioassays.

With potential ESI ion source contamination and the associated ionization suppression in mind, one could raise questions against the concept of direct ESI MS readout in biochemical assays. Because of the continuous influx of proteins, buffers, and, eventually, other constituents of the bioassay into the ESI MS source, in several cases an on-line bioassay is not truly compatible with ESI MS readout. However, from the bioassay perspective, important benefits are achieved with MS readout compared with fluorescence readout. Most importantly, MS-based bioassays do not rely on the availability of fluorescent probes, tracers, or substrates. There are evident problems with fluorescent labeling because ligands, substrates, or other bioassay components must be chemically modified to incorporate the fluorescent label required. With MS readout, native ligands or substrates can be used. When, in addition, the bioassay is coupled on-line to LC separation in a continuous-flow format for bioaffinity assessment in mixtures, the MS can be used to simultaneously measure drug target–ligand/substrate interaction and identify the ligands/substrates [35]. For this purpose, the mass spectrometer is used in two modes:

1. selected-ion monitoring (SIM) or selected-reaction monitoring (SRM) to detect and quantify the free ligand concentration (for ligand-binding assays) or the product formed (for enzymatic drug targets); and

2. full-spectrum operation, eventually involving data-dependent switching to MS² acquisition, to identify the eluting ligands.

When looking in more detail at mass spectrometers that could be used for full-spectrum operation (mode 2), the advantages and disadvantages are the same as when performing normal LC–MS(−MS) analysis. One has to bear in mind, however, that when combining both bioassay monitoring (mode 1) and analyte/ligand identification (mode 2), the analyte/ligand identification part is hampered by the post-column bioassay dilution and buffer conditions that are less favorable for typically used positive ESI ionization (low organic modifier concentration at a bioassay-compatible pH of ~6 to 7.5). For the bioassay part (mode 1), usually a robust and convenient mass spectrometer should suffice (e.g. ion-trap or quadrupole) but, because the analyte identification part (mode 2) demands good sensitivity, resolution, and the possibility of analyte fragmentation, often a hybrid mass spectrometer is the first choice. When one deals with a bioassay with a very low rate of enzymatic product formation or a low concentration of enzyme in the bioassay, a triple-quadrupole mass spectrometer, which enables very specific and sensitive product monitoring, could be considered. Until now however, in most cases a Q-TOF (or ion-trap–time-of-flight) mass spectrometer has been the instrument of choice enabling mode 1 with sufficient sensitivity and specificity and mode 2 with sufficient sensitivity and resolution and the possibility of analyte fragmentation.

Figure 3 shows a typical example of results obtained with HRS methodology employing MS readout. In this
Applications of on-line bioassays with MS readout

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the neurotransmitter acetylcholine, producing choline and acetic acid. AChE is found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity serves to terminate synaptic transmission. As such, AChE is the target of many Alzheimer’s drugs, nerve gases (e.g. Sarin), and insecticides.

An on-line bioassay with MS readout for the identification of AChE inhibitors was described by de Jong et al. [39]. The native substrate acetylcholine was used and the product choline was monitored on-line by MS readout. Inhibitory compounds prevent product formation in the reaction coils before MS. Figure 4 shows the MS traces of both the substrate and the product in the on-line substrate conversion assay, and the MS trace of the injected inhibitor galantamine. It also shows the process of starting up the bioassay in the first 20 min of the chromatograms. The MS is operated in such a way that both inhibitor...
identification and on-line enzyme activity monitoring are achieved.

Phosphorylation and de-phosphorylation of proteins and peptides, and the formation and breakdown of cyclic phosphate molecules, e.g., cAMP and cGMP, are pivotal for most endogenous signaling processes [40]. Protein phosphatase and kinase enzymes are involved in these processes. Post-column on-line affinity-based analysis of phosphorylated peptides and proteins was described by Krabbe et al. [41, 42], who used a ligand-exchange reaction between eluting phosphopeptides/proteins and the metal–ligand complex iron(III)–methylcalcein blue, which releases the tracer ligand methylcalcein blue. The reaction is monitored by MS, switching between SIM and data-dependent MS–MS, enabling both quantification of the tracer and identification of the phosphopeptides. This proof-of-principle study also demonstrated that the approach, although elegant, lacks sufficient sensitivity for studies in which very low concentrations of phosphopeptides are involved, for example phosphoproteomics studies.

Different approaches to an on-line inhibition assay of a protease/peptidase were reported by de Boer et al. [37, 43, 44], who used the enzymatic conversion of a peptide substrate by cathepsin B into two products (Z-FR and AMC) as a model system. Product formation was continuously monitored by direct on-line ESI MS (Fig. 3). The enzymatic conversion rate was reduced on injection of an inhibitor, resulting in negative peaks in the MS readout. By combining SIM analysis of the products and full-spectrum analysis, unknown inhibitors can be detected and characterized, using the extracted-ion chromatogram (EIC), as shown in Fig. 5, where a fungal extract containing bioactive compounds is profiled. Correlation between SIM and EIC traces enables retention time and peak shape correlation between bioaffinity and chemical information (m/z of the inhibitor) [37]. In a follow-up study, high-temperature LC, enabling lower organic modifier concentrations, was demonstrated [43]. As the ESI MS source in direct monitoring of a biochemical reaction mixture is prone to contamination, miniaturization of the bioassay can be highly advantageous. Reduction of the flow-rate of the bioassay to the ESI MS source by a factor of 25–50 was achieved by use of microfluidics-based continuous flow [44].

![Fig. 5](image-url)
On-line bioaffinity analysis with parallel MS detection

As discussed above, direct MS readout of bioassays is not always attractive, especially because of potential ionization suppression effects. However, it must be emphasized that on-line implementation of MS detection in HRS applications is necessary for compound identification. In that case, on-line MS detection is performed in parallel with the bioassay readout based on, e.g., fluorescence detection. This general setup is depicted in Fig. 2b. It features a post-column split with part of the column effluent being directed to the continuous-flow bioassay and another part to ESI MS (or MS^n) for simultaneous chemical characterization of the eluting bioactive compounds. The parallel setup enables direct correlation of chemical and biological information (in a similar way as demonstrated for direct MS readout in Fig. 5). For analysis of bioactive mixtures, this setup is a powerful and versatile alternative to traditional fractionation processes followed by microplate bioassays.

In this section, various examples and applications of on-line bioaffinity analysis with parallel MS detection are reviewed. In this HRS format, ligand-binding and substrate conversion bioassays are most conveniently incorporated. For this, fast and straightforward bioassays are needed in order to allow sensitive detection and limited post-column band broadening in the reaction coils. However, a good percentage of targets with corresponding biochemical assay formats can be implemented efficiently in HRS systems. Examples include nuclear receptors, protein kinases, proteases, phosphodiesterases and phosphatases, and metabolic enzymes. In these cases, HRS can often give very rapid, robust and complete bioaffinity profiles of mixtures containing bioactive compounds.

Enzymatic conversion by AChE and binding to AChBP

Before the development of the MS-based on-line AChE bioassay, another, colorimetry-based, on-line bioassay was used [45] to enable the identification of inhibitors in extracts of the narcissus “Sir Winston Churchill” [46]. The alkaloid unguinorine was found to have a mild inhibitory effect on AChE. A more sensitive AChE bioassay was achieved by the use of a substrate which is hydrolyzed to a highly fluorescent product [47]. Using this approach, various bioactive plant extracts from bulbs of Galanthus nivalis, Eucharis amazonica, Crinum powelli, and Nerine bowdenii were screened and compounds with strong AChE inhibitory activity were detected and characterized by parallel MS detection. The same HRS system was used by Marques et al. to screen microsomal metabolic incubations of the first-generation Alzheimer’s drug tacrine for bioactive metabolites [48]. Parallel MS and off-line NMR after fraction collection was performed to characterize the metabolites. This example demonstrates that in some cases MS and MS^n characterization is insufficient for full identification of unknown bioactive compounds. The correlation between bioaffinity and MS-based chemical information obtained from HRS may direct fractionation. This improves the efficiency of purification and further structure elucidation, e.g., by NMR (see below).

Recently, an on-line bioaffinity screening approach related to the acetylcholine binding protein (AChBP) was described [49]. AChBP is a relevant model protein for the family of nicotinic acetylcholine receptors, important receptors possibly involved in many brain-related diseases, for example Alzheimer’s and Parkinson’s disease, ADHD (attention deficit/hyperactivity disorder) and chronic pain-related disorders. The bioaffinity detection is based on a specially developed tracer ligand the fluorescence of which is enhanced in the binding pocket of the protein. A schematic representation of the on-line bioassay principle is given as inset in Fig. 2b. Parallel MS detection was performed next to a parallel dual bioassay, one to assess the bioaffinity of the eluting ligands and the other a “blank” bioassay performed to detect native fluorescence properties of eluting compounds. The objective of the bioassay was analysis of small focused medicinal chemistry-like libraries and it enabled estimation of pK_i values of known compounds after injection at only one concentration, even for ligands in a mixture [49].

Bioassays with soluble receptors

HRS screening of mixtures for receptor affinity can be performed in different bioassay setups. For instance, a fluorescently labeled receptor can be used in conjunction with an immobilized affinity column at the end of the reaction coil, as used in antigen–antibody interactions (see above) [26] and in the screening of ligand mixtures for the urokinase receptor [50].

Alternatively, a homogeneous continuous-flow bioassay (without filtration or affinity column separation steps) can be used, as, for instance, demonstrated for estrogen receptor binding. In this case, fluorescence enhancement with the natural fluorescent estrogenic tracer ligand coumestrol is monitored [51]. Eluting ligands that displace coumestrol provide negative peaks on the baseline. Eventually, especially to increase sensitivity, a restricted access column can be used at the end of the reaction coils to remove the fluorescent non-receptor-bound coumestrol fraction from the solvent stream. As an alternative to fluorescence enhancement, detection based on fluorescence polarization (FP) has also been reported [52].
In an MS-based HRS setup, an estrogen receptor (ER) bioassay is especially interesting in the analysis of natural compounds such as phytoestrogens [27, 53], environmental samples, screening for endocrine-disrupting compounds [54] and metabolic mixtures, and profiling bioactive drug metabolites [55–57].

Various parts of the pomegranate fruit (Punica granatum; Punicaceae), e.g., juice, seed oil, and peel extract, have been shown to exert suppressive effects on human breast cancer cells in-vitro. A crude mixture of pomegranate peel extract was separated by HPLC followed by on-line bioaffinity analysis for the ER-β with parallel MS and MS–MS detection [53]. Three estrogenic compounds (luteolin, kaempferol, and quercetin) were detected and identified by comparing the molecular masses and negative-ion atmospheric-pressure chemical ionization (APCI) MS–MS spectra with data from an estrogenic compound library.

In order to efficiently assess the contribution of drug metabolites to the overall therapeutic and adverse effects, profiling of bioactive metabolites in metabolic mixtures is important in drug discovery. The power of HRS in this area was first demonstrated for metabolites of the nonsteroidal antiestrogen tamoxifen, used in the hormonal therapy of human breast cancer and, moreover, as a chemopreventive agent for women with an enhanced risk of developing breast cancer. Tamoxifen is known to be metabolized into multiple mono and dihydroxylated and N-demethylated derivatives, some of which have a much higher bioaffinity than tamoxifen itself. Metabolites in metabolic mixtures of tamoxifen were identified with simultaneous bioaffinity profiles for the ER-α [55]. For production of metabolic mixtures, incubations with microsomal liver fractions containing the most important cytochrome P450 isoenzymes involved in phase I metabolism are usually conducted. The results found were in accordance with many separate studies from the past, based on conventional screening directed at the bioaffinity of specific metabolites only. Subsequently, the HRS system was combined with parallel at-line bioreactors that enabled simultaneous metabolic CYP incubations of antiestrogenic drugs followed by on-line solid-phase extraction (SPE) trapping, washing, and elution to the HRS part of the system [54, 56]. As a result, metabolic schemes could be constructed, including bioaffinity information per metabolite.

Metabolic profiling with MS and MS–MS detection often does not lead to complete structure elucidation of the metabolites. HRS was therefore incorporated in a wider approach capable of efficiently profiling metabolites for their individual bioaffinity in combination with total structure elucidation by high resolution MS with an ion-trap–time-of-flight hybrid MS (IT-TOF), fractionation by preparative LC, and off-line NMR spectroscopy [57]. Dual parallel bioassays for ER-α and ER-β were included in the approach. Metabolites of known estrogenic drugs were generated using conventional CYP incubation (with human liver microsomes) and genetically engineered BM3 mutants (mutants of bacterial CYPs) [58, 59]. A typical HRS result obtained by use of this system is shown in Fig. 6. Bioactivity chromatograms from the parallel (dual) ER-α/ER-β bioassays are shown for metabolic mixtures (from human liver microsomes) of α-estradiol and β-estradiol injected at two concentrations. Finally, the bottom chromatograms show EIC traces from parallel MS detection of the mixture of α-estradiol metabolites.

Bioassays for phosphate-related ligands or substrates

As already indicated, phosphorylation and de-phosphorylation processes, and formation and breakdown of cyclic phosphate molecules (e.g. cAMP and cGMP) are pivotal for most endogenous signaling processes [40].

An HRS system for detection of phosphodiesterase (PDE) inhibitors in natural extracts, based on the conversion of the fluorescent substrate Mant-cGMP (2′,5′-cyclic monophosphate) into the highly fluorescent Mant-GMP was developed by Schenk et al. [22]. The applicability of the system was shown by analyzing a plant extract spiked with two PDE inhibitors. Quantification of the active compounds was by evaporative light-scattering detection, thus enabling estimation of the IC50 values of the bioactive compounds directly from the crude extract.

A generic on-line bioassay for phosphate-consuming or releasing enzymes was developed and applied to measurement of continuous phosphate release from alkaline phosphatase and to detection of tetramisole in a plant extract as an inhibitor of the enzyme [60]. A fluorophore-labeled phosphate-binding protein with strong fluorescence enhancement on binding to inorganic phosphate was used for readout, and parallel MS for confirmation of identity of the inhibitor.

In the vast majority of signaling pathways, protein kinases play pivotal roles in transmitting ligand-mediated signals by (de)phosphorylating proteins. Therefore, protein kinases are important targets for inhibition screening. The MAP kinase p38α (p38) is important in some types of cancer and inflammation processes and is regarded as a (potential) drug target. The development of an HRS system based on fluorescence enhancement in the (ATP) binding pocket of protein kinase p38 was demonstrated by Falck et al. [61]. Parallel MS enabled efficient correlation between bioaffinity for p38 kinase and identity of the inhibitor compounds.
Bioassays for proteases and peptidases

Proteases (and peptidases) are important in many biological processes, for example different hormonal processes in which cleavage of peptides or proteins changes the bioaffinity of proteins involved, in protein homeostasis of the body, and in many physiological reactions, from food digestion to blood-clotting and apoptosis. Various HRS approaches to assay protease activity or inhibition have been reported. In addition to the systems with direct MS readout, already discussed, systems with parallel MS have also been reported.

The angiotensin-converting enzyme (ACE) is an exopeptidase that is important in blood pressure regulation. An HRS system with parallel MS was developed for screening of ACE inhibitors [62]. Accurate-mass data, MS–MS fingerprints, and protein database search procedures were used to identify bioactive peptides found in mixtures, such as hydrolyzed milk samples, digests, or fermentation products.

Fig. 6 As metabolism of drugs can result in the formation of pharmacologically active metabolites, it renders them essential for early consideration in drug discovery and development programs. The figure shows HRS analysis used to rapidly enable the identification of individual estrogenic ligands in metabolic mixtures with simultaneous detection of their biological activity. Shown are analyses of metabolic mixtures, injected at two different concentrations depicted as superimposed solid and dotted bioaffinity chromatograms, of α-estradiol and β-estradiol. Parallel analysis of bioaffinity towards two ER subtypes (α and β) gives selectivity information. The m/z traces of expected and unexpected metabolites are plotted at the bottom of the figure to enable retention time and peak shape correlation in order to correlate bioactivities with identities. The m/z traces of the mother compounds are not shown. The second-last bioactive peak is from α-estradiol and the last one (longest retention time) is from β-estradiol. Metabolism was from human liver microsomes.
HRS screening for inhibitors of serine proteases, based on a fluorescently labeled peptide tracer that yields increased fluorescence on cleavage was recently demonstrated [63]. In order to keep the modifier concentration in the bioassay constant during gradient elution, a counter gradient was used that was parked in a semi-preparative LC column.

For analysis of the proteolytic enzyme activity of individual proteases in mixtures, the on-line combination of size-exclusion LC and ion-exchange chromatography under non-denaturing conditions and a bioassay has been developed by Schebb et al. [64]. Bioassay readout by UV detection is based on the protease cleavage of a p-nitroaniline-labeled peptide, releasing the colored p-nitroaniline. The method was applied to the characterization of the crude venom of the pit viper *Bothrops moojeni* and extracts of the pathogenic amoeba *Acanthamoeba castellanii*. It is a tool for serine protease screening in general by fast ESI MS–MS identification of active samples followed by the separation and isolation of active sample constituents in on-line bioaffinity analysis mode.

**Off-line and at-line bioaffinity analysis with parallel MS detection**

Although a great variety of bioassays can be converted into HRS systems with parallel MS detection, HRS is not always feasible, for instance because of the bioassay conditions demanded. It is difficult, or impossible, to use the HRS approach for bioassays requiring poorly soluble constituents, membrane-bound targets, and/or very long incubation times. Although use of gas-segmented post-column systems to reduce post-column band broadening effects has been demonstrated [65], such an approach provides only a very limited extension of the incubation time. Also, cellular bioassays [66–68] are currently out of reach. Targets present at very low concentrations and targets that require indirect means of detection are cumbersome to analyze with HRS methods. In such cases, the use of off-line or at-line techniques, which are still capable of correlating bioaffinity/bioactivity and chemical information on bioactive compounds in a mixture, can be considered [29]. Some examples are reviewed in the next section.

**Off-line bioaffinity analysis**

In the conventional approach to bioaffinity or bioactivity screening, which involves fractionation of the column effluent and subsequent plate reader-based bioassays, often using fluorescence readouts [69], MS is increasingly recognized as a universal, label-free tool for monitoring enzymatic reactions [70, 71], or determining the affinity of small molecules for a target protein [72]. MS is, therefore, increasingly being used for off-line screening of small molecules. A fully integrated platform with online sample cleanup, capable of sampling rates as fast as ~5–10 s per sample, is the so-called “RapidFire” technology [73–75]. The method involves rapid SPE purifications from assays conducted in 96 or 384-well plates after incubation. Extracted substrate(s) and/or product(s) from the incubations are rapidly eluted from the SPE cartridge into a triple-quadrupole mass spectrometer in order to quantify the substrate and product [76]. Figure 7 shows a schematic overview of the technology in combination with some typical results from a well plate analyzed. Bioassays for the screening of CYP inhibition [77], antibacterial substances [78], and others [79, 80] have been reported. The advantages of such an approach are the possibility of measuring not only one formed product, or converted substrate, but basically both, or even multiple products in a label-free manner. Until now, the technology has only been applied to pure compound screening.

A more-or-less generic approach for protein kinase screening involves use of a Caliper microfluidics platform based on parallel capillary electrophoretic separations and detection by laser-induced fluorescence [81–84]. In theory, the microfluidics platform could be placed on-line with MS, enabling label-free screening.

Another interesting off-line screening approach is based on the MALDI Flash Quant technology, basically a MALDI triple-quadrupole system operated in SRM mode to overcome interferences from the MALDI matrix [85, 86]. This technology has been used to determine the binding of small molecules to the murine GABA (gamma-amino-
butyric acid) receptor mGAT 1 [87], to determine the inhibition of protein kinase A (PKA) [88], and, in a multiplexed system, to investigate the enzyme activity of a kinase and an esterase in a single analysis [89].

At-line bioaffinity analysis

At-line bioaffinity screening combines the traditional fractionation approach with on-line bioaffinity analysis [29]. This technique involves mixing of the necessary biochemicals with the column effluent just before the fractionation process (Fig. 2c) and microfractionation, that is fractionation in a seconds rather than minutes time frame, to retain the high-resolution chromatographic separation. This enables correlation of the bioaffinity data with the chemical data from the MS which is positioned parallel to the microfractionator. As the bioassay is performed virtually off-line in 384 or 1536-well plates, free choices in incubation time, necessary biochemical additives, and readout formats can be made, because separation and incubation are independent. Moreover, drying and mixing issues are circumvented as the microtiter plates already contain the homogenous bioassay mixture with the fractionated compounds ready for incubation before plate reader-based bioaffinity analysis. Additional post-column splits in both on-line and at-line approaches can be used to run several biochemical assays in parallel. At-line bioaffinity screening has so far been demonstrated for screening of PKA inhibitors [29] and antibacterial substances [90]. In both cases, reconstructed high-resolution bioaffinity chromatograms could be plotted and compared with the MS chromatograms for bioaffinity-to-identity correlation. In the latter case, a regio-isomeric mixture of N-alkylated neomycin derivatives was separated, mixed with bacterial inoculum, incubated for 18 h, and, after addition of readout reagent, the antibacterial effect of the eluting substances was determined. Elucidation of the structures of the different (bioactive) regio-isomers could be accomplished by MS analysis on an IT-TOF MS instrument.

Conclusions and perspectives

A wide variety of bioassay formats for different biological targets and assay types have been successfully transformed into on-line or at-line bioassays with direct coupling to separation for mixture analysis. Additional bioassay formats involve pre-column and on-column techniques. Although all these hyphenated screening approaches can rapidly deliver comprehensive bioaffinity information with correlated compound identities, one must remember that after identification of the bioactive compounds found, traditional approaches have to take over. The hyphenated screening approaches are not suited to subsequent purification and/or synthesis of the bioactive compounds. There are three other main reasons why traditional approaches must be used after HRS-based identification of bioactive compounds in mixtures. First, most research facilities are adapted to traditional and integrated workflows of analyzing pure compounds from the hit stage to the lead development phase. Second, advances in more traditional screening now even enable elaborate and comprehensive “high content” analysis of single compounds that hyphenated bioassay-analytical screening methods can not yet compete with. Third, traditional techniques are applicable to a wider range of assays, from easy ligand-binding bioassays to high-content cellular biological assays with multiple readouts. Nevertheless, as a first and important step in finding and identifying bioactive compounds, HRS and related techniques discussed in this paper can play an important role, especially in the dereplication of bioactive mixtures. When an HRS strategy has been developed, bioactive mixture profiling can be performed more rapidly and in a more convenient fashion. Drug target classes that are most conveniently incorporated in on-line screening processes are, for instance, nuclear receptors, protein kinases, proteases, phosphodiesterases, phosphatases, and metabolic enzymes. Enzymes that are able to rapidly convert substrates into fluorescent products and receptors for which ligands are available that show fluorescence enhancement in the binding pocket are most suitable in this regard. For other enzyme and receptor drug targets, on-line MS-based bioassays or at-line screening methods are good alternatives, e.g., for bioassay formats for which only native substrates or ligands are available, bioassays that demand long incubation times, and other difficult bioassay formats, for example those involving membrane-bound drug targets and cellular assay formats. Although it is difficult to directly incorporate on-line and at-line approaches in traditional drug-discovery processes, efficient incorporation is certainly possible with modifications.

In summary, on-line and at-line strategies used to profile bioactive mixtures provide powerful tools for mixture analysis, capable of rapidly and straightforwardly correlating bioaffinity with compound identity. They do, however, need some development time and effort per drug target to be screened for and flexibility in terms of implementation into traditional drug-discovery workflows.

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