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Bioassay-coupled Chromatographic Analysis of Medicinal Natural Products: A Review

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ABSTRACT

Medicinal plants have found immense use in drug discovery over the years as several chemically available drugs today are either directly from plant secondary metabolites or have their templates from such. Recent advances in the application of bioassay-coupled chromatographic analysis of plant extracts have led to reliable characterization of unknown samples with regards to their activity profile as well as the compounds responsible for observed activity. This paper reviews the progress made in the field of bioassay-chromatography hyphenated techniques for natural products analysis, notably medicinal plants. Different bioassays including microbial, biochemical, micro-chemical and cell based assays as well as several chromatographic and spectroscopic techniques such as Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Liquid Chromatography (LC), Over-Pressured Layer Chromatography (OPLC), Gas Chromatography (GC), Nuclear Magnetic Resonance (NMR), Fourier Transform Infra-red (FTIR) spectroscopy, Mass Spectrometry (MS), Surface Enhanced Raman Spectroscopy (SERS), Diffuse Reflectance Infrared Fourier Transform (DRIFT), etc. are discussed in this paper.

Introduction

Plants both marine and terrestrial contain underutilized pool of secondary metabolites which could be an important source of new and unique agents that may be of immense therapeutic potential.[1] However, the main challenge of natural product drug discovery is the identification of active compounds from complex matrices. Several strategies which involved the study of bioactivity only after the compounds have been isolated and characterized, have been used in time past for the discovery of active compounds but these have changed considerably in the last few decades due to their high cost and laborious methodologies.

Current strategies therefore involve bioassay-guided chromatographic experiments in which active components are isolated and identified. This is a result of the fact that chromatographic separation of the whole plant extract will not provide information about any observed biological activity while bioassay alone of the whole extract will also not give any information on the exact component responsible for the activity.[2] The process of separating components before biological analysis is also costly, requires great efforts and complex analytical system as well as database for the detection and identification of large number of substances.[3] Thus, the combination of efficient separation technique with bioassay seem to be the way forward and current advances made in the field of the application of bioassays in direct combination with chromatographic techniques has led to adequate characterization of unknown samples with respect to their profile of activity.[3] Bioautography, which refers to the hyphenation of chromatographic analysis with biological detection[4] can be done in both highly developed as well as small research laboratories with minimum sophisticated equipment.[5]

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Planar chromatography, basically thin layer chromatography (TLC) and paper chromatography (PC) are used for bioautography even though other advanced chromatographic tools such as high performance thin layer chromatography (HPTLC), over pressured layer chromatography (OPLC) and planar electrochromatography (PEC) have been introduced.[6]

The combination of bioassays, with separation methods and spectroscopic techniques has become a very attractive tool in the analysis of medicinal plant samples.[7] These analytical techniques are composed of chromatographic methods that place all necessary detectors in a single system, such that most relevant information (activity, spectrometric and spectroscopic) are obtained in a single run. Bioassay-coupled chromatography has also found application in biological finger printing of herbal samples.[8] Herbal finger printing involves a set of characteristic chromatographic and spectroscopic signals which lead to unambiguous sample identification upon comparison with reference(s). However, since traditional chromatographic analysis could only provide qualitative and quantitative information, compounds present in low concentration which may be responsible for the biological activity of the sample may not be detected.[8] Thus, the need to add a system which involves bioassay coupled with chromatography and spectroscopy.

In the search for active compounds from highly complex sample mixtures such as in the field of natural product research, target analysis only is ineffective.[3, 9] Combination of a database with high sophisticated hyphenated and comprehensive online methods are also not necessarily the key to the search for bioactive molecules as any disruption at a small link in the chain can force the whole process to a stop[3] thus reinforcing the need for bioassay-coupled chromatography. Bioassay-coupled chromatography also has the ability to reduce thousands of compounds in a complex mixture to a few important bioactive ones as well as reduce the rate of false positive seen in several experimental methods by complementing existing methodologies and providing specific information.[3] The application of hybrid chromatography in the analysis of plant samples have been discussed.[8] Characteristic points in the coupling process are bioassay, separation and detection.

Methodology

The search keywords used for this review include medicinal plants, natural products, bioassay techniques, chromatographic analysis, hyphenated techniques, secondary metabolites, and structure elucidation. The search was performed in scientific database such as Science direct, Pubmed central and Google scholar, and PubMed central Canada. For this review, 75 references were consulted.

Biological assay

Bioautographic assays are the key to screening of complex botanical samples for specific natural constituents.[10] A number of microbiological and biochemical assays has been transferred to planar chromatography like HPTLC even though this field of research is still largely underexplored despite the evident advantage of linking directly to bioactive compounds.[3]

Microbial assay

Coupling of planar chromatography with microbiological assays is an old practice.[11] Three bioautographic methods have been used: contact bioautography, direct TLC bioautography and immersion/agar overlay bioautography.[12, 13] Usually, developed TLC plates are immersed in suspension broth of microorganism and then incubated after which visualization is done directly on the plate. However, an improvement in the efficiency of the process leading to sharply bounded zones following 24 hours of incubation was recently achieved.[14] The detection of bioactive compounds in complex samples by bacterial assays using the directly bioluminescent marine bacteria *Vibrio fischeri* has been reported.[15-17] Many enzyme substrate reactions that show the viability of microorganism are suited for use in HPTLC bioassay. For instance, in the detection of algicides [18] and fungicides.[19, 20]

Biochemical assay

Biochemical assays are analytical *in vitro* procedures used for the detection, quantification and/or study of the binding or activity of biological molecules such as enzymes.[21, 22] An early part of drug discovery experimental process usually involves screening large number of compounds for activity using biochemical assays in an ultrahigh-throughput format.[23, 24] Recent advances in biochemical assay techniques have been because of contributions from both chemistry and biology, especially molecular biology, molecular genetics as well as engineering and information technology.[25-27] Biochemical assays already transferred to the plate include tests for;

- Anti-cholinesterase compounds using either the Ellman reagent or the fast blue salt B[28, 29]
- α- and β-Glucosidase inhibitors[30, 31]
- Xanthine oxidase inhibitors[32, 33]
- Tyrosinase inhibitors[34]
- Detection of estrogenic compounds[4]

Micro-chemical assay

Chemical derivatization can also be linked to activity for instance in assaying for antioxidants and radical scavenging. Several bio-autographic methods coupled with spectroscopy which led to identification of active constituents have been reported. This include: bleaching under UV 366 nm or white light after spraying the developed plate with β-carotene solution,[35] DPPH (2,2-dipheryl-1-Picrylhydrazyl) radical scavenging assay,[36] ABTS (3-ethylbenzthiazoline-6-suulphonic acid) radical scavenging assay[37] and β-carotene detection.[35]

Cell test System

There is complex interaction between drug and organism at multiple levels. This interaction cannot be predicted using biochemical assays only thus the need for cell-based screening assays as more biologically relevant option to predicting the response of organism.[23] Also, at some point in the drug discovery process, it will become important to know the cellular toxicity of the molecule of interest. Eukaryotic cell culture is an acceptable test model to get initial information on toxicity.[23] Choosing a cell-based screening assay method however requires an understanding of the endpoint being measured, correlation with cell viability, and the limitations of the assay chemistries.[23] A recent study describes a new platform for the fast and efficient screening for bioactive compounds in complex natural mixtures using a cell-based assay coupled with chromatography.[38]

Chromatography

Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC)

The most streamlined option for coupling chromatography with bioassay is through planar chromatography[3] since thin layer chromatography is one of the most popular separation techniques and all the limitation of column derived method can be avoided by the open, offline and planar format of TLC and HTPLC. Advantages include less effort and data handling is adjustable to different detector options and simple modular instrumentation. Usually, TLC is hyphenated with appropriate bioassay to enable direct *in vitro* activity study of an extract that has previously been separated into its components on the plate. Coupling of TLC with bioassay and a spectroscopic technique like UV, using UV densitometric scanning,[39, 40] MS using TLC-MS interface[41] will result in the identification of the targeted active substances in the plant extract. It has the advantage of good availability, ease of application and cost effectiveness. Also, the use of various derivatization reagents has made detection highly flexible. Thus, direct bioautographic assay on TLC plate has made thin layer chromatographic screening a powerful tool for fast identification of active compounds in crude plant extracts.[1]

High performance thin layer chromatography (HPTLC) coupled with bioassay is a simple and rapid technique for the discovery of bioactive compounds present in complex extracts of natural products.[42] Advantages of this technique include simplified sample preparation, short time of analysis, low cost of operation, simultaneous analysis of several samples, option for multiple detection, matrix- tolerance and compatibility to biological assays.[41, 1] The HPTLC setup can be used for many project and bioassay at any time without additional capital cost. One other major advantage is the eluent-free detection made possible by this system since chromatography is distinctly separated from the detection step.[3] Moreover, in HPTLC all the samples are collected in the plate after separation such that composition of unknown samples can be assessed for bioactivity first and only active ones are then subjected to further characterization and structure elucidation. The disadvantage of HPTLC however is the reduced separation power when compared to HPLC or GC. HPTLC has been used in the study of polyphenols from *Citrullus lanatus*,[43] *Rosa hybida*,[44] *Diplazium esculentum.*[45] However, it may not be useful for high sensitive volatile and oxidation-prone bioactive components.[41] Useful detail information on the background and practical performance of HPTLC-MS has been reported.[46, 47]

Liquid Chromatography (LC)

Offline and online separation of complex mixtures can be done by Liquid chromatography including Low pressure LC, high performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UPLC), counter current chromatography (CCC) and other variants.[48] All these can be coupled with bio-assay and spectroscopy. Disadvantages of LC however includes the use of organic solvents and other additives which are not compatible with most bioassay and fast separation which often do not match the time frame required for many bioassays.[48] Attention is however still on the use of liquid chromatography nuclear magnetic resonance (LC-NMR) and LC-MS (mass spectrometry) for direct and rapid screening of medicinal plant extracts.[49]

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) became more relevant in the last decades due to the overlook of TLC potentials in analytical chemistry.[50-52] However, coupling HPLC with bioassay has shown some drawbacks which include the need to remove all traces of disinfectant used to rinse the flow system after use to prevent the growth of biofilms, so as not to disrupt the bioassay results. Challenges with column derived coupling also include capital cost, complexity of instrumentation for routine operation, dealing with large amount of data generated and finding single eluent that is optimal for all detectors.[11] Also, HPLC eluent may require post-chromatographic aqueous dilution tolerable for the specific bioassay, Peak broadening usually occurs except for bioassay with fast reaction times, complex and laborious instrumental set up are required, sample preparation need to be adjusted to HPLC requirements, limiting to the online system in the sample throughput by sequential sample analysis, time dependent effect on bioassay cannot be achieved and limit of detection (LOD) is worse in HPLC enzyme inhibition.[3]

Gas Chromatography (GC)

Gas chromatography is a separation technique used for volatile compounds because of its efficiency and excellent detection sensitivity when combined with MS. It has the advantage of being associated with

high data library which allows tentative identification of compounds.[48] It is not however compatible with many biochemical systems but it has found application in the analysis of fragrance and flavor on GC-olfactometry.[53]

Structure Identification and Elucidation

Following proper isolation using appropriate chromatographic technique, the next step is the characterization and elucidation of the unknown isolated bioactive compounds. Usually, hyphenation is selected to provide the most relevant information as required. Bioassay coupled chromatography has often been hyphenated with several spectroscopic equipment such as Attenuated Total Reflection/Fourier Transform Infrared (ATR-FTIR). In this case spectra can be recorded directly from zones of interest on an HPTLC plate through the versatile TLC-MS interface.[54, 55] Nuclear Magnetic Resonance (NMR) has been coupled with several bioautography.[56, 57] Even on analytical scale, it has been used for structure confirmation.[58] Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy is also a well-known technique for characterization.[59] It has found some application in natural product research[60] and has been coupled with chromatography.[61, 62] Surface Enhanced Raman Spectroscopy (SERS) has also been coupled with chromatography[63] and could find some applications in natural product research.[64] UV/VIS/FLD spectrometry coupled with chromatography is also a useful technique in bioactive components identification.[65-68] It is usually coupled with planar chromatography from which images can be captures directly on the plate. Most of these experiments can be measured directly from analytical plates. Important samples are then directed to mass spectrometer (MS) or high-resolution mass spectrometer (HRMS) for structure confirmation.

Specific examples

Reversed phase HPLC separation coupled to NMR-MS and bioassay was used to discover triterpenoidal saponin with significant anti-helminthic activity from *Bacopa Monniera*.[69] Seven new AChE inhibitor were isolated from the dichloromethane extract of the aerial parts of *Blumea gariepina* (Asteraceae) using the diazotization method[70] while an indole alkaloid was isolated from *Tabernaemontana austrialis* through Ellman reaction.[71] Twelve (12) compounds including phenolic acids, phthalides and terpene glycoside were identified by HPLC coupled with DAD and ESI–MS from *Paeonia lactiflora* and *Ligusticum chuanxiong*.[72] Antioxidant (DPPH) bioassay coupled with LC-MS was used to identify carnosol, rosmanol, carnosic acid, methyl carnosate, and some flavonoids such as cirsimaritin and genkwanin from *Rosmarinus officinalis*.[73] The on-line separation and structure elucidation of naphthodianthrones, flavonoids, and other constituents from an extract of *Hypericum perforatum* L. using high performance liquid chromatography (HPLC) coupled on-line with ultraviolet-visible, nuclear magnetic resonance (NMR), and mass spectrometry (MS) has also been reported.[74] Thirteen (13) compounds including ferulic acid, Z-ligustilide, E-ligustilide, Z-butylidenephthalide, E-butylidenephthalide, 3-butylphthalide, 3-butylidene-4-hydroxyphthalide, senkyunolide A, 6,7-epoxyligustilide, senkyunolide F, senkyunolide H, senkyunolide I, and 6,7-dihydroxyligustilide were identified using gas chromatography–mass spectrometry (GC–MS) coupled with pressurized liquid extraction (PLE) from *Angelica sinensis*.[75]

Conclusion

The role of medicinal plants in drug discovery is quite immense. This makes the need for the development of analytical techniques which could provide adequate information on the activity, toxicity as well as the bioactive compounds very important. Also, the fact that separation is usually challenging and compounds are usually isolated in very small amounts, it is necessary to develop analytical processes that can detect and fully identify bioactive lead compounds in small quantities. Thus, bioactivity-coupled chromatographic separation in hyphenation with appropriate spectroscopic technique is the way forward in order to achieve rapid discovery of potential drug leads.

Conflict of interest

The author declares no conflict of interest.

Declaration of liability

The author takes responsibility for the content of this article.

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