

Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading

doi:10.12980/JCLM.3.201514J66

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Recent trends in rapid dereplication of natural product extracts: an update

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PEER REVIEW

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Comments

This paper is a review about the methods of dereplication of phytochemicals. The authors report examples concerning herbal drugs and a lot of literature data.

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ABSTRACT

Natural products are the prominent sources of drugs to combat various infectious diseases. The rapid progress of multi-resistance and pan-resistant pathogens to various medicines represents today a major challenge. The search for novel natural products requires a quick and efficient approach to distinguish novel compounds from the known ones, a process called dereplication. Dereplication strategy is generic and time-saving, avoids isolation/purification of known compounds, enables an efficient liquid chromatography peak annotation of most of the studied compounds and can be well adapted for plant chemotaxonomy, phytochemical screening and metabolite profiling. In this review, recent developments of hyphenated techniques towards dereplication of active compounds, chemotaxonomy, metabolite profiling and rapid detection of novel compounds in medicinal plant extracts are presented.

KEYWORDS

Dereplication, Phytochemical screening, Metabolite profiling, Hyphenated techniques

1. Introduction

For decades natural products continued to offer templates for the development of novel scaffolds of drugs. Nevertheless, much time is wasted during the search for novel natural products on isolation, purification and spectroscopic identification of already identified compounds. This can be simplified and the process becomes more effective if it is supported by rapid dereplication techniques which are capable of identifying the known compounds efficiently. In recent years due to the progress of methods for dereplication of natural product extracts, studies on isolation, chemotaxonomy,

biosynthesis, chemical fingerprinting, quality control of herbal products, speeding-up the lead compound identification in the early stages of the drug discovery process and metabolomics have become much simpler and effective[1-3]. In the search for novel natural products, a process known as dereplication is vital to efficiently distinguish previously isolated, characterized and tested compounds from novel compounds. Currently, dereplication is much easier than ever due to the rapid development of modern hyphenated techniques such as gas chromatography-mass spectrometry, liquid chromatography-photodiode array, ultrahigh performance liquid chromatography (HPLC) coupled with

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Article history:

Received 9 Sep 2014

Received in revised form 19 Sep, 2nd revised form 11 Dec 2014

Accepted 21 Jan 2015

Available online 6 Feb 2015

time-of-flight mass spectrometry, liquid chromatography–mass spectrometry, liquid chromatography–fourier transform infrared spectroscopy, liquid chromatography–nuclear magnetic resonance (LC-NMR), liquid chromatography–nuclear magnetic resonance–mass spectrometry, liquid chromatography–circular dichroism and NMR metabolomics[3-12]. Here in this paper, the recent developments for conventional dereplication techniques has been reviewed.

2. Ultrahigh performance HPLC coupled with time-of-flight mass spectrometry

Ultrahigh performance HPLC coupled with time-of-flight mass spectrometry is one of hyphenated techniques that drew the attention of researchers for metabolite profiling of natural product extracts. The liquid chromatographic retention peak, high mass accuracy and molecular formula of the known compounds saved in the library (shift tolerance of ± 0.05 min and an exact mass tolerance of ± 0.05 Da) are mostly used as reference parameters to screen out the known constituents of the extracts[13]. A series of studies done on six *Lippia* species (Verbenaceae) and determination of previously isolated phenolic compounds from *Lippia salviaefolia* and *Lippia lupulina* are some of the recent examples that applied this technique[13-22]. The merit of the technique relies on its ability to have detailed information of the chemotaxonomic relationship among species of the same genus and identify major constituents of respective species on comparative basis.

3. Liquid chromatography–mass spectrometry (LC-ESI-MS technique)

The Web of Science database listed 132 and 93 publications in the period 2010-2011 only on the application of ESI and LC-ESI-MS, respectively, in plant based research[23]. Although some natural products only show poor fragmentation pattern in ESI-MS, the fragmentation pattern can be helpful in dereplication, mainly when dealing with glycosylated compounds.

Dereplication of phytochemicals in the leaves extract of *Melicope vitiflora*[24], acid-soluble phytochemicals in the leaves extract of *Ruta graveolens*[25], bark and leaves extract of *Facelina coronata*[26], stem bark of *Taxus wallichiana*[27] and stem bark of *Elaeocarpus chinensis*[28] are among some of the recent examples that applied this approach. In a related study metabolic profiling of various tissue cultures of *Taxus* species, with the aim to increase the production of taxol and its related derivatives, this technique was used as a main tool. Among these, the works of Madhusudanan *et al.*[27,29,30] and Zhao and Yu[31] are the prominent ones. Earlier works of Madhusudanan *et al.* revealing ammonium cationization at low cone voltage followed by analysis of respective $[M+NH_4]^+$ and $[M+Na]^+$ simplified the analysis of taxoids in the partially purified extracts of *Taxus wallichiana*[27,29,30]. A related work on the cell cultures of *Taxus chinensis* revealed that the same method can be utilized to identify taxane constituents[31]. The significant observation of this latest work suggests that acetyl substituents tend to produce ammonium adduct ions peak whereas multi-hydroxy taxanes give protonized molecular ion peaks in positive ion mode ESI-MS[31].

4. LC-NMR technique

LC-NMR experiments can be operated in both continuous-flow and stop-flow modes. The main prerequisites for on-line LC-NMR are the continuous-flow probe and a valve is installed before the probe for recording either continuous-flow or stopped-flow NMR spectra. The HPLC control unit is connected to the data acquisition system of the NMR for the harmonization of the different operations. A sensitive detector, such as UV, is usually coupled with up-stream in order to

trigger the stop-flow measurements.

Reversed phase silica gel are used, employing a binary or tertiary solvent mixture with isocratic or gradient elution modes. In order to overcome the interference of solvent protons on NMR spectrum, solvent signal suppression is mandatory which can be achieved by presaturation, soft-pulse multiple irradiation or water suppression enhancement[1,32,33]. Stop-flow HPLC-NMR is used to elucidate structures of new compounds prior to (or instead of) their time consuming isolation[34]. The recent progress in pulse field gradients and solvent suppression, the improvement in probe technology and the construction of high field magnets have given a new stimulus to LC-NMR and the method has now rapidly developed to the level of one of the reliable technique in the analysis of phytochemicals in plant mixtures. The use of iron magnets in initial trials but the use of superconducting solenoid magnet was quickly recognized[1,32,33].

Recent advancements with LC-NMR demonstrated multiple technological advancements, like use of strong field magnets, microprobes and cryoprobe technology to improve instrument sensitivity and resolution equipped with a valve or a loop collector for stop flow experiments[35-37]. Cryogenic cooling helps in the detection of submicrogram quantities since decreasing the temperature increases the response[36,37]. The major challenge of the requirement of high volumes of expensive deuterated mobile phase solvents is effectively handled through on-line solid phase extraction (SPE) units that are embedded in-between LC and NMR. Several recent reports in the analysis of phytochemicals have appeared in past decade[38,39].

One of the drawbacks of this technique relies on the unreliability of comparing chemical shifts of the NMR spectra obtained with mixed composition of solvents with the chemical shifts of known compounds available in the literature. The recently introduced new technique, HPLC-SPE-NMR, allows an optimum HPLC separation and NMR analysis giving a reliable chemical shifts of the desired analytes to be compared with published chemical shifts data. The later approach is still in its infant stage and has its own limitations as well. In this context, coupling LC-MS with NMR would allow unequivocal identification of compounds and co-elution of isomers, if there is, it will not be a challenge for identification of phytochemicals as in the case of LC-MS-MS coupling. Nevertheless, lack of sensitivity and automation of interpretation is still a challenge. The necessary sensitivity for online NMR (>1 μ g per compound of interest) can be achieved by HPLC with deuterated solvents or by on-line SPE-coupling[35-37,40].

5. HPLC-SPE-NMR technique

This technique is one of the popular techniques developed past decade. The SPE unit facilitates the solvent exchange from the mobile phase of the HPLC component to a deuterated NMR solvent. Early works of Albrecht[41] provided the breakthrough for several following researchers developing LC-SPE-NMR techniques for rapid analysis of mixtures[42-44]. Originally the LC-SPE-NMR was famous with a flow cell volumes ranging from 60-250 μ L with on-flow experimental set up initially, but later progressed to stop-flow LC-NMR experimental step up[44]. Though this method has several interesting applications, the method still has some drawbacks. The back flush from SPE is strongly influenced by both the elutropic power and hydrogen bonding capacity of the NMR solvent which limits the wide use of less polar solvents such as $CDCl_3$ and highly viscous solvents such as $DMSO-d_6$ or C_5D_5N . Multiple trapping strategies might be a solution to collect dozen micrograms of sample to be transferred from SPE to the NMR spectrometer. It has also been suggested that the method can be taken as a preferred technique for the analysis and identification of unstable metabolites mainly for those that convert to equilibrium mixtures.

6. LC-NMR-MS technique

This technology takes advantage of the rapid and sensitive screening capabilities of MS, which can pinpoint metabolite peaks of interest in complex mixtures for further structural analysis by NMR spectroscopy. The most common way of interfacing LC to both MS and NMR is the parallel mode, where the eluent is split to give two parallel flows. The balance between the two split flows can be adjusted using a splitter. Since NMR is less sensitive compared to MS, a typical split ratio is 95:5 for NMR vs. MS. The LC-NMR-MS technique can be used in both on-flow and static conditions, although in the latter case the MS would be idle most of the time while waiting for NMR data acquisition to complete. Because of the high sensitivity and rapid scanning ability of the MS, sometimes MS data can be acquired on-line during the chromatographic run[45]. The first application of LC-NMR-MS in the field of natural products was presented in 1999[46]. Since then, the method has been used couple of times in studying plant metabolites and it was soon realized that the combination of the NMR and MS might be the most powerful analytical tool in plant-products analysis[47-50]. Parallel to the necessary hardware developments, a variety of automation procedures and software packages have become available for LC-NMR and LC-NMR-MS, allowing such analyses to be performed in a convenient, reproducible and precise manner.

7. MS with mass defect filter (MDF)

The recently developed MDF technique has revolutionized the field of metabolite profiling, screening and identification in drug discovery and development. This technique depends on elemental constituents, not on molecular weight or mass fragmentation. MDF is a post data acquisition processing technique that takes advantage of high resolution MS technology[51]. The MDF approach attempts to discriminate metabolite ions from matrix ions based on similarity of the mass defect values of a drug and its metabolites and the difference in elemental composition between drug related and matrix related ions. Conventionally, high resolution MS technology was employed for the determination of metabolite molecular formula and their accurate mass fragments and for detecting common metabolites based on their predicted mass shifts. MDF enables high resolution MS to be utilized to detect both common and new metabolites because of narrow ranges of changes when a 4-place decimal points of high resolution LC-MS data can be accurately obtained regardless of changes in molecular mass, fragmentation patterns, or isotope patterns of metabolites[50,51]. This technique was applied in recent study to determine steroidal alkaloids from a methanolic extract of *Fritillaria thunbergii*, and glycyrrhizin analogs, coumarin, and flavonoid analogs from a methanolic extract of *Radix glycyrrhizae*[52].

8. Chiral LC-circular dichroism-NMR (LC-CD-NMR)

Chiral HPLC is one of the powerful methods for estimating optical and chemical purity of chiral compounds[53]. Nevertheless, much time and effort are required to prepare authentic samples. Recently, chiral LC-CD-NMR technique is proved to require only crude chiral compounds that include enantiomers as minor impurities and also capable of identifying target enantiomers in crude compounds without authentic samples. In this hyphenated technique, the CD unit is inserted between the column and photodiode array unit of UV detector of a conventional LC-NMR system. By chiral LC-CD-NMR, the enantiomer peak can easily be identified by an opposite sign of

the CD cotton effect curve and an identical ^1H NMR spectrum to that of the main component. Using NMR as a detector, this method is superior in ability to discriminate enantiomers from other isomers indistinguishable by MS.

Recent studies proved that chiral LC-CD-NMR is a suitable solution in establishing best chiral HPLC conditions superior to other modern chromatographic methods. Recent works of Tokunaga *et al.* examined pyridylalanine derivative mixture by chiral LC-CD-NMR with three chiral columns and proved that the enantiomer could be identified in each column without need for authentic samples, on the basis of the NMR and CD data[54]. Chiral HPLC conditions can be optimized using a crude chiral compound that includes an enantiomer as a minor impurity. A related study by Bringmann *et al.*[9-11] proved that HPLC-CD hyphenation was permitted to unswervingly distinguish between the enantiomers of two regioisomers (type I and II) of the ubiquitously occurring plant-derived phytoprostane B1 which occurs in a racemic form due to the formation by a non-enzymatic free-radical mechanism[9].

9. Conclusion

Use of hyphenated techniques is by far the most powerful strategy, by which an analyst can study complex mixtures of natural products. Compared to the classical use of UV, MS and NMR spectroscopy applied to pure natural products, integration of all these techniques in their hyphenated forms (LC-UV, LC-MS and LC-NMR) in a single set up allowed complete characterization of different metabolites in a mixture during a single analysis. LC-hyphenated techniques are playing an important role to support a simple and effect phytochemical analysis. Preliminary information about the content and nature of the constituents of crude plant extracts can easily be archived before a large numbers of samples are processed protecting unnecessary isolation of known compounds. Once the novelty or utility of a given constituent is established, it is then easy to process the plant extracts in the usual manner to obtain samples for full structure elucidation and biological or pharmacological testing. In practice, however, many factors may hinder on-line detection and structure determination of an unknown plant metabolite and often only partial structure information will be obtained.

An application of LC-ESI-MS/MS and HPLC analysis demonstrated a rapid identification of a number of taxoids in extracts of *T. chinensis* cell culture. The analytical methodology provided a rapid, conventional and reliable tool to profile a group of taxol-polarity-related taxoids produced by *Taxus* cell culture. It is also suited for taxoid routine analysis in plant or other complex matrix without complicated sampling. For the on-line *de novo* structure determination of natural products, LC-NMR plays a key role and allows the recording of precious complementary on-line structure information when LC-UV-MS data are often insufficient for unambiguous peak identification. In LC-NMR, the on-flow techniques are proved to be more efficient. Nevertheless, for detailed structure elucidation of minor peaks stopped flow and loop-storage method are necessary. When sample concentration is limiting, sample manipulation techniques such as SPE, multiple trapping and the use of a cryogenic probe are proved to be helpful.

On the contrary to UV or MS, NMR remains a rather insensitive detection method and the need for solvent suppression has restricted the observable NMR range. Improvements in sensitivity are expected in the years to come. Moreover, much effort is needed on the chromatographic side to improve efficiency of pre-concentration, high loading and time-slicing. The inability of LC-NMR to provide ^{13}C NMR spectrum with reasonable amounts of sample is still an

important limitation of this technique for natural product analysis and on-line identification of unknowns. Promising advancements have also been observed in hyphenation of NMR with CD and gas chromatography devices to address stereochemistry issues and facile identification of volatiles, respectively.

These impressive technological developments opened a new field of investigation in pharmacognosy and phytochemistry, where a rapid chemical screening with minute amounts of plant material becomes practically feasible. It is also good to remind that a combination of various techniques such as LC-UV and LC-MS-MS or LC-MSn database have to be built up. Integration of high-resolution on-line LC bioassays is also a vital strategy since they will permit an efficient localization of the bioactive compounds in the crude extracts and their subsequent full or partial identification on-line. The integration of such technologies with highly sensitive bioassay that can be coupled on-line or used at-line after microfractionation holds much promise for advances in the fields of chemotaxonomy, plant metabolomics and natural products analysis, which could lead to investigations of more novel leads to drug discovery.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Much time is wasted during the search for novel natural products on isolation, purification and spectroscopic identification of already identified compounds. It is not easy to study the natural substances and the current techniques do not resolve all problems. The development of modern hyphenated techniques can be very helpful.

Research frontiers

The paper reports the development of the modern hyphenated techniques that are the cutting edge in the field of natural substances.

Related reports

In recent years due to the progress of methods for dereplication of natural product extracts, studies on isolation, chemotaxonomy, biosynthesis, chemical fingerprinting, quality control of herbal products, speeding-up the lead compound identification in the early stages of the drug discovery process and metabolomics have become much simpler and effective

Applications

When a method is unable to analyze some phytocomplexes, the integration of some technologies can be adapted to studies on natural substances. Dereplication strategy allows researchers to make phytochemical screening also with minute amounts of plant extract.

Peer review

This paper is a review about the methods of dereplication of phytochemicals. The authors report examples concerning herbal drugs and a lot of literature data.

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