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Multidimensional separations in the pharmaceutical arena

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The introduction of novel, powerful and rapid multidimensional separation and characterization methods has produced revolutionary global changes at the genome, proteome and metabolome level, bringing about a radical transition in our views of living systems, at the molecular level. The age of proteomics and metabolomics demands high-resolution multidimensional separation techniques. Multidimensional gas and liquid chromatography techniques, in addition to capillary and microchip electrophoresis methods, offer increased resolution and sensitivity, while also affording adequate throughput and reproducibility to meet the demands of the modern pharmaceutical industry. Coupled with MS, these techniques provide not only separation but also reliable identification of the sample components. The resolving power of these methods has proved to be superior over individual one-dimensional approaches, enabling the comprehensive separation of complex biological mixtures, with excellent resolution and reproducibility. High capacity computer systems that are capable of rigorous qualitative and quantitative analysis of the separation profiles allow the establishment and mining of large databases. Examples of various modern multidimensional separation techniques, and their integration with MS, are reviewed, here, with respect to pharmaceutical analysis.

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▼ Entering the age of proteomics and metabolomics from the genomics era, we expect to see high-resolution, multidimensional bioanalytical techniques being used in an integrated and automated fashion, to solve formidable separation problems and enable high performance analysis of complex mixtures of biomedical interest in the pharmaceutical and biotechnology industry. The resolving power of these techniques is superior over conventional one-dimensional approaches, and it is possible to reproduce the separation of complex sample mixtures, containing thousands of components. Multidimensional separation methods, in conjunction with modern informatics technologies, have demonstrated successful pattern recognition and complex image analyses in different areas of drug discovery. The coupling of various orthogonal separation methods, such as gas chromatography (GC), liquid chromatography (LC), capillary and microchip electrophoresis (CE and ME) to each other and/or to MS and NMR is an emerging and promising approach in separation science, and the latest developments in this field are thoroughly discussed here. MS and NMR are regarded, here, as separation techniques, in the sense that multiple components can be selectively detected.

Gas chromatography

Comprehensive two-dimensional gas chromatography (GC×GC) is rapidly gaining importance for the analysis of complex samples in the modern pharmaceutical industry. For trace-level analysis, for example, drug metabolites in complex biological samples, the peak capacity of conventional GC fails to separate all of the individual constituents [1,2]. Multidimensional GC was first reported as a technique for analyzing a single, or a few fractions, from the first dimension (heart-cutting) and transfer them to the second column for consequent analysis, enabling $n_1 + n_2$ resolution [3] (n_1 and n_2 are the number of the separated peaks in the first and second dimensions, respectively). However, in GC×GC, instead of just a few selected peaks, all separated peak clusters are transferred to a second column for further separation, providing orthogonal resolution on a comprehensive n_1 x n_2 basis. Figure 1a represents the comprehensive GC×GC system. The first dimension is usually a 15–30 m long high-resolution capillary column (internal diameter (ID) of 250–320 μm) with a non-polar stationary phase, supporting mainly boiling point-based separation. The modulator is the interfacing device between the first and second

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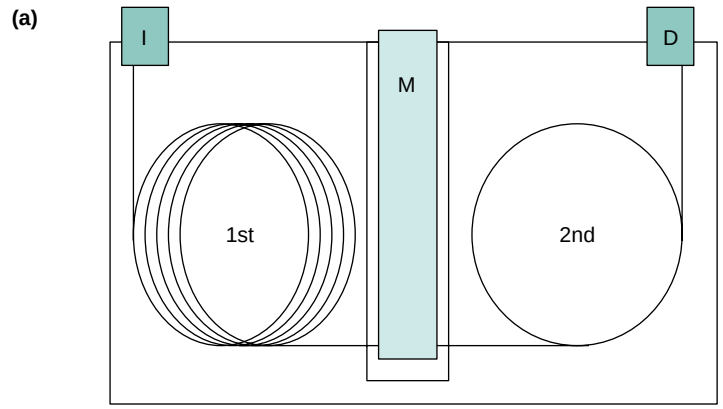
dimensional columns. Here, fractions from the first column are refocused and injected to the second column. The second GC capillary is significantly shorter than the first one (1–2 m, and ID = 50–100 μm), with a polar phase to provide true orthogonal separation. Separation time in the first dimension is 1–2 hours but separation in the second column takes only seconds, under essentially isothermal conditions. There are various types of modulators, including dual-stage thermal desorption modulators [4], cryogenic trapping and focusing [5], and the recently developed cryogenic jet system [6]. To maintain resolution in the first dimension and avoid the loss of ordered structures (wrap-around), four or more modulations are necessary for each band that is separated in the first column [7], and separation in the second column should be finished before the next pulse is injected. Fast separations require rapid detection systems with small dead volumes and high data acquisition rates, such as the fast flame ionization detector (FID) [8] or micro electron-capture detector (μECD) [9]. Figure 1b is a representative 3D view of a GC×GC–FID chromatogram. Comprehensive two-dimensional GC has been applied to drug analysis in doping control with 10–100 μg L^{-1} detection limits [10]. Analysis of enantiomers was accomplished by a combination of cyclodextrins × BP-20, or DB-5 × cyclodextrins columns [11].



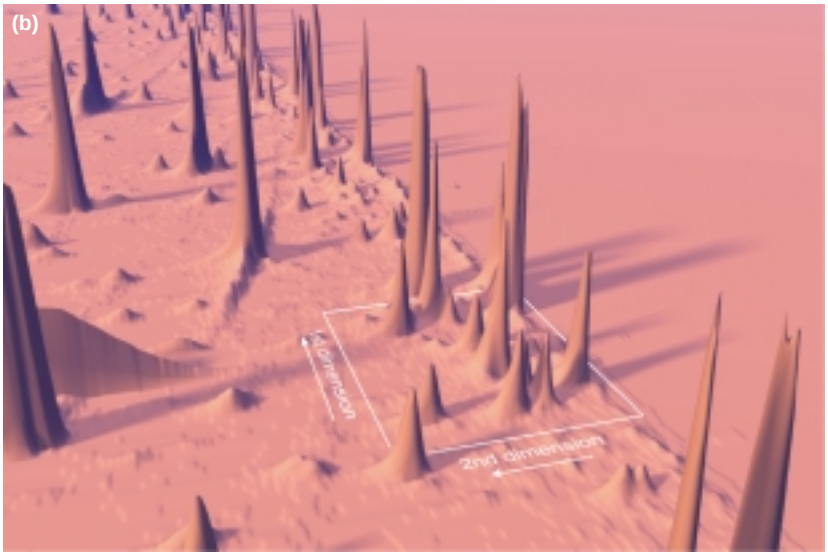


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Figure 1. (a) Schematic of a GC×GC system. D, detector; I, injector; M, modulator; 1st, GC oven with first-dimension column; 2nd, (separate) GC oven with second-dimension column. **(b)** Representative 3D view of a GC×GC-FID chromatogram. With permission from Ref [2].

GC–MS [12] has been in routine use for many years, so only a couple of techniques for fast analyses in drug monitoring are mentioned here. To fulfill the high detection rate requirement, time of flight (TOF) MS is preferred, because it can acquire >50 spectra per second. The most frequently used GC–MS interfaces are open coupling and direct coupling [13]. Guetens *et al.* [14] have recently discussed the use of capillary GC–MS in anticancer drug monitoring. Others have reported chemical profiling of 4-methylenedioxy-methamphetamine (MDMA) tablets by GC–MS analysis [15]. Maurer [16] extensively reviewed GC–MS procedures for the detection and quantification of drugs and their metabolites, relevant to clinical and forensic toxicology, doping control or biomonitoring, using negative ion chemical ionization. It should be noted that immunoassays for drugs of abuse are confirmed by GC–MS to avoid cross-reactivity [17]. GC–MS is a powerful tool for the investigation of genetic metabolic diseases (through analysis of organic acids and acylcarnitines) [18] and it has been successfully applied in the detection of hypocholesterolemia and Smith-Lemli-Opitz syndrome [19]. Volkman *et al.* analyzed microalgae samples by GC×GC–TOF–MS and found several sterols, steroidal ketones and stearanes [20]. Recently introduced high-capacity computer systems, capable of rigorous qualitative and quantitative analysis of the separation profiles are necessary for processing the large amount of data that is produced by the GC×GC–MS approach.

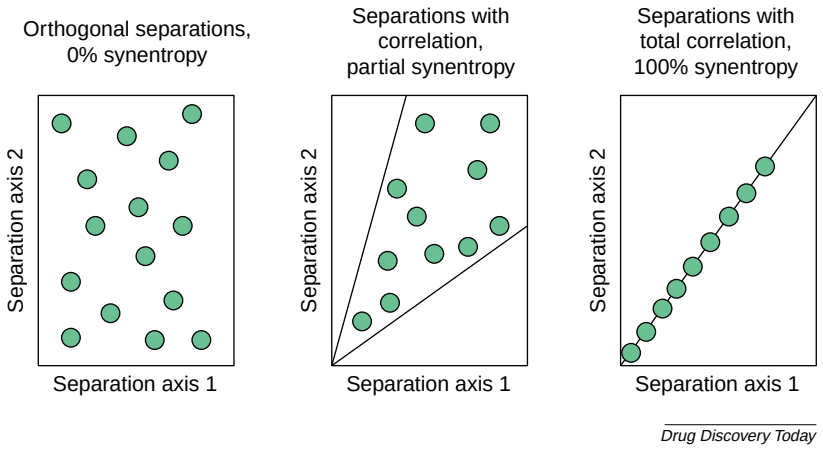

Figure 2. Degrees of orthogonality and synentropy, and their effect on the use of multiple dimensions of chromatographic space.

Liquid chromatography

HPLC is an essential analytical and preparative chromatographic tool, used in the pharmaceutical industry. The goal of multidimensional HPLC is to obtain a net increase in separation power (serial coupling) or in overall throughput (parallel arrangement). Multidimensional HPLC requires computerized control of the chromatographic instrumentation and valves for the transfer of analytes from one dimension to another, for the delivery of the appropriate solvent gradient to each column and for data acquisition. The full range of HPLC detectors, such as photodiode array (PDA), fluorescence (FL), MS, evaporative light scattering (ELSD) and, more recently, NMR, are coupled to multidimensional separation systems. The extra information that is provided by MS enables compromises in efficiency of the chromatographic separations when non-isobaric compounds are to be measured in a sample. There are many elegant reviews that detail the applications of LC–MS to pharmaceutical analysis [21,22,23,24], thus, we focus here on the advances in coupling multiple liquid chromatographic systems to improve resolution and sensitivity.

HPLC×HPLC

Much like the development of the comprehensive GC×GC methods (discussed above), multidimensional HPLC was first used to transfer specific peaks to the second dimension. During the 1990s, the different mechanisms of selectivity in ion exchange and size exclusion chromatography were used to increase the number of sample components that could be separated by comprehensive chromatographic coupling [25]. Proteome analysis is now highly dependent on the multidimensional separation of peptides by chromatography and MS [26,27,28]. The application of comprehensive HPLC×HPLC to small-molecule analyses has also progressed, although more slowly, since the demonstration of a separation of alcohol ethoxylates on aminopropyl silica columns, coupled to reverse-phase C_{18} columns [29]. Improvements in comprehensive HPLC×HPLC systems are being guided by optimization of the parameters that define the degree of correlation between separations in different dimensions [30]. These include the percentage of synentropy, which is a measure of the percentage of two-dimensional informational entropy that is contributed equally from each dimension [31]. Zero percent synentropy indicates that each separation dimension is operating in an independent fashion, whereas, 100% synentropy indicates that the separation dimensions strongly correlate, resulting in little or no difference in resolving power. Another parameter that is used to measure performance is the degree of orthogonality between the different separation dimensions [32]. Higher degrees of orthogonality lead to greater peak capacity and better resolution in the same separation time as a system with low orthogonality. Synentropy and orthogonality can be plotted to assess resolving power, as shown in Figure 2.

Recent studies have examined the orthogonality that is achieved when a wide variety of reverse and specially bonded phases of HPLC columns were coupled together. Venkatramani *et al.* developed an automated orthogonal two-dimensional LC system, using commercially available pumps and software [33]. The primary column was alternately coupled to two additional secondary columns, to sample up to 50% of the total flow of the first column into the second dimension. The primary column was eluted for approximately one hour, with sampling to the secondary columns occurring every 20 s. Maintenance of similar solvent strengths in both columns throughout the HPLC run, and the use of smaller columns in the second dimension prevented the occurrence of wrap-around effects. The system produced a single chromatogram from the primary column and many sub-chromatograms from the secondary dimension. The peaks in the secondary dimension chromatograms were recombined with the primary dimension chromatogram to generate either contour plots or 3D peak plots. Separation in the two dimensions was shown to be highly orthogonal, resulting in resolution of a greater range of compounds, than that achieved by a single column. To establish the parameters that are necessary to separate

poorly resolved diastereomers, the fractions generated from a C_{18} column were injected onto a carbon-clad zirconia column (CCZ) [34]. This demonstrated a high degree of orthogonality between the hydrophobic (C_{18} column) and molecular orientation (CCZ column) separation mechanisms. This study revealed that the conditions necessary to resolve diastereomers could be identified in an offline manner. A commercially available example of a multidimensional HPLC system is the Sepbox®, produced by Sepiatec (http://www.sepiatec.com). This system uses reverse-phase (RP) and solid-phase extraction (SPE) columns to separate and trap compounds [35]. The SPE columns take the place of fraction collectors after the first pass purification; the semi-pure bands, retained on the SPE columns, are then transferred to a bank of HPLC columns that are arranged in parallel. Different aspects of this technology have been optimized for natural product purification from crude extracts and for the analysis of combinatorial libraries.

To capitalize on the ionic character of cefmatilen (a beta lactam antibiotic) and seven of its metabolites, cation and anion exchange pre-columns were coupled to five RP HPLC columns for the determination of urine and plasma concentration of these compounds [36]. Detailed programming of the valves and pumps to transfer the desired bands from the initial ion exchange columns to the RP columns enabled simultaneous quantification of the cefmatilen metabolites.

SPE×HPLC

There are many examples of coupled HPLC systems that transfer selected peaks or bands of interest from one column to another. SPE separates the desired analytes from a liquid matrix by passing the biological fluid matrix, such as plasma, urine or bile, through a column containing a packing that retains the analytes and allows the proteins and other highly polar components to pass through to waste. Removal of highly polar matrix components leads to improved sensitivity by reducing ion suppression in the MS source. Online SPE–HPLC systems are typically plumbed with two pumps and switching valves, such that the matrix containing the analyte is passed through the SPE column and the non-adsorbed components are directly sent to waste. Reversing the flow through the SPE column back-flushes the analyte from the SPE column onto the much higher resolution HPLC column for separation of the components of interest (Figure 3). The plasma concentration of the calcium channel antagonist nifedipine were quantified using this technique [37]. To reduce the fouling of the SPE cartridges and the loss of the sites for analyte retention, restricted access media (RAM)

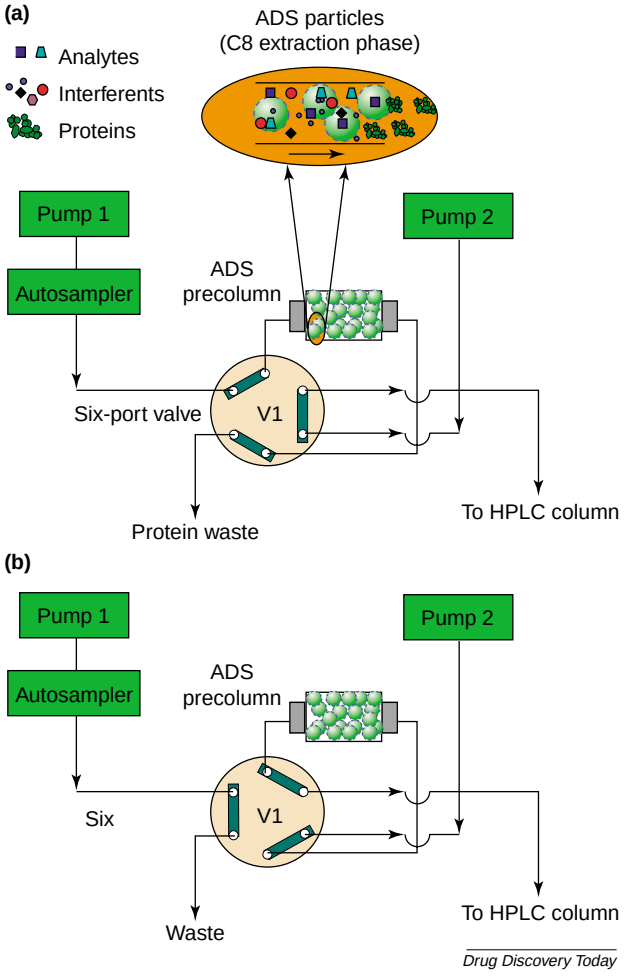

Figure 3. Representative flow path in a SPE-HPLC system. With permission from Ref. [38]

columns are employed. These columns work with a dual mechanism of size- and polarity-based selectivity; they typically have large pore sizes to permit biomolecules to pass through without reaching the retention sites of the C_4, C_8 or C_{18} phase, on which the small organic molecules of interest are retained. A widely used phase in RAM SPE columns is the alkyl-diol-silica phase (ADS). The verapamil concentration, metabolism and pharmacokinetics in rat plasma samples were determined by injecting centrifuged plasma onto a RAM-HPLC system [38]. Mislanova *et al.* [39] employed a C_8 ADS RAM SPE column which was back-flushed onto a teicoplanin-based Chirobiotic T chiral separation phase HPLC column. This system enabled the direct determination of the enantiomeric ratio of (*R*)- and (*S*)-propranolol with FL detection in 25 min, to provide higher precision, accuracy and sensitivity, by comparison with an offline liquid–liquid extraction method to prepare the sample for the teicoplanin HPLC column. Subsequently,

Xia and co-workers [40] at Merck (http://www.merck.com) further developed the enantiomeric analysis of propranolol to include two alternating RAM–SPE columns, coupled to a Chirobiotic T HPLC column with a triple quadrupole mass spectrometer as the detector. RAM columns have been used for the online SPE of amoxycillin [41], metyrapone [42] and pantoprazole [43].

A novel immunoaffinity column-based method was recently demonstrated for the analysis of the retinoic acid analog AM-80 in human plasma. Antibodies that recognized AM-80 were bound to TSK gel tresyl 5PW and the column was used to capture AM-80 from plasma [44]. Subsequently, the retinoic acid analog was transferred to a C_{18} column for separation to provide detection levels of 0.5 ng mL^{-1} of AM-80 in plasma.

Parallel HPLC systems

The need for increasing analytical and preparative chromatographic throughput has driven the development of parallel HPLC systems. Multiple channel autosamplers and novel data tracking software for flow injection analysis LC–MS [45] have led to the development of a method to split the stream in a dual column parallel preparative HPLC instrument that is capable of mass-based fractionation [46]. An interface to multiplex as many as eight streams of solvent from eight parallel HPLC columns into the ionization source of a single mass spectrometer was commercialized by Micromass and Waters (MUX-technology™; http://www.micromass.co.uk). Single and triple quadrupole instruments can reproducibly sample four streams; however, for eight streams, a TOF-based mass spectrometer is required to rapidly sample the full mass range. Merck has implemented a four-channel MUX-technology™ to quantitatively monitor drug concentrations in biological samples [47]. In addition to using a single pump with a flow splitter in their parallel systems, the back pressure of each line was regulated by additional HPLC pumps to ensure even flow through all of the columns. Preparative systems, using multiplexed detection, have also been developed for mass-based fractionation [48], and this method is being extended to parallel supercritical fluid chromatography [49].

Capillary electrophoresis and microchips

Capillary electrophoresis

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are analytical techniques that employ narrow bore capillaries to perform electric field-mediated separation of drugs, drug metabolites and natural product extracts, complementing separations from GC–MS and LC–MS [50]. The two main advantages of capillary-based techniques are the low buffer consumption and minute sample requirement. In pharmaceutical analysis, the methods used are, chiefly, open tubular techniques, such as capillary zone electrophoreis (CZE), micellar electrokinetic chromatography (MEKC), capillary isoelectric focusing (cIEF), and capillary isotachophoresis (cITP). Combination of CE with MS adds extra separation dimensions. Primarily, quadrupole mass spectrometric detectors are coupled to CE, however, TOF, ion trap, triple quadrupole and Fourier transform–ion cyclotron resonance (FTICR) mass spectrometers have been used recently [51–57]. CE readily supports electrospray ionization (ESI) [51], which transforms ions in solution into ions in gas phase and is, therefore, useful for the mass analysis of polar compounds, including pharmaceuticals and their metabolites. One of the main challenges of associating CE with MS detection via electrospray ionization is the volatility and the ionic strength of the buffers that should be applicable to both systems. The ionic strength of the buffers has noticeable effects on sensitivity. The main ESI interface types connecting CE to MS are coaxial liquid sheath flow, liquid-junction and sheathless coupling [52], shown in Figure 4. Figure 4a depicts the coaxial liquid sheath flow interface [53], where the CE capillary is surrounded by a steel tube that delivers the sheath liquid from a syringe pump to close the electric circuit for both the CE and spray voltages. The nebulizing gas (sheath gas) that is delivered through the third concentric outer tube assists in spray formation. The recently developed liquid-junction interface [54] (Figure 4b) connects the CE capillary with the electrospray tip through a small gap (10 –20 μm) that is filled with CE buffer, to decouple the CE and ESI processes. The disadvantage of this setting is the band broadening and concomitant loss of separation efficiency, caused by the interface gap. Application of a chemically etched porous liquid junction within the separation capillary alleviates this problem [55]. Figure 4c represents a sheathless interface [56]. In this particular setting, the end of the separation capillary is pulled to form a sharp fine-point needle to accommodate a stable spray at low flow rates. As this particular interface provides comparable flow rates for both the CE and ESI, it has better sensitivity if coupled with a nano-electrospray source [57].

Microfabricated separation devices

Integrated microfabricated analytical devices offer enhanced separation performance in drug discovery and HTS [58]. Miniaturized separation systems feature reduced reagent consumption, improved performance and multifunctionality, through the use of an interconnected network of channels. Multidimensional analysis on a single glass chip, using open channel electrochromatography (OCEC) and CE, have already been implemented as first and

second dimensions, respectively [59]. The effluent sampling rate from the first dimension into the second one was optimized to perform representative CE analysis of the OCEC separation products. A similar microfabricated two-dimensional device [60] combined MEKC and CE for the analysis of peptide mixtures, reaching peak capacity in the 500–1000 component range. A prototype three-dimensional Polydimethylsiloxane microfluidic system for multidimensional electrophoresis was demonstrated in George Whitesides' laboratory [61]. Microfabricated multidimensional separation systems have great potential to serve in special military and space-research applications. Microfluidic systems can also be connected to MS [62,63], enabling automated sample delivery and enhanced mass spectrometric efficiency by integrating sample processing, enrichment or clean-up, and fractionation before detection. These devices can transport the analyte fluid electrokinetically or by pressure, and generate electrospray via an attached capillary or more complex emitter couplings. A microfabricated, electrically permeable, thin glass septum was also developed to generate electrospray by electro-osmotically pumping the solutions through field-free channels, past the point where the CE electric potential was applied [64]. A new design for high-throughput microfabricated CE–ESI–MS with automated sampling from a micro-well plate has been reported by Barry Karger's group [65]. The assembly combined a sample loading port, separation channel, and a liquid junction MS coupling with a miniaturized sub-atmospheric electrospray interface. Microfluidic devices were found to significantly reduce dead-volume and sample consumption, and increase MS detection sensitivity by 1–2 orders of magnitude. Lion *et al.* [66] integrated a membrane-based desalting step in a microfabricated disposable polymer injector for MS analysis of small drugs, peptides and proteins. The nanospray microfluidic system was fabricated by plasma etching in plastic; it enabled direct MS coupling and high detection sensitivity [67]. A microdialysis junction device was employed to realize a two-dimensional separation system with on-line coupling of capillary isoelectric focusing with transient isotachophoresis zone electrophoresis [68].

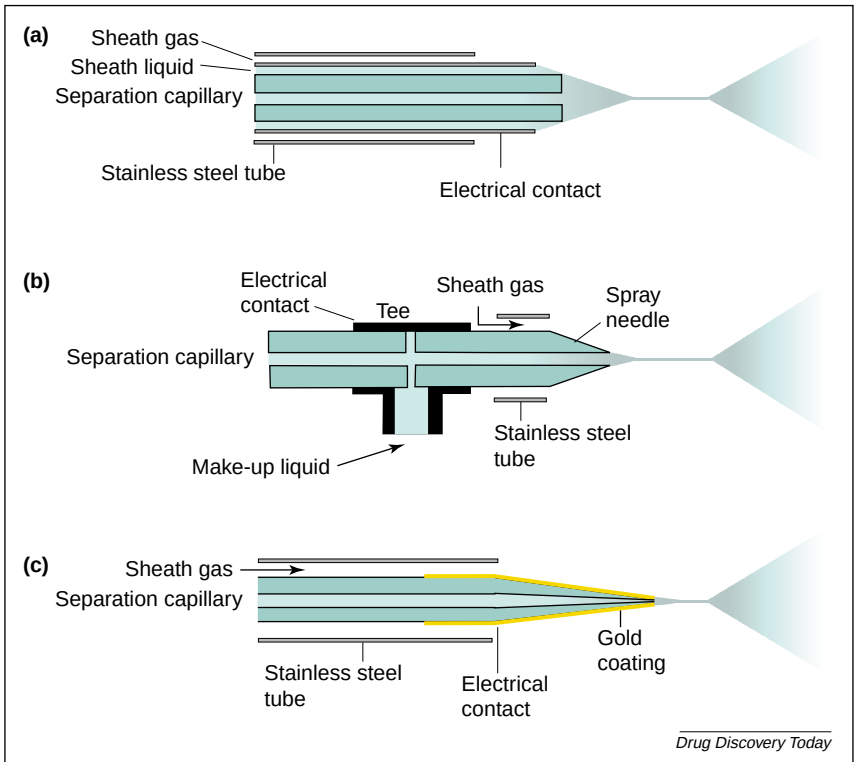

Figure 4. Schematic representation of electrospray ionization CE–MS interfaces. **(a)** Sheath-flow interface, **(b)** Liquid junction and **(c)** Sheathless interface. Adapted, with permission, from Ref. [52].

Although ESI is usually the ionization mode of choice with electric field-mediated capillary separation techniques, CE can also be associated with matrix-assisted laser desorption/ionization (MALDI) MS [69]. Off-line coupling with MALDI–TOF and TOF–TOF–MS was demonstrated [70] through vacuum deposition onto standard stainless steel target plates, allowing decoupling of the CE separation from the MS analysis. The same group also reported on-line coupling, using a continuous vacuum deposition interface with CE [71]. Automated MALDI–MS analysis was reported by Ekstrom *et al.* [72], using a porous, microfabricated digestion chip, integrated with a sample pre-treatment robot and micro-dispenser for transferring reaction products to the target plate. Others performed chemical reactions on a sub-nanoliter scale in a parallel fashion, opening up new opportunities in HTS, particularly when combined with MALDI–TOF–MS [73].

Other multidimensional combinations

Simultaneous on-line NMR and MS detection can provide complimentary data and can combine the power of LC–MS and LC–NMR to specifically identify components [74]. NMR–MS interface is achieved by computer-controlled post-LC-column splitter, which directs 90–95% of the flow to the NMR and provides appropriate dilution and make-up flow for optimal MS ionization. The system

also comprises a loop storage mode, where portions of the sample can be stored in a delay loop before transfer to NMR. By this means, chromatographic peaks can be trapped on-line, in multiple loop cassettes, stored and then automatically transferred to the NMR flow probe. Additionally, LC–MS can operate routinely during longer NMR measurements. LC–NMR–MS with automated loop storage mode have provided a versatile analytical platform for complex mixture analysis. In the future, development of MS-directed LC–SPE–NMR–MS systems, equipped with cryogenic cooling of the NMR radio frequency coils, should provide a powerful analytical tool for drug discovery. Recently, Corcoran and Spraul have published a detailed review on the topic, discussing numerous applications in drug discovery [75].

NMR is a ubiquitous and indispensable tool for elucidating molecular structures and dynamics, and for determining impurities and so on. The non-destructive nature of NMR 'compensates', in part, for its low sensitivity, and it is often used to study molecular binding and to screen potential drug candidates. Continuous improvements in sensitivity and the introduction of small-volume (1 μL) NMR probes for high-resolution spectra has enabled coupling between CE or CEC and the information-rich detection of NMR spectroscopy. Sweedler and co-workers described the first application of a microcoil NMR probe as an on-line detector for CE [76]. Later, Bayer *et al.* reported the first CEC–NMR experiments, conducting a comparison the results with those of CE–NMR and LC–NMR [77]. A technique using cITP to concentrate analytes from nL-volume samples generated a two-orders-of-magnitude increase in mass sensitivity ^{1}H NMR [78]. A comprehensive and detailed overview of theory, instrumentation and applications of the CE–NMR system has been published recently [79].

Conclusions

Modern drug discovery relies on libraries of synthetic and naturally produced compounds to screen against disease-relevant targets. Multidimensional separations have undoubtedly powerful potential, in this respect – they generate comprehensive data for drug development. It is believed that the greatest potential of multidimensional separations lies in identifying individual molecules or groups of molecules (metabolites, peptides, proteins and so on) that are down- or up-regulated in specific disease states or by drug treatment. Other important advantages of multidimensional separations, over 'one-dimensional' approaches, are the higher level of information that the data can provide, in cases where multiple components are measured, and the higher level of sensitivity that is achievable with MS detection, in cases where one particular component is of interest; sometimes both of these benefits can be realized. The sensitivity gain in MS detection after multidimensional separation is a result of removing the interfering components that, otherwise, might suppress ionization of component of interest.

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References

1 Phillips, J.B. and Xu, J.Z. (1995) Comprehensive multi-dimensional gas chromatography. *J. Chromatogr. A.* 703, 327–334
2 Dalluge, J. *et al.* (2003) Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool. *J. Chromatogr. A.* 1000, 69–108
3 Schomburg, G. (1995) Two-dimensional gas chromatography: Principles, instrumentation, methods. *J. Chromatogr. A.* 703, 309–325
4 Liu, Z.Y. and Phillips, J.B. (1991) Comprehensive two-dimensional gas chromatography using an on-column thermal modulator interface. *J. Chromatogr. Sci.* 29, 227–231
5 Marriott, P.J. *et al.* (2000) Comparison of thermal sweeper and cryogenic modulator technology for comprehensive gas chromatography. *J. High Resolut. Chromatogr.* 23, 253–258
6 Ledford, E.B. (2001) Transverse thermal modulation. PCT/US01/01065
7 Ong, R.C.Y. and Marriott, P.J. (2002) A review of basic concepts in comprehensive two-dimensional gas chromatography. *J. Chromatogr. Sci.* 40, 276–291
8 Beens, J. *et al.* (1998) Quantitative aspects of comprehensive two-dimensional gas chromatography (GC×GC). *J. High Resolut. Chromatogr.* 21, 47–54
9 Korytár, P. *et al.* (2002) High-resolution separation of polychlorinated biphenyls by comprehensive two-dimensional gas chromatography. *J. Chromatogr. A.* 958, 203–218
10 Kueh, A.J. *et al.* (2003) Application of comprehensive two-dimensional gas chromatography to drugs analysis in doping control. *J. Chromatogr A.* 1000, 109–124
11 Shellie, R. and Marriott, P.J. (2002) Comprehensive two-dimensional gas chromatography with fast enantioseparation. *Anal. Chem.* 74, 5426–5430
12 Kitson, F.G. *et al.* (1996) *Gas Chromatography and Mass Spectrometry, a Practical Guide*, Academic Press
13 de Hoffmann, E. and Stroobant, V. (2001) *Mass Spectrometry* (2nd edn), Wiley
14 Guetens, G. *et al.* (2002) Hyphenated techniques in anticancer drug monitoring. II. Liquid chromatography-mass spectrometry and capillary electrophoresis-mass spectrometry. *J. Chromatogr. A.* 976, 239–247
15 Gimeno, P. *et al.* (2002) A contribution to the chemical profiling of 3,4-methylenedioxymethamphetamine (MDMA) tablets. *Forensic Sci. Int.* 127, 1–44
16 Maurer, H.H. (2002) Role of gas chromatography-mass spectrometry with negative ion chemical ionization in clinical and forensic toxicology, doping control, and biomonitoring. *Ther. Drug Monit.* 24, 247–254
17 Vandevenne, M. *et al.* (2000) Detection time of drugs of abuse in urine. *Acta Clin. Belg.* 55, 323–333
18 Clayton, P.T. (2001) Applications of mass spectrometry in the study of inborn errors of metabolism. *J. Inherit. Metab. Dis.* 24, 139–150
19 Roux, C. *et al.* (2000) Role of cholesterol in embryonic development. *Am. J. Clin. Nutr.* 71, 1270S–1279S

20 Volkman, J.F. *et al.* (1998) Microalgal biomarkers: a review of recent research developments. *Org. Geochem.* 29, 1163–1179
21 Cheng, X. and Hochlowski, J. (2002) Current application of mass spectrometry to combinatorial chemistry. *Anal. Chem.* 74, 2679–2690
22 Lim, C-K. and Lord, G. (2002) Current developments in LC-MS for pharmaceutical analysis. *Biol. Pharm. Bull.* 25, 547–557
23 Kostiainen, R. *et al.* (2003) Liquid chromatography/ atmospheric pressure ionization- mass spectrometry in drug metabolism studies. *J. Mass Spectrom.* 38, 357–372
24 Clarke, N. *et al.* (2001) Systematic LC/MS metabolite identification in drug discovery. *Anal. Chem.* 73, 431A–439A
25 Bushey, M.M. and Jorgenson, J.W. (1990) Automated instrumentation for comprehensive two-dimensional high-performance liquid chromatography of proteins. *Anal. Chem.* 62, 161–167
26 Wehr, T. (2003) Multidimensional liquid chromatography in proteomic studies. *LC GC* 16, 154–158
27 Washburn, M.P. *et al.* (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 19, 242–247
28 Wagner, K. *et al.* (2002) An automated on-line multidimensional hplc system for protein and peptide mapping with integrated sample preparation. *Anal. Chem.* 74, 809–820
29 Murphy, R.E. *et al.* (1998) One- and two-dimensional chromatographic analysis of alcohol ethoxylates. *Anal. Chem.* 70, 4353–4360
30 Giddings, J.C. (1995) Sample dimensionality: a predictor of order-disorder in component peak distribution in multidimensional separation. *J. Chromatogr. A.* 703, 3–15
31 Patrick, J. *et al.* (1996) Informational orthogonality of two-dimensional chromatographic separations. *Anal. Chem.* 68, 682–689
32 Venkatramani, C.J. *et al.* (1995) Separation orthogonality in temperature-programmed comprehensive two-dimensional gas chromatography. *Anal. Chem.* 68, 1486–1492
33 Venkatramani, J. and Zelechnonok, Y. (2003) A automated orthogonal two-dimensional liquid chromatograph. *Anal. Chem.* 75, 3452–62
34Gray, M. *et al.* (2002) Two dimensional reversed phase reversed phase separations. Isomeric separations incorporating C18 and carbon clad zirconia stationary phases. *J. Chromatogr. A.* 975, 285–297
35 God, R. *et al.* (1999) Online coupling of HPLC and solid phase extraction for preparative chromatography. *G. I. T. Laboratory Journal* 3, 188–191
36 Nishino, I. *et al.* (2000) Determination of a new oral cephalosporin, cefmatilen, hydrochloride hydrate, and its seven metabolites in human and animal plasma and urine by coupled systems of ion-exchange and reversed phase high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* 749, 101–110
37 Yritia, M. *et al.* (2000) Quantitation of nifedipine in human plasma by on-line solid-phase extraction and high-performance liquid chromatography**.** *J. Chromatogr. A.* 870, 115–119
38 Walles, M. *et al.* (2002) Application of restricted access material (RAM) with pre-column switching and matrix solide phase dispersion (MSPD) to the study of the metabolism and pharmacokinetics of verapamil. *Anal. Bioanal. Chem.* 374, 1179–1186
39 Mislanova, C. and Stefancova, A. (2001) Comparison of two different approaches of sample pretreatment for stereoselective determination of (R,S)-propranolol in human plasma. *Journal of Trace and Microprobe Techniques* 19, 163–170
40 Xia, Y.Q. *et al.* (2003) Automated online dual column extraction coupled with teicoplanin stationary phase for simultaneous determination of (*R*)- and (*S*)-propranolol in rat plasma using liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 788, 317–329
41 Cass, Q.B. *et al.* (2003) Determination of amoxycillin in human plasma by direct injection and coupled-column high-performance liquid chromatography. *J. Chromatogr. A.* 987, 235–241
42 Cassiano, N.M. *et al.* (2002) Determination of the plasma levels of metyrapone and its enantiomeric metyrapol metabolites by direct plasma injection and multidimensional achiral-chiral chromatography. *Chirality* 14, 731–735
43 Cass, Q.B. *et al.* (2001) Enantiomeric determination of pantoprazole in human plasma by multidimensional high-performance liquid chromatography. *J. Chromatogr. B. Analyt Technol Biomed. Life Sci* 766, 153–160
44 Itoh, M. and Kominami, G. (2001) On-line immunoaffinity extraction followed by high-performance liquid chromatography and radioimmunoassay for a novel reinobenzoic acid, AM-80, in human plasma. *J. Immunoassay Immunochem.* 22, 213–223
45 Wang, T. *et al.* (1998) A new ultra-high throughput method for characterizaing combinatorial libraries incorporating a multiple probe autosampler coupled with flow injection mass spectrometry analysis. *Rapid Commun. Mass Spectrum.* 12, 1123–1129
46 Zeng, L. and Kassel, D.B. (1998) Development of a fully automated parallel HPLC/ mass spectrometry system for the analytical characterization and preparative purification of combinatorial libraries. *Anal. Chem.* 70, 4380–4388
47 Morrison, D. *et al.* (2002) An evaluation of a four-channel multiplexed electrospray tandem mass spectrometry for higher throughput quantitative analysis. *Anal. Chem.* 74, 1896–1902
48 Rongda, D. *et al.* (2002) High-throughput mass-directed parallel purification incorporating a multiplexed single quadrapole mass spectrometer. *Anal. Chem.* 74, 3055–3062
49 Ripka, W.C. *et al.* (2001) High-throughput purification of compound libraries. *Drug Discov. Today* 6, 471–477
50 Rathore, A. and Guttman, A. eds (2003) *Electrokinetic Phenomena*, Marcel Dekker
51 Fenn, J.B. *et al.* (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246, 64–71
52 Choudhary, G. *et al.* (2000) Use of on-line mass spectrometric detection in capillary electyrochromatography. *J. Chromatogr. A.* 887, 85–101
53 Smith, R.D. *et al.* (1988) Improved electrospray ionization interface for capillary zone electrophoresis-mass spectrometry. *Anal. Chem.* 60, 1948–1952
54 Lee, E.D. *et al.* (1988) On-line capillary zone electrophoresis-ion spray tandem mass spectrometry for the determination of dynorphins. *J. Chromatogr.* 458, 313–321
55 Janini, G.M. *et al.* (2003) A sheathless nanoflow electrospray interface for on-line capillary electrophoresis mass spectrometry. *Anal. Chem.* 75, 1615–1619
56 Olivares, J.A. *et al.* (1987) On-line mass spectrometric detection for capillary zone electrophoresis. *Anal. Chem.* 59, 1230–1232
57 van Brocke, A. *et al.* (2001) Recent advances in capillary electrophoresis/ electrospray-mass spectrometry. *Electrophoresis* 22, 1251–1266
58 Khandurina, J. and Guttman, A. (2002) Microchip based HTS analysis of combinatorial libraries. *Curr. Opin. Chem. Biol.* 6, 359–366
59 Gottschlich, N. *et al.* (2001) Two-dimensional electrochromatography/capillary electrophoresis on a microchip. *Anal. Chem.* 73, 2669–2674
60 Rocklin, R.D. *et al.* (2000) A microfabricated fluidic device for performing two-dimensional liquid-phase separations. *Anal. Chem.* 72, 5244–5249
61 Chen, X. *et al.* (2002) A prototype two-dimensional capillary electrophoresis system fabricated in poly(dimethylsiloxane). *Anal. Chem.* 74, 1772–1778
62 Li, J. *et al.* (1999) Integration of microfabricated devices to capillary electrophoresis-electrospray mass spectrometry using a low dead volume connection: application to rapid analyses of proteolytic digests. *Anal. Chem.* 71, 3036–3045
63 Zhang, B. *et al.* (1999) Microfabricated devices for capillary electrophoresis-electrospray mass spectrometry. *Anal. Chem.* 71, 3258–3264
64 Lazar, I.M. *et al.* (2000) Novel microfabricated device for electrokinetically induced pressure flow and electrospray ionization mass spectrometry. *J. Chromatogr. A.* 892, 195–201

65 Zhang, B. *et al.* (2001) High-throughput microfabricated CE/ESI-MS: automated sampling from a microwell plate. *Anal. Chem.* 73, 2675–2681
66 Lion, N. *et al.* (2002) Integration of a membrane-based desalting step in a microfabricated disposable polymer injector for mass spectrometric protein analysis. *Electrophoresis* 23, 3583–3588
67 Gorby, V. *et al.* (2002) Microfabricated polymer injector for direct mass spectrometry coupling. *Proteomics* 2, 405–412
68 Mohan, D. and Lee, C.S. (2002) On-line coupling of capillary isoelectric focusing with transient isotachophoresis-zone electrophoresis: a two-dimensional separation system for proteomics. *Electrophoresis* 23, 3160–3167
69 Karas, M. (1987) Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Process.* 78, 53–63
70 Rejtar, T. *et al.* (2002) Off-line coupling of high-resolution capillary electrophoresis to MALDI-TOF and TOF/TOF MS. *J. Proteome Res.* 1, 171–179
71 Preiser, J. *et al.* (1998) On-line MALDI-TOF MS using a continuous vacuum deposition interface. *Anal. Chem.* 70, 5278–5287
72 Ekstrom, S. *et al.* (2000) Integrated microanalytical technology enabling rapid and automated protein identification. *Anal. Chem.* 72, 286–293
73 Litborn, E. *et al.* (2000) Parallel reactions in open chip-based nanovials with continuous compensation for solvent evaporation. *Electrophoresis* 21, 91–99
74 Watt, A.T. *et al.* (2003) Metabolite identification in drug discovery. *Curr. Opin. Drug Discov. Devel.* 6, 57–65
75 Concoran, O. and Spraul, M. (2003) LC-NMR-MS in drug discovery. *Drug Discov. Today* 8, 624–631
76 Wu, N. *et al.* (1994) Nanoliter volume sample cells for 1H NMR: application to online detection in capillary electrophoresis. *J. Am. Chem. Soc.* 116, 7929–7930
77 Pusecker, K. *et al.* (1998) On-line coupling of capillary electrophochromatography, capillary electrophoresis and capillary HPLC with NMR spectroscopy. *Anal. Chem.* 70, 3280–3285
78 Kautz, R.A. *et al.* (2001) Sample concentration and separation for nanoliter volume NMR spectroscopy using capillary esotachophoresis. *J. Am. Chem. Soc.* 123, 3159–3160
79 Jayawickrama, D.A. *et al.* (2003) NMR detection in capillary electrophoresis and capillary electrochromatography. In *Electrokinetic Phenomena* (Rathore, A. and Guttman, A.), Marcel Dekker (in press)