On-Line Microdialysis Coupled to Analytical Systems

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Abstract

In vivo sampling of interstitial fluid using microdialysis (MD) fibers has become a standard and accepted procedure. The resulting small volume samples, often with low concentrations of the analyte of interest, present a particular challenge to analytical methods. Rapid developments of analytical techniques with high sensitivity accelerate their combination with MD. On-line MD-based analytical systems are receiving increasing attention because they can provide near real-time data prior to the off-line system. The purpose of this review is to provide information for on-line MD–analytical systems. Special emphasis has been given to the main progression on methodologies of each method during recent years. The advantages, limitations, and adaptivity are also discussed. These methods include on-line MD–liquid chromatography, on-line MD–capillary electrophoresis, on-line MD–mass spectrometry, and on-line MD–biosensor.

Introduction

Microdialysis (MD) is a well-established approach for the continuous monitoring of substances both in vivo and in vitro. Previous studies in our laboratory have proved it an ideal sampling technique, causing minimal perturbation to physiological processes (1). The fast development of this analytical technique facilitates the combination of MD sampling off-line with a suitable separation technique and detector; thus an "MD-based analytical system" is realized and widely utilized. However, off-line analytical strategies for mixture have commonly required incorporation of sample preparation steps for scale-up, extraction, fractionation, storage, and multiple instrumental analysis of each fraction. These steps can be time-consuming, progress-slowing, and can sometimes lead to component degradation (2,3). In order to overcome these drawbacks, on-line MD–analytical systems appeared, in which dialysate is directly injected into the analytical apparatus that usually contains a separation device and a detector. It offers a number of advantages over off-line systems. Figure 1 shows a schematic of the on-line MD–analytical system concept. It usually contains a microdialysis sampling device, a suitable separation device (sometimes without separation device), a detector, and a system for data analysis.

The main advantages the on-line system possesses are shown as follows (4,5): (*i*) providing immediate feedback on treatment effects, which allows near real time detection of analytes with subminute temporal resolution; (*ii*) eliminating problems related to fluid transfer and storage, including sample mislabeling or loss; (*iii*) avoiding problems associated with the handling of small volume samples, because nanoliter scale sample has been commonly analyzed; (*iv*) reducing the exposure of dialysate to air, aimed at avoiding degradation of some unstable substances such as 5-hydroxytryptamine and malondialdehyde; (*v*) providing simplified sample preparation and automated analysis; (*vi*) generating analytical information while the experiment is still processing; and (*vii*) providing a high recovery, good reproducibility, and good stability.

Here we review the main kinds of on-line MD–AS, including on-line MD–liquid chromatography (LC), on-line MD–capillary electrophoresis (CE), on-line MD–mass spectrometry (MS), and on-line MD–biosensor (B). Each method is reviewed according to the following scheme; (1) advantages and limitations; (2) recent developments; and (3) application.

Methods

On-line MD–LC

On-line MD–LC has been well-validated and widely employed by combining with UV, MS, fluorescence detection (FD), and

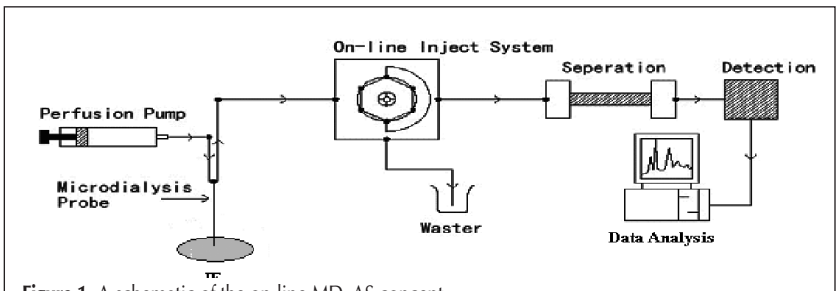

Figure 1. A schematic of the on-line MD–AS concept.

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electrochemical detection (EC), etc. in biochemical and pharmaceutical research (4,6–8).

Advantages and limitations

On-line MD–LC has the characteristics of simplicity and popularity compared with other methods. Although the temporal resolution cannot be comparable to that of on-line MD–CE, the on-line device using the LC method is a more common and less sophisticated instrument (9). Additionally, as MD samples are protein-free, they can directly be injected into the chromatographic system without any further treatment (10,11).

One difficulty encountered with the on-line system is how to maintain the continuity provided by MD, which represents an instantaneous picture of the in vivo system. Two problems are involved in order to keep synchronism. On one hand, the next sampling cannot be injected until the previous one has been completely eluted to avoid sample carry-over (4). On the other hand, all of the dialysate must be collected and analyzed, which is particularly important for pharmacokinetics (PK) investigations where several of the calculated parameters depend on the area of the concentration-time curve (AUC). Once the collection requirement is met, AUC is simply the sum of the individual dialysis samples, as these are integral over the collection time interval (12,13). This problem can be improved by using two sample loops instead of only one, in which one loop is filled with dialysate while the other is injected into the chromatograph (12,14).

On-line injection valve is commonly utilized in on-line MD–LC systems. It is a key to continuous, automatic, and dynamic sampling and analysis, especially for the "separation-based" method, as the proceeding is most likely affected by the time of separation. The initial design of the dual six-port injection valve system is shown in Figure 2 (12,14). In Position A, path a which is filled with the next dialysate sample shows the sample injection process, and path b shows the sample analysis process. In Position B, path b shows the sample injection process, and path a shows the sample analysis process. The two processes are alternated to ensure the continuity of sampling and analysis. Later, single six-port injection valves appeared to overcome the complexity of the dual six-port injection valve system; however, they are not able to maintain continuous dialysate collection. As shown in Figure 3, Position A is the dialysate flow path and Position B is the chromatographic flow path. The versatile 10-port injector is a significant addition to the previously mentioned two and has been increasingly employed because it is not only concise in design but also maintains continuous dialysate collection and analysis. It employs two sample loops alternating between the dialysate flow path and the chromatographic flow path. When analysis of dialysate in one sample loop is in progress, the next dialysate sample is injected through the other sample loop. The plumbing and the alternating principle between two positions of this system is shown in Figure 4.

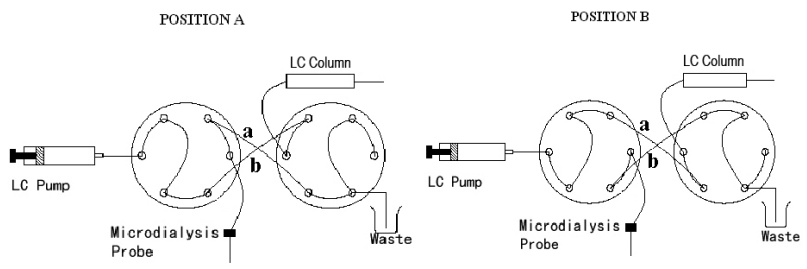

Figure 2. Schematic diagram of the dual six-port on-line injection valve system.

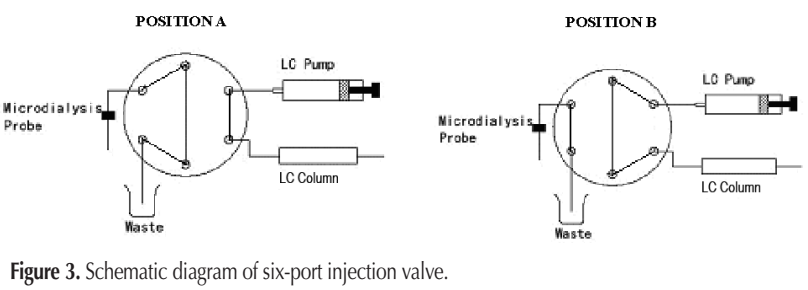

Figure 3. Schematic diagram of six-port injection valve.

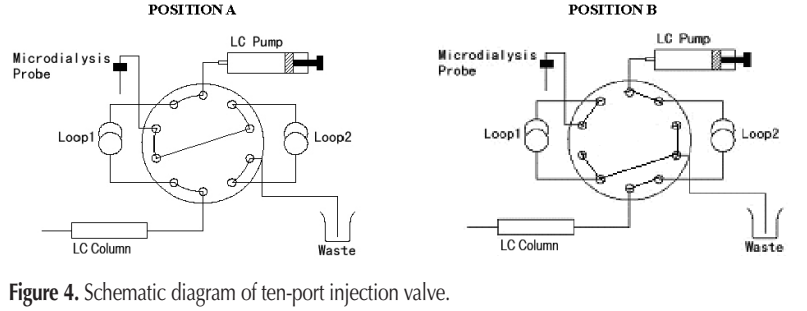

Figure 4. Schematic diagram of ten-port injection valve.

Problems brought by the on-line injection systems are that valuable sample will easily be lost between injections as the dialysate is shunted to waste during the separation, and will be possibly contaminated due to incomplete flushing of the injection valve. The investigation has been undertaken to make some substantial improvements. For example, microbore or capillary columns are employed at much lower flow rates aimed at gaining better temporal resolution and prolong flushing time.

Recent developments

Compared to conventional LC (15), microbore (MB) columns with a 20-fold smaller cross section than the conventional 4.6 mm i.d. column induces a tremendous increase in the sensitivity, a larger decrease in detection limits, and also provides a corresponding increase in sample concentration as well as a decrease in band broadening (16). Now it has been shown to be a suitable technique for on-line MD systems. This is because in on-line MD–LC systems, the dialysate is collected over some fixed time interval to provide the sample for chromatographic analysis, while the high temporal resolution of MD is lost

Table I. Applications of On-Line MD–Analytical Systems

Analytical method	Substance	Reference
MD–HPLC–UV	5-HT	(30)
	Melatonin	(30)
	Phthalate esters	(7)
	Carbohydrate enzyme	(36)
	Nitric oxide	(25)
	Arbutin	(37)
	Fluconazole	(33)
		(34)
	Ginkgolide A, B	(38)
	Acetylcholine	(31)
	Malondialdehyde	(32)
	Diclofenac	(35)
	Dopamine	(29)
MD–LC–MS	Acetylcholine	(6)
	Morphine	(18)
	Peptide	(39)
	Complex protein/peptide mixtures	(40)
	Meropenem	(21)
MD–HPLC–EC	Ropivacaine	(19)
MD–HPLC–FD	Ascorbic acid	(8)
	Histamine	(4)
MD–HPLC–CL	Levodopa	(41)
MD–HPLC–HGAAS	Arsenic	(42)
MD–CE–LIFD	Dopamine	(43)
	Peptide	(45)
	Amino acid	(45)
		(46)
		(47)
		(48)
	Noradrenaline	(44)
	Gamma-aminobutyric acid	(44)
	Glutamate	(44)
		(49)
	Aspartate	(44)
		(49)
	Taurine	(51)
	D-serine	(50)
MD–SPME–CE–UV*	Beta-lactoglobulin A	(52)
MD–SPME–CE–UV	Ovalbumin	(52)
MD–MS	Non-covalent complexes	(53)
MD–CIEF–MS	Peptide	(54)
MD–CIEF–RPLC–MS	Complex protein/peptide mixtures	(55)

and becomes dependent upon the sample volume requirements of the chromatographic system. MBLC systems are commonly employed to minimize the sample volume required and therefore increase the temporal resolution of the experiment (12,17).

However, one thing worth noting is the dead volume in a microbore column, which strongly influences the overall performance of LC with respect to sample diffusion and consequent peak broadening (17). A UniJet electrochemical cell to minimize the dead volume for the microbore column was introduced by Chaurasia et al. (16). The cell serves as an end fitting to the microbore column instead of using connecting tube and adapter. It has not only provided less dilution of samples due to its smaller internal volume, but also exhibited a two-to-three-fold higher sensitivity due to its unique radial flow pattern.

The detector is one of the main parts in on-line LC systems. LC–UV is the most common detection method for its high sensitivity, achieving 0.001AUFS, low noise, and especially wide adaptivity. Substances can be detected by UV if they only have

Table I. (Continued) Applications of On-Line MD–Analytical Systems

Analytical method	Substance	Reference
MD–ESI–FTICR–MS	Complex protein/peptide mixtures	(56)
MD–B	D-lactic L-lactic acids	(58)
	Glucose	(60)
		(61)
	Glutamate	(59)
	Choline	(59)
	Dopamine	(57)
MD–IF–B	Glucose	(62)
	Lactate	(62)
		(60)
MD–FI–EC	Glucose	(63)
	Lactate	(63)
MD–FI–CL	Metronidazole	(64)
MD–GFAAS	Terbutaline sulfate	(65)
	Metal ion	(66)
	Mg	(67)
MD–FAAS	Calcium	(68)
MD–Micro-flow CL	Glucose	(69)
MD–FD	Ergometrine maleate	(70)
MD–Mid-infrared spectroscopy	Urea	(71)
MD–Enzyme fluorometric detection	Glutamate	(44)

* SPME: solid-phase microextraction.

absorption between 200~400nm. FD is another sensitive method used, less popular than UV due to low quantities of fluorescent materials. However, just for this reason, it has excellent specificity and selectivity and is used frequently in some special areas. Moreover, the very low detection limitation makes FD extremely suitable for trace analysis, which meets the requirement of small sample volume collected by MD. EC, with high sensitivity and good selectivity, is also utilized for electro-active substances. EC is more vulnerable to gradual changes in sensitivity and signal-to-noise ratio. The selection of detectors in experiments mainly depends upon the analyte character and the experimental goal.

In the last few years, on-line MD–capillary LC (cLC)–MS systems emerged as promising and effective approaches for small volume samples with low concentrations (6,18–20). Packed capillary column LC is suitable because it is able to reduce the chromatographic dilution in columns and also improve ionization efficiency via lower flow rates. An on-line coupling of MD to a packed capillary column switching LC system (0.2 mm i.d.) and MS detection was developed by Bergstrom et al. for the measurement of free concentrations of ropivacaine and metabolite from spiked plasma samples (19). Good linearity was achieved from 0.1 to 100 nM (R^2 were 0.9999 for all analytes). A current report showed improvements in in vivo monitoring of ACh based on on-line MD–cLC–MS (6). Data from this study exhibited high temporal resolution (data points at 2.4 min intervals). The pre-column is usually employed in these systems for pre-concentration of the analytes, and is also essential for desalting the sample prior to MS analysis.

Applications

On-line MD–LC is applied more popularly than other on-line detection systems. Three areas of usage are currently being investigated: (1) PK investigations of drugs; (2) monitoring of chemical changes in vivo; (3) determining the concentration in other areas. Relevant applications of these assays are assembled in Table I (recent 5 years).

Tsai and co-workers evolved a series of PK research in this decade using on-line MD–MBLC–UV systems. In the latest research, a carbapenem antibiotic Meropenem was assayed in the dialysate using a LiChrosorb RP-18 column (mobile phase: 50 mM monosodium phosphoric acid–methanol [80:20, v/v, pH 3.0]) with a UV detector (21). The area under the concentration–time curve and elimination half-lives of meropenem were approximately 6144 ± 1494 min mg/mL and 61 ± 17 min, respectively. Peak areas of meropenem were linear (R^2 > .995) over a concentration range of 0.1~500 mg/mL. Additionally, this method also had a quantitative limit of 0.1 mg/mL. Other drugs investigated including cefazolin, cephalexin, chlorogenic acid, catecholamines cefmetazole, cefuroxime, ceftriaxone, buspirone, etc.

This on-line system has also been shown to be a powerful technique for near real-time monitoring of chemical changes in vivo, especially for intracranial monitoring of neurochemical substances from the extracellular fluid of the brain. One of the fields in which on-line MD–LC has been found widespread use is that of ischemia. Lactic acid levels, pyruvate levels, and the lactic acid–pyruvate ratio of the brain have been advocated for estimation of the severity of cerebral ischemia. All of the compounds mentioned have been proven well monitored by this system in several researches (4,22–24). Moreover, a recent study is designed to elucidate the dynamic changes of nitric oxide (NO) production in the perilymph after transient cochlear ischemia, Significant increases in the oxidative NO metabolites, nitrite (NO[2][–]) and nitrate (NO[3][–]), were measured using an in vivo on-line MD–HPLC system (25). The NOx levels in the scala tympani were significantly increased on day 1 after ischemia/reperfusion, and had returned to the basal level by day 7. Of note is the observation that the time-course pattern of the NOx changes in the cochlea was parallel to the changes in the expression of iNOS.

Other researches can also be seen, such as the determination of aniline and 2-chloroaniline in polymer industrial wastewater (26). The retention times of aniline and 2-chloroaniline are 6.14 min with 0.13% RSD (n = 3), and 12.19 min with 0.13% RSD (n = 3), respectively. The reproducibility of quantitative detection for 100 mg/L was 3.86 and 2.36% RSD for three determinations of aniline and 2-chloroaniline, respectively. Sarafloxacin residues in fortified and incurred eggs were also analyzed by on-line MD–HPLC–programmable-FD system including an automated trace enrichment of dialysates (27,28). Overall recoveries of 87~102% for sarafloxacin residues were obtained from samples fortified over a range of 1~100 ng/g, and the limits of detection and quantitation were 0.2 and 1 ng/g, respectively.

On-line MD–CE

On-line MD–CE is an ideal method for the analysis of samples with small sample volume, rapid analysis, great resolution power, and low cost (72). In most cases, affinity capillary electrophoresis, rapid micellar electrokinetic capillary chromatography (48), and capillary zone electrophoresis (CZE) have been exploited to improve both the sensitivity and selectivity of the method.

Advantages and limitations

CE, with its high mass sensitivity, potential for high-speed separations and only nanoliters of samples being required, is well-suited for such on-line measurements (73,74). The applications of on-line MD–CE have allowed temporal resolution to be improved to better than 10 s (75). Additionally, as only nanoliters of a sample are injected, the direct coupling of MD to CE permits

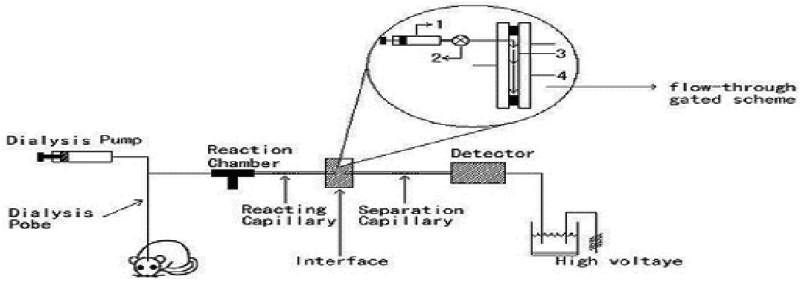

Figure 5. Diagram of a flow-through gated system: 1, gating-flow pump; 2, gating valve; 3, interface channel; and 4, interface.

the use of low perfusion flow rates, which results in higher relative recoveries (76,49).

Another potential adaption of on-line MD–CE is high throughput analysis of complex samples, which can make some improvements in sample preparation. The use of fast CE for high throughput analysis is hindered by a requirement for cumbersome sample preparation methods. For instance, the determination of small organic compounds in tissue samples may require pretreatments such as deproteination; usually precipitation of proteins is applied. When CE is coupled with MD, sampling and deproteination is achieved by inserting MD probes, to generate a sample stream free of particulates and macromolecules (9,77).

The injection method for operation of the MD–CE system should be under consideration. It is usually based on a "flow-through gated scheme", which allowed dialysate to be automatically injected into the separation capillary (43,72,73,76). The interface commonly consists of a Lucite block that holds the outlet of the reaction capillary and the inlet of the separation capillary aligned with a 75 µm interface channel between them. During a separation, a gating flow of electrophoresis buffer is pumped at a low flow rate through the interface channel between the capillaries. This flow prevents derivatized dialysate from entering the separation capillary. When performing an injection, the gating flow is stopped by a pneumatically actuated gating valve. While the gating flow is stopped, the injection voltage is applied (73,78). A diagram of the flow-through gated scheme is shown in Figure 5. In order to minimize the number of external connections required to eliminate dead volumes, a great deal of scientific research has been devoted to the fabrication of microdevices.

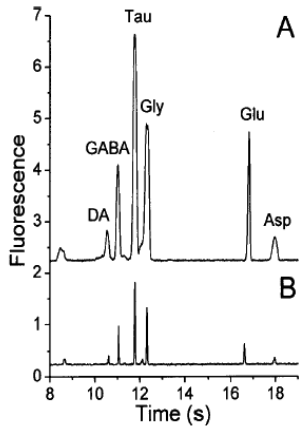

Figure 6. Effect of injection on peak widths for dopamine (DA), GABA, taurine (Tau), glycine (Gly), glutamate (Glu), and aspartate (Asp). The injections were 200 ms at 20 kV and 200 ms at 2 kV for electropherograms (A) and (B), respectively. The separation buffer was 40mM borate (pH 10.5) in both cases with no cyclodextrin (10).

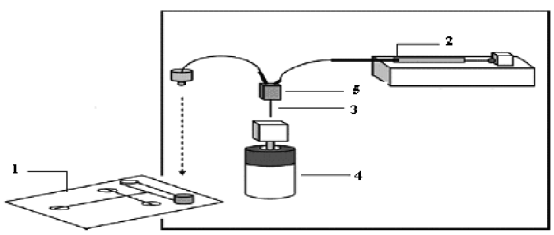

Figure 7. Schematic of the MD–microchip CE setup: 1, microchip device; 2, perfusate syringe; 3, MD probe; 4, sample vial; and 5, tubing.

Recent developments

CE should be coupled to different detection methods: UV, EC, laser-induced fluorescence detection (LIFD), or MS. The present research is focused especially on the applications of CE coupled with LIFD. On-line MD–CE–LIFD performs both derivatization and separation on-line to obtain results in real time. The first reported on-line MD–CE with LIFD involved the separation of an investigational antineoplastic, from its main metabolite, SR-4317 (79). Today it has been introduced as an appropriate technique for neurotransmitter and endogenous active substance determination.

LIFD, with its excellent performance, is required in CE analysis for small volume samples collected by MD in a short time. Its use of lasers as an excitation source greatly improves the detection sensitivity (48). The temporal resolution of the on-line MD–CE–LIFD system in a recent in vivo Dopamine Monitoring research was evaluated by rapidly moving the probe between two vials containing stirred solutions of dopamine (100 and 200 nMm, respectively) at 37ºC while recording electropherograms. It was concluded that the temporal resolution for monitoring would be the electropherogram collection interval of 60–90 s (43). MS detection also gives high sensitivity, but its high cost prevents more widespread application as a routine analytical technique. EC detection has favorable sensitivity, but it still has poor concentration sensitivity and difficult manipulation (7,79). Moreover, the on-line MD–CE–LIFD system also provides a low detection limit. On the basis of the report from O'Brien et al., using the commercial instrument with UV absorbance detection, the limit of detection for D-serine was 13.5 µM. This could be improved to 270 nM using LIFD detection (76).

The background signal caused by the laser scatter offers a particular challenge to on-line MD–CE–LIFD systems, as it usually limits sensitivity in on-column detection. In order to reduce this influence, some improvements have been made in designs. For instance, a sheathflow cuvette was incorporated. Bowser et al. (73) described an electropherogram of six amino acid standards obtained with the new sheath-flow cuvette detector, but with separation and injection conditions similar to those reported in a prior report (79), as shown in Figure 6A. Gains in sensitivity made through the use of the sheath-flow cuvette allowed a reduction in injection volume, which allowed a substantial improvement in separation efficiency, as illustrated in Figure 6B. Peak widths were approximately 30 ms and efficiencies were approximately 500,000 plates compared to 200,000 plates used in reference 79. Action should always be taken to improve the sensitivity of on-line MD–CE–LIFD systems.

One more thing that should be considered is the on-line derivatization that must occur very rapidly, in seconds, especially in microchip electrophoresis devices. The ortho-phthalaldehyde/2-mercaptoethanol (OPA/2ME) system is commonly selected for on-line derivatization because the reaction is rapid and because OPA itself is not fluorescent. Sandlin and coworkers

reported a microfluidic chip coupled to MD that incorporated a reactor channel for pre-column derivatization of amino acids with OPA (80), Recently, naphthalene-2, 3-dicarboxyaldehyde (NDA), which has been found to be more stable than OPA, is increasingly applied for rapid amino acid and peptide analysis. Huynh and coworkers utilized on-column NDA/2-mercaptoethanol (NDA/2ME) derivatization for a three-component mixture of Arg, Gly–Pro, and Asp on-chip (45). It has been shown that NDA/2ME is more suitable for on-column and post-column derivatization of amino acids and peptides.

Another tendency is miniaturization of on-line MD–CE systems. Tubing and connectors in conventional on-line MD–CE can contribute to increased lag times and band broadening (72). The advent of microchip/microfluidic systems has enabled the simplification and miniaturization of analytical instrumentation, minimizing the number of external connections required to eliminate dead volumes (81). With such techniques, probes may be operated at low flow rates where the recovery approaches 100%, allowing quantitative monitoring while avoiding the difficulties of in vivo calibration (46).

The first reported coupling of an MD sampling probe to a microchip CE device was designed by Huynh et al. (72). Later studies integrated derivatization and detection components on-chip to create a portable segregation-based sensor. Sandlin et al. used on-line microfluidic MD–CE for in vivo monitoring of primary amine neurotransmitters in rat brains (46). Recently, the microchip system exhibited a high degree of integration and incorporation with electrophoretic separation, LIFD, and on-chip sample gating and injection, and then the device was connected to an external MD device. A schematic representation is shown in Figure 7. The microchip device described here consists of a glass layer containing etched microfluidic channels that are plasma sealed with a layer of poly-dimethylsiloxane. The tubing serves as a fluidic connection to the microchip and the other end of the tubing is connected to either a perfusate syringe or microdialysis probe (45).

One challenge in coupling MD on-line to a chip-based analytical device is that typical flow rates used in MD vary from 0.1 to 2 µL/min, which is much higher than the picoliters per minute of electroosmotic flow encountered in a chip. However, the sample stream must enter and exit the device without disturbing the sample injection, separation, derivatization, and detection. To accomplish this, some strategies should be developed to accommodate the continuously flowing MD sampling rate (46). Harrison's group was the first to integrate a large sample introduction channel with low flow resistance, allowing real-world samples to enter and flow through the chip (72). A limitation of the design was sample leakage from the injection channel to the separation channel. Chen's group introduced a hydrodynamic plug which pumped sample flow into the separation channel using a gated injection scheme (82,83). Voltage was applied to a single reservoir and floated for 1–5 s to allow discrete sample injection prior to separation. However, all of those strategies have not been consummated and should be constantly improved.

Applications

The current tendency to make on-line MD–CE easier and affordable for basic and clinical research will continue. There will also be more emphasis on in vivo chemical monitoring, especially the concentration change of neurotransmitters in the brain, where they are released and reuptaken in fractions of a second. A large number of neurochemical constituents of the brain can be detected. It should be noteworthy that the study of amino acids is active, including gamma-aminobutyric acid, glutamate, l-aspartate, etc. (39,46,48,73,76,78,84). For example, a recent in vivo research monitored amino acids by microdialysis sampling with on-line derivatization by NDA and rapid MEKC (48). This method allowed 17 amino acid derivatives to be resolved in less than 30 s. On-line injections could be performed at 30 s intervals for in vivo samples, and multiple injections of 1 µM amino acid mixtures derivatized on-line indicated that peak heights had RSD of 3~7% (n = 15~20), depending on the amino acid. Migration times had RSD of 0.2~0.4% over short times (10 min), and increased to 0.3~0.9% for a 2 h in vivo recording session. Detection limits were from 10 to 30nM for the amino acids.

So far, the application of on-line MD–CE in enantiomer determination has been developed. It is regarded as the most effective means of obtaining a rapid separation of D, L-aspartate, and D-serine, where chiral analytes are derivatized with a chiral reagent to form diastereomers, which can be separated in achiral media (9,76). With 7.5 mM cyclodextrin in the electrophoresis buffer, the enantiomers of interest could be resolved in 3 s with an electric field of 2500 V/cm over a separation length of 15 mm. Relevant MD applications of these assays are assembled in Table I.

On-line MD–MS

The development of on-line MD–MS systems was stimulated by the ever-increasing demand for the capability of handling samples with improved sensitivity and higher throughput (85). Among MS techniques, the electrospray ionization approach (ESI–MS) has received much attention for its compatibility with liquid separation techniques such as LC and CE (40,86–87).

Advantages and limitations

Compared to other detection techniques, the high selectivity of MS (MS–MS) detection and its ability to conclusively identify the analytes based on their molecular weight makes MS an attractive choice for the analysis of MD samples (85). It is suitable for handling the analysis of small samples with low concentrations of analytes frequently present in MD samples. Moreover, MD sampling can remove part of the salts and impurities because of the size-exclusion nature of the membrane.

To date, on-line MD–MS or on-line MD–LC–MS is found to be not always straightforward for the non-volatile ions in the perfusate, requiring systematic cleaning to avoid loss of sensitivity (19). To this purpose, a volatile buffer (88) or water (89–90) has been used as perfusate in some in vitro experiments instead of a physiological buffer. However, this method can change the osmotic pressure at the probe and may affect the extraction efficiency; moreover, it cannot mimesis the biological environment, which is essential to samples in vivo. Bergstrom et al. developed a packed capillary column switching liquid chromatographic system (0.2 mm i.d.) to on-line measure free concentrations of ropivacaine and metabolite from spiked plasma samples (19). In this system, a deuterated internal standard was added

on-line by the middle valve and the third valve operated both a pre-column, for desalting of the physiological buffer used in the sampling procedure, and a separation column. The capillary pre-column provided effective on-line desalting of the high ionic strength perfusate. Later, they employed the similar packed capillary mini-column in the on-line MD–MS to study liver damage (85).

Recent developments

With the growing application of on-line MD–MS for the studies of complex biological processes, especially for identification of multiple proteins and characterization of DNA, substantial improvements are required in separating systems for achieving excellent resolving power, ultrahigh speed, and ultrasensitive analysis of complex protein/peptide mixtures (40,87). The recent research has aimed at the development of multidimensional separation schemes (usually two-dimensional). In these systems, MD junction was employed as an effective clean-up strategy (56).

In addition, many mass spectrometers use two or more mass analyzers for tandem MS (MS–MS). Apparatus and parameters associated with the methods for the on-line integration of MD sampling with MS–MS using thermospray and electrospray interfaces were studied by Kerns et al. (2). It was found to provide useful levels of sensitivity, efficiency, linearity, and structural identification data. Consequently, short sampling times and continuous analysis were readily obtained.

Applications

The present research is focused on exploiting novel bio-analytical approaches with ultrahigh speed and sensitivity required for identificating and quantitating multiple proteins and DNA segments and assaying biological samples (91–94). On-line MD–MS has been widely used for this purpose. The present investigation is undertaken to introduce it in the analysis of complex protein/peptide mixtures, especially in proteomics research (55). An MD-crossed immunoelectrofocusing (CIEF)–transient capillary isotachophoresis (CITP–CZE)–ESI–Fourier transform ion cyclotron (FTICR)–MS system was described in one report for the separation and characterization of proteins in complex mixtures (56). A total of 1174 unique proteins, corresponding to 26.5% proteome coverage, were identified from the cytosolic fraction of *S. oneidensis*, while requiring < 500 ng of proteolytic digest loaded in the CIEF capillary. Reproducibility of peptide separation and identification was examined by performing multiple runs of identical *S. oneidensis* tryptic digest samples. For two analyses performed on consecutive days, the RSD of the peak height, peak area, and migration time in transient CITP–CZE–ESI–FTICR–MS were around 5–10%.

The characteristics of suitability for handling the analysis of small samples with low concentrations and real-time determination made the on-line MD–MS a potential method for endogenous substance monitoring. On-line MD–cLC–MS has been applied recently to monitor endogenous acetylcholine (ACh) from the rodent brain with high temporal resolution (data points at 2.4 min intervals) and sensitive limits of detection (0.04 nM or 8 amol injected) (12).

There has been a growing interest in the area of neuropeptides monitoring. MD sampling has provided direct measurement of extracellular peptide levels in vivo; however, the microliter samples made analytical measurements difficulty. One of choice is radioimmunoassay (RIA) because it has excellent specificity and can achieve detection limits of 100 amol in microliter fractions, but RIA results can be ambiguous due to crossreactivity of the labeled antibody with similar peptides, and also usually is limited to the measurement of only a single peptide per animal. MD–LC–MS is attractive because it offers the possibility of detecting peptides with sequence specificity and can be used, in principle, for any peptide. Several endogenous neuropeptides have been investigated, including Met-enkephalin8 and neurotensin. Haskins and his group utilized on-line MD–LC–MS to monitor and discover endogenous neuropeptides from the globus pallidus of anesthetized male Sprague-Dawley rats (20). The result showed that it was useful for monitoring known peptides (basal dialysate levels of Met-enkephalin and Leu-enkephalin were 60 ± 30 and 70 ± 20 pM, respectively), studying processing of endogenous peptides, and characterizing novel peptides (revealed 11 novel peptides) that are potentially neuroactive.

In pharmaceutical research experiments, the system was applied successfully to study drug metabolite and protein binding profiles. On-line MD–packed cLC–tandem MS was reported for measuring free concentrations of ropivacaine and metabolite from spiked plasma samples (19). The free fractions of ropivacaine (200 nM total concentration) and its metabolite (20 nM total concentration) in spiked plasma samples were 12 ± 3 and 47 ± 5% (± standard deviation for day-to-day variations, n = 3), respectively. Others, like penicillins G and V, cocaine, and benzoyl ecgonine were also reported (2). Relevant MD applications of these assays are assembled in Table I.

On-line MD–B

On-line MD–B systems have gained widespread use for on-line determination or monitoring of a wide range of analytes including glucose, lactate, amino acids, glycerol, etc. (57,59,60,62,95,96). The apparatus typically consists of a syringe pump, a MD probe, and a sensor or enzyme reactor, followed by an EC detector.

Advantages and limitations

On-line MD–B is more quantitative and provides a higher conversion of analytes, a better stability, a longer lifetime, and easier calibration compared to an implantable modified microelectrode. It can provide a high level of sensitivity by exploiting the very high-defined rates of mass transfer to its surface to greatly enhance the Faradaic component of their current response. Additionally, the small size also allows it to be operated efficiently in small solution volumes (97).

Recent developments

The present study is focused on fabricating a micro-device that usually involves a MD sampling interface, a sensor element, and a fluidic connection between these components. Minimization of the response time is achieved as a result of suppression of the system dead volume, which is considered to be a solution to the problems the conventional device met (59,97).

Some strategies have been taken to improve the performance (e.g. linearity, selectivity, and stability) of the miniaturized on-line MD–B, and as a result, different designs of microdevices have been reported in recent years. A miniaturized flow-through MD-B with a cell volume of only a few nanoliters was developed in one laboratory (101). Another miniaturized on-line monitoring system based on a microfabricated multi-enzyme silicon sensor chip with flow channels integrated on a chip was also reported (98). When the system was equipped with an MD probe with lower flow rates of 0.5 microl/min, an over 90% recovery and long-term stability (a slower decrease of approximately 0.3% per h for both sensors under continuous operation over 24 h) could be achieved.

MD can also be incorporated into a flow-injection (FI)–B system with the ability to provide on-line sample dilution. Once the injection valve is introduced into the system, the multiple sensors used in the fabrication of the device have the potential for multi-analyte determinations (39). In an on-line MD–FI–dual B, the dialysate from the MD tube is delivered to a sample loop of the six-way autoinjector and then automatically injected into the FI line with a dual enzyme electrode suitable for the simultaneous of D-lactic and L-lactic acids (58).

Applications

On-line MD–B has been widely used for monitoring a number of compounds, both in vitro and in vivo. The possibility of using this system as an in situ clinical monitor is of particular interest (95). Applications of transcutaneous MD and monitoring of glucose have been described for adults to provide better insulin management regimens for diabetics (60,99). It was found that transcutaneous glucose was a good index of plasma glucose in adults, so it can be used as personal monitors for blood glucose levels. The measurement of lactate, a marker for oxygen deficiency, is also used in the intensive care unit to monitor patients' conditions. The GlucoDay portable medical device, which was used to continuously measure glucose levels, is already present in the European market. Recently, a novel continuous lactate monitoring system has been developed (100). It is capable of recording subcutaneous lactate every 3 min and the response of the biosensors remained stable, showing a limited drift of 8% within 60 h during prolonged monitoring periods. Preliminary results have also shown a shelf life of approximately 10 months.

There is increasing interest in recent years in the use of on-line MD–B for continuous and on-line monitoring of cerebral extracellular fluid. The variations of glucose, lactate glutamate, and pyruvate in the brain were determined to provide information on cerebral energy metabolism and have become a diagnostic tool in acute human brain insults, such as stroke, head trauma, or ischemia after subarachnoid hemorrhage (59,62,101). A recent report has shown a system using slow MD (0.5 µL/min), fast sampling, and FI–B for glucose and lactate to on-line monitor of ischemic events and metabolic changes, following reperfusion in striatum of freely moving rats subjected to endothelin-1 induced, transient focal cerebral ischemia. The high-time resolution (1 min) provided detailed information on lactate rise times and duration of low glucose (62). Relevant MD applications of these assays are assembled in Table I.

Other on-line MD-analytical systems

Apart from the on-line systems mentioned previously, there has been a growing interest to employ other analytical methods, including on-line MD–enzymatic assays (EA) (102,103), on-line MD–flame atomic absorption spectrometry (FAAS) (66–68), on-line MD–chemiluminescence (CL) (64,65,69), and on-line MD–mid-infrared sensor (104). Among these methods, the first three have received much more attention.

On-line MD–EA is a rational and effective strategy for identifying specific compounds in MD samples, especially when high temporal resolution is required. These methods include on-line flow MD–EA–FD, on-line flow MD–EA–amperometric detection (AD), and on-line sequential MD–EA–AD. Recently, an on-line micro-flow MD–EA–AD was proposed for the detection of trace amounts of the neurotransmitter L-glutamate released from rat brain cells (102). This method has gained relative widespread use for the determination of numerous endogenous metabolites.

On-line MD–FAAS is presented as a novel concept for automatic micro sampling and continuous monitoring of metal ions with minimum disturbance of the sampling site. This system is useful not only in auto-sampling in situ throughout the experimental process, but also in monitoring the dynamic changes of minerals in extremely low concentrations and small sample volumes at near real-time. An on-line MD–graphite furnace atomic absorption spectrometry system was designed for dynamic monitoring of extracellular Mg in gerbils subjected to transient focal cerebral ischemia with the detection limit 0.03 microg/L (67). Another direct, rapid, and continuous in vivo monitoring of diffusible calcium in the blood of

Table II. A Summary of On-Line MD–Analytical System Methods

Method	Advantages	Recent developments	Application
MD–LC	Simplicity and popularity	MB–LC MD–cLC–MS	Widespread use
MD–CE	Small volume sample required Rapid analysis Great resolution power	MD–CE–LIFD Miniaturization	In vivo chemical monitoring
MD–MS	High mass sensitivity	Multidimensional separation Tandem MS	Complex biological processes neuropeptides monitoring
MD–Biosensor	Excellent selectivity High sensitivity	Miniaturization MD–FI–B	Clinical monitor In vivo chemical monitoring

living rabbit systems has been developed, also using MD coupled on-line with FASS (68), gaining a low detection limit (3.66 mg/L) and good precision (RSD = 6.2%, n = 50). In the future, this system will gain relatively widespread use for continuous metal ion monitoring.

On-line MD–FI–CL systems have been developed and demonstrated considerable potential for in vivo on-line monitoring, achieving improved resolution and reliability. This system is usually employed for in vivo glucose determination (69,106), and also provided a reliable and simple technique for the study of drug-protein interaction. The interaction of metronidazole and human serum albumin was studied using this system by Chen et al. (64). The binding of terbutaline sulfate to bovine serum albumin was also studied in vitro (105).

Conclusions and Future Directions

In contrast to other sampling methods, MD sampling, which is considered an invasive technique, provides continuous sampling of compounds with minimal perturbation to the system under study. The advent of on-line MD–analytical systems provide good time resolution, high levels of relative recovery, and eliminated the need to pretreat, collect, and store tissue samples over the off-line approach. An issue to be considered in on-line systems is the connection between MD and the analytical technique. Fortunately, recent advances in valve and injection systems have facilitated the development of on-line techniques.

On-line MD coupled to an analytical system including LC, CE with different detectors, or directly coupled to MS or biosensor has been successfully developed. On-line MD–LC has been generally accepted as a prevalent and well-accepted method, though its temporal resolution cannot be comparable to that of on-line MD–CE. Both on-line MD–CE and MD–MS have high mass sensitivity and good temporal resolution. However, problems with the two previously mentioned methods should be under consideration, such as salt contamination within on-line MD–MS. On-line MD–biosensor has been emphasized for its application in several special fields; for example, glucose monitoring.

Different methods along with individual advantages and limitations, recent develop-ments, and application are described in this text. Important progress has been made during a short period in this area, such as micro-chip design and versatility of detectors. Un-doubtedly, these improvements have had an important bearing on the development of on-line MD systems. The main conclusion is concisely listed in Table II.

It should be considered that the use of on-line MD–based analytical systems is still in the developmental stages, with the continuous integration of the sampling and analytical techniques to improve time resolution and synchronism. Furthermore, the problems brought by connection will be eliminated to some extent. In addition, miniaturization of the analytical systems will extend their applications into other areas, and facilitate their utilization in clinics.

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