Research at the Biomedical Analysis Group of the University Utrecht, The Netherlands

“Driven by analytical questions from biomedical research and development, the Biomedical Analysis Group will continue to design novel, improved and complementary techniques for pharmaceutical analysis and bioanalysis.”

The Biomedical Analysis Group is part of the Department of Pharmaceutical Sciences at Utrecht University (UU), The Netherlands. The group participates in the Academic Biomedical Centre, which unifies the strong expertise in life science and biomolecular research at UU in the fields of (veterinary) medicine, pharmacy, biology and chemistry. The UU has denoted drug innovation as one of its major focus areas.

The Biomedical Analysis Group designs and applies novel analytical methodologies for pharmaceutical and biomedical purposes. Requirements in the pharmaceutical, biomedical and clinical fields are starting points for our research strategy, which is directed towards the investigation of innovative analytical concepts with particular attention on sample pretreatment, separation and detection using state-of-the-art instrumentation. The group's research focuses on advanced separation techniques and their combination with MS. Special emphasis is put on capillary electromigration techniques and nano-LC. The group explores the wide potential of CE for achieving new or improved possibilities for drug and biomolecular analysis. CE is a microscale technique providing fast and efficient separations and requiring only very small amounts of sample and solvents. The mechanism of CE offers unique selectivity and separation performance. CE is also particularly useful for highly polar compounds that may not be easily covered by other separation techniques. The potential of monolithic materials for nano-LC to accomplish fast and efficient separations for peptides and proteins is another research theme.

The group's research started in 1995 at the University Centre for Pharmacy in Groningen, The Netherlands, with the development of new methods for drug-impurity profiling based on CE. The efforts quickly expanded to the analysis of new protein therapeutics and DNA (genotyping). The move of the authors

to UU in 2001 strongly boosted the development and use of new CE–MS methodologies, in which the group now holds a leading international position. Method development has been directed towards (chiral) drugs, peptides, (glyco)proteins, carbohydrates and metabolites and has triggered various collaborations with the pharmaceutical industry, hospitals, instrument companies and academic groups. At present, our particular research focus is on drug profiling, characterization of protein pharmaceuticals and metabolomics. The main lines of research are outlined in the next section.

Research profile

■ Drug profiling by CE–MS

CE is very suitable for the analysis of ionogenic drugs and their impurities. The separation mechanism of its most widely used mode, capillary zone electrophoresis, allows analyte differentiation according to charge-to-size ratio, thus yielding valuable orthogonality with common separation techniques such as reversed-phase LC. Special attention is paid to electrokinetic chromatography (EKC) as both charged and neutral drug compounds can be separated. EKC methods often involve the use of nonvolatile pseudo-stationary phases, such as micelles and cyclodextrines, which may interfere with ESI. Several successful EKC–ESI–MS approaches have been developed and their applicability for impurity profiling of drugs was demonstrated (**FIGURE 1**). The group was one of the first to introduce alternative ionization techniques, such as atmospheric pressure photoionization, to CE–MS and EKC–MS. Unlike ESI, these techniques do not suffer from signal suppression by nonvolatile constituents and are particularly useful for less polar compounds. The different ionization techniques have been evaluated systematically and were shown to provide complementary information on drug impurities.

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■ Characterization of biopharmaceuticals by CE-MS & CE-fluorescence

When compared with conventional drugs, the characterization of protein pharmaceuticals poses a great challenge due to their complex properties. Proteins may comprise different isoforms and highly related impurities. Protein structural integrity is essential for activity and, therefore, degradation, protein (un)folding and formation of aggregates should preferably be monitored. CE is an attractive tool for the

separation of intact proteins, offering fast and efficient separations. Moreover, CE can be conducted in 'biocompatible' buffers under conditions that allow analysis of proteins in their native state.

Methodologies for efficient CE-MS of proteins have been developed by the group. Capillary-coating strategies involving non-covalently bound polymers are used to prevent the adverse adsorption of proteins to the capillary wall. TOF-MS, which presents the high mass resolution and accuracy needed for native protein analysis, has been coupled to CE, providing the possibility of identifying highly related degradation products in biopharmaceuticals (**FIGURE 2**). CE-MS interfacing schemes, with and without the use of sheath liquids, are designed and compared for protein analysis in order to achieve sub-nanomolar sensitivities. The coupling of capillary isoelectric focusing (CIEF) and MALDI-MS using spotting technologies has been accomplished and applied to the analysis of biopharmaceuticals. Another line of research involves the development of selective wavelength-resolved fluorescence detection of protein pharmaceuticals in CE. Making use of native protein fluorescence, information on protein purity and conformation can be obtained simultaneously.

■ SPE-CE

The concentration sensitivity of CE is relatively low, due to the intrinsically small injection volumes (1–100 nL). The usefulness of CE for the analysis of low-abundant compounds in biofluids can be greatly improved by applying electrophoretic and/or chromatographic pre-concentration techniques. The group has paid particular attention to the online and inline combination of SPE with CE. In the online mode, the SPE column has been coupled to the CE system using new valve-based interfacing techniques providing solvent-switching capabilities. In the inline mode, the SPE material is an integrated part of the CE capillary, avoiding dead volumes and loss of analyte; however, the introduction of the biological matrix into the capillary can disturb the separation. Both coupling approaches have been evaluated and applied to the determination of neuropeptides in CSF, yielding detection limits in the low nanogram/milliliter range. Powerful bioanalysis is achieved by combining SPE-CE with MS comprising sample handling, separation and selective detection in one hyphenated system (**FIGURE 3**).

Figure 1. Impurity profiling of the drug amiodarone by nonaqueous electrokinetic chromatography with ESI-MS detection. Extracted-ion chromatograms at indicated m/z values are shown. A total of 12 impurities are revealed with an overall relative content of 0.47% (area/area). Background electrolyte: 10-mM ammonium formate, 0.75-M formic acid and 10-mM HDAS- β -cyclodextrin in methanol. Reproduced with permission from [1] © Wiley-VCH Verlag GmbH & Co. KGaA.

The metabolome is the complete set of endogenous low-molecular weight metabolites in cells, tissues and body fluids and metabolomics is the comprehensive analysis of these metabolites. Metabolomics provides insight into the (patho) physiological state of an individual as endogenous metabolites represent real end points of biochemical pathways. Given that the overall status of an individual is reflected by his or her metabolic state, metabolomics has the potential to have a great impact upon medical practice by providing useful biochemical data.

- Monolithic LC columns

Monolithic LC columns contain a single piece of stationary phase that displays high permeability and low resistance to mass transfer. Monolithic columns are very suitable for the separation of peptides and proteins. Both silica-based and polymeric materials are studied for the separation of protein digests and protein mixtures. Integrated systems based on monolithic columns, immobilized enzymes and MS detection have been developed. Particularly, the possibility of relatively long monolithic columns for the separation of complex protein digests has been demonstrated (**FIGURE 5**). Short monolithic columns appear to be well suited for the fast analysis of more simple mixtures.

Figure 4. CE-MS workflow for metabolomics study of urine of patients with CRPS. CE capillary coated with polybrenopoly(vinylsulfonate).

BGE: Background electrolyte; CRPS: Complex regional pain syndrome; DA: Discriminant analysis; FA: Formic acid; MVA: Multivariate analysis; PCA: Principle component analysis; PLS: Partial least squares.

Peak capacities of different columns are compared with MS identification scores such as the number of identified peptides and protein sequence coverages.

Future perspective

Driven by analytical questions arising from biomedical research and development and together with partners from the pharmaceutical industry and institutes, the Biomedical Analysis Group will continue to design novel, improved and complementary techniques for pharmaceutical analysis and bioanalysis. New challenges in drug profiling, such as the determination and identification of genotoxic and immunogenic impurities, will require highly specific and sensitive analytical methodologies. Protein therapeutics will play an increasingly important role in contemporary drug development and production. As a result, there is a growing demand for separation–detection methodologies that allow quality assessment of intact proteins. New analytical approaches are needed that allow the accurate and reliable determination of the potential differences among biosimilars (i.e., generic therapeutic proteins) and the characterization of new biopharmaceuticals, such as therapeutic antibodies, vaccines and drug–carrier constructs. We will develop CE methodologies for these purposes applying

Figure 5. Base peak chromatograms of a nine-protein digest mixture. Separated on polystyrene-divinylbenzene monolithic 250 x 0.2-mm internal diameter column using a 150-min gradient of 1–31% acetonitrile in water (both acetonitrile and water containing 0.5% formic acid).

appropriate interfacing schemes to combine different CE modes with advanced mass analyzers and fluorescence detectors. Next to ESI, other ionization techniques, such as MALDI, will be used and optimized for CE–MS, providing high sensitivity and structural information for target compounds.

The potential of CE techniques for selective and sensitive bioanalysis of compounds that are difficult to analyze with other methods will be further explored. Particular attention will be paid to the profiling of highly polar metabolites in biofluids, the possibilities of achieving enantioselectivity among endogenous compounds using chiral selectors and the determination of protein therapeutics in plasma or serum. As the sensitivity of CE–MS is still limited when compared with LC–MS, improvement of detection limits by electrokinetic and chromatographic preconcentration

will be further investigated. Selective SPE using (bioaffinity) phases will be pursued and combined with CE and LC. Comprehensive profiling of metabolites will be a major goal using a few CE methods applying appropriate coatings. Comparisons will be made with novel LC stationary phases suited for polar compounds and complementarity will be tested for clinical samples.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

- The research carried out by the Biomedical Analysis Group of the University Utrecht involves the development and evaluation of advanced separation–detection techniques for pharmaceutical analysis and bioanalysis.
- The group's main area of research is exploring the wide potential of CE–MS, including various ionization modes to achieve new and improved methods for the analysis of (bio)pharmaceuticals.
- Improved resolution and reproducibility in CE is pursued by the use of selective buffer additives, capillary coatings and nonaqueous electrolytes.
- For more sensitive bioanalysis, online approaches for preconcentration using electrokinetic and (affinity) chromatographic principles are developed.
- Nano-LC with monolithic stationary phases is studied and used for protein and peptide analysis.
- Application areas in pharmaceutical analysis are drug profiling and characterization of biopharmaceuticals.
- Bioanalytical application areas are metabolic profiling of biofluids and bioanalysis of (bio)pharmaceuticals.
- The group collaborates intensively with the pharmaceutical industry, instrument companies, hospitals and other academic groups.
- Future challenges include the further improvement of sensitivity and selectivity of CE–MS and (nano-)LC–MS systems and the integration of sample handling, efficient separation and selective detection.

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