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Liquid chromatography–mass spectrometry with ionspray and electrospray interfaces in pharmaceutical and biomedical research

A. P. BRUINS
University Centre for Pharmacy, A. Deusinglaan 2, 9713 AW Groningen (Netherlands)

ABSTRACT

Electrospray and ionspray techniques use samples that exist as ions or ion–molecule complexes in solution. After the dispersion of the solution into an electrically charged aerosol, the sample ions may escape from the solution into the gas phase in a region that is at atmospheric pressure. The sample ions are transported into the mass analyser which is operated under a high vacuum. Liquid chromatographs can be coupled to electrospray and ionspray interfaces. Flow injection or continuous infusion of a sample solution (both without the use of a separating column) may be preferred over on-line liquid chromatography–mass spectrometry in certain applications. Electrospray or ionspray is applicable to polar or ionic samples. Weakly polar and apolar samples are not ionized under electrospray or ionspray conditions. Applications of the techniques are in the fields of drug metabolism, natural product analysis and the determination of high molecular weights through the observation of multiply charged ions.

INTRODUCTION

The majority of samples in biomedical analyses are polar and thermally labile. Liquid chromatograpy (LC) can handle such samples. One-line LC–mass spectrometry (MS) is the logical choice if the advantages of LC are to be combined with the power of MS. The conventional gas phase ionization techniques that are routinely used in MS [electron impact (EI) and chemical ionization (CI)], require that the sample is present in the vapour phase, which can only be achieved through the input of a sufficient amount of heat. Although the time spent in the hot zones in moving-belt, particle-beam and thermospray LC–MS interfaces is short, limitations are imposed on the analysis of thermolabile samples.

Many samples of biological origin exist as ions in solution. Others can easily associate with ions in solution, such as H^+, Na^+, NH_4^+, acetate and Cl^-. Such samples are difficult to volatilize and ionize by EI or CI. The goal of newly developed ionization techniques for LC–MS is the transport of sample ions or ion–molecule complexes from the liquid phase (the effluent of the liquid chromatograph) into the mass analyser, without passage through the hot zones [1].

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Ions can be taken from the condensed phase into the gas phase or vacuum with the help of mechanical energy [fast atom bombardment (FAB), secondary ion mass spectrometry (SIMS)], electrical energy (field desorption [2], ion evaporation [3]) or photons.

On-line LC–MS with FAB or SIMS requires the continous transport of a sample solution to the liquid target surface as achieved in continuous-flow FAB, pioneered by Ito *et al.* [4].

The separation of ions from the condensed phase under the influence of electrical forces has been studied extensively in field desorption MS [2] and in electrohydrodynamic ionization from glycerol solution [5], but neither have been adapted for routine on-line LC–MS. The pioneering work by Iribarne *et al.* [3] on ion evaporation from small electrically charged water droplets had far-reaching consequences for on-line LC–MS. Present day LC–MS interfaces that use the ion evaporation principle are electrospray [6,7] and ionspray [8].

The operating principle of electrospray and ionspray is the dispersion of a sample solution into an electrically charged aerosol. In the case of electrospray, a phenomenon described by Zeleny in 1917 [9], nebulization takes place by the exposure of a liquid surface to a high electric field. In an electrospray LC–MS interface [10] a solution of the analyte is introduced into dry air or nitrogen at atmospheric pressure through a metal capillary tube that is held at a potential of several kilovolts relative to the walls of the ion source. The build-up of charge at the liquid surface creates such an instability in the liquid that coulomb repulsion forces are sufficient to overcome the surface tension: small (< 1 μm diameter) charged droplets separate from the liquid emerging from the capillary tube. The technique works best with flow-rates in the range below 5–10 μl/min and hence needs microbore columns or a split of the effluent from the liquid chromatograph. Dispersion of liquids by electrical forces alone, as is the case with electrospray, becomes more difficult if the percentage of water in the eluate is high. In the case of ionspray, the nebulizing action of the electric field is assisted by a high-velocity gas flow.

The ionspray LC–MS interface is essentially a concentric pneumatic nebulizer, exposed to an electric field. The nebulizing capillary is usually floated at 3 kV relative to the walls of the ion source. The input of mechanical energy in the nebulization step makes higher flow-rates (40 μl/min, compatible with 1 mm I.D. microbore columns) and higher percentages of water in the sample solution feasible. However, the best sensitivity is still obtained with flow-rates around 5 μl/min.

Once an electrically charged aerosol is formed, evaporation of solvents from the very small droplets takes place. The size of the droplets decreases, and the electric field at the liquid surface of the droplets increases. As a result, the liquid surface becomes so unstable that microdroplets containing a few ions, or ions with one or more shells of solvent, separate from the droplets [3,11]. A final desolvation of each microdroplet or solvated ion produces sample ions that are transported into the mass analyser.

Electrospray and ionspray ionization are carried out at atmospheric pressure for a number of reasons. First, the evaporation of solvents from droplets is more efficient at high pressure, due to the effective transfer of heat. Second, a reduced pressure combined with high electric fields gives rise to strong electrical discharges. In a discharge, both positive and negative-charge carriers are formed, that will recom-

bine with droplets having the opposite charge. As a result droplet charge is neutralized and the formation of sample ions from charged droplets can no longer take place. Third, the gas flow through a pneumatic nebulizer cannot be accommodated by the standard vacuum system of a mass spectrometer.

As often, new methods build upon experience gained a decade or more ago. In the case of LC–MS with atmospheric pressure ionization (API), analytical applications were published by Horning *et al.* in 1974 [12]. Their original API source was very sensitive, but in its original design it showed two drawbacks: ions were drawn into the mass analyser through a small 25 μm I.D. orifice, which was prone to plugging by dust particles or non-volatile materials; in addition, the expansion of ions and polar components of the LC eluent, such as water, tended to produce big cluster ions due to strong cooling in the free jet expansion stage.

Manufacturers of analytical mass spectrometers have not produced API sources as standard accessories for their instruments. Interest in LC–API-MS remained dormant for nearly 10 years until Henion *et al.* at Cornell University [13] revitalized the technique in co-operation with SCIEX, a manufacturer of API mass spectrometers designed for monitoring air quality. This instrument has a 100-μm orifice combined with fast cryogenic pumping. The penetration of dust and water vapour into the free jet expansion stage is prevented by a dry nitrogen gas curtain [14]. Fig. 1 shows the construction of an ionspray LC–MS interface with a SCIEX ion source. Basically ions are pushed to the right towards the small conical orifice by the voltages applied to various parts. At the same time dust and neutral molecules are pushed to the left by the flow of dry nitrogen gas. At the entrance of the conical orifice ions are drawn into the vacuum system of the mass spectrometer, together with a part of the dry nitrogen gas flow. In this simple and elegant construction, blockage of the orifice and the formation of clusters from ions and water molecules are prevented. The electrospray ion source constructed by Whitehouse *et al.* [10] makes use of a similar counter-current flow of dry nitrogen.

An API source with an electrospray or ionspray interface as described here can operate at room temperature, but an increased temperature (up to 80°C) may be advantageous for the handling of a high percentage of water in the eluent. In either case the temperature is probably low enough to prevent thermal degradation of the most difficult samples.

The early efforts on electrospray MS were directed at improvements of the hardware and the investigation of multiply charged ions of polyglycols [15]. The ionspray interface was used together with microbore LC for the support of environmental and biomedical studies. The determination of sulphonated azo dyes in waste water [16], the metabolism of steroids in race horses [17], contamination in pharmaceuticals [18] and the real-time monitoring of the products of an enzyme reaction [19] have demonstrated the versatility of the electrospray or ionspray ionization technique.

The emphasis by Wong *et al.* [15] on multiply charged ions was received with scepticism when the complicated spectra of polyethylene glycol oligomers were shown. A significant breakthrough was achieved with the presentation of series of multiply charged ions of pure samples, in particular proteins [20]. The ability to obtain a molecular weight in the range up to 100 000 and more with a simple quadrupole mass spectrometer has led to an overwhelming interest in electrospray and ion-

spray techniques. Manufacturers that were not convinced of the potential of API and electrospray/ionspray a few years ago have now modified their instruments.

Another new application of electrospray is the combination of capillary zone electrophoresis (CZE) with MS, developed by Smith *et al.* [21]. Electrophoretic separation techniques have been the theme of three *International symposia on High-Performance Capillary Electrophoresis* (Boston, MA, 1989; San Franciso, CA, 1990 and San Diego, CA, 1991) where attention was focused on the efficient separation of samples of biochemical, medial, pharmaceutical and biotechnological origins. CZE–MS of dynorphins may serve an a representative example from this new field [22].

Electrospray and ionspray are suited for polar and ionic compounds, but are not effective inonization techniques for weakly polar and apolar samples. Such weakly polar samples can still be ionized if the atmospheric pressure ion source can be adapted for use in the atmospheric pressure chemical ionization mode (APCI) instead of the electrospray mode [23].

EXPERIMENTAL

A NERMAG R3010 triple quadrupole mass spectrometer was modified for API [24]. The original transfer line for the gas chromatograph was removed. The API source is mounted on a 10 cm I.D. tube and flange which has replaced the gas chromatography inlet flange. Three vacuum stages are used [25]. The first stage is pumped by a 35 m^3/h rotary pump (Alcatel 2033). The second and third stages are the original ion source housing and analyser regions, each pumped by the original 700 l/s oil diffusion pumps. The pressures in the first, second and third stages are 1.7, $2 \cdot 10^{-3}$ and $1 \cdot 10^{-5}$ mbar, respectively.

Ions are drawn into the first stage through a 0.3 mm I.D. nozzle orifice in a 1 mm thick stainless-steel disk. The central portion of the beam of gas and ions is admitted into the second stage through a 1.4 mm I.D. skimmer orifice located 4 mm downstream from the nozzle. Ions are guided into the mass analyser by the use of a radio frequency only quadrupole mounted in the original source housing of the NERMAG R3010. The nozzle orifice is protected from particulate matter and solvent vapour by a dry nitrogen gas curtain [14] as shown in Fig. 1.

A Jasco FAMILIC 100N syringe pump and a Jasco injector with a 0.3-μl loop was used for the flow injection experiments. Methanol was used as the eluent at 5 μl/min. Samples were injected as solutions in methanol. A flow-rate of 5–10 μl/min of

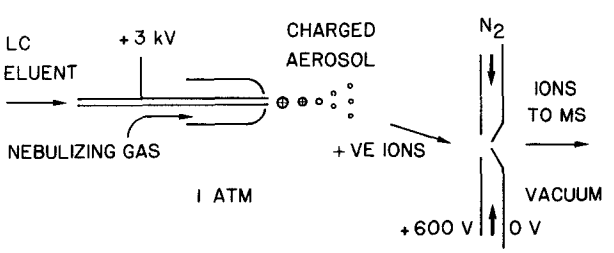

Fig. 1. Ionspray LC–MS interface in atmospheric pressure ion source with dry nitrogen gas curtain.

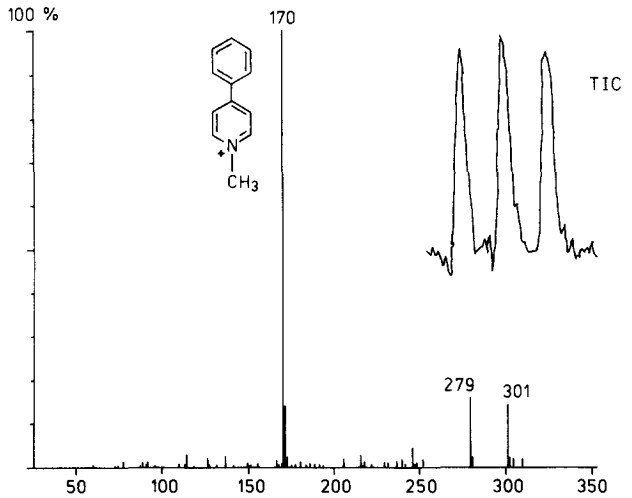

Fig. 2. Electrospray mass spectrum of MPP^+, with total ion current (TIC) trace (m/z 30–380) of three repeated 3-ng injections; m/z 279 and 301 are the MH^+ and $M \cdot Na^+$ ions of dibutyl phthalate. Sample courtesy of H. Rollema (University Centre for Pharmacy, Groningen, Netherlands).

the effluent from 2 and 4.6 mm I.D. columns was introduced into the ionspray interface via a splitter in on-line LC–MS experiments.

The instrument is entirely dedicated to LC–API-MS with ionspray for the support of research on drug metabolism, natural products and newly synthesized compounds.

RESULTS

Fig. 2 shows the electrospray sprectrum of MPP^+, a neurotoxin formed from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a byproduct of a designer

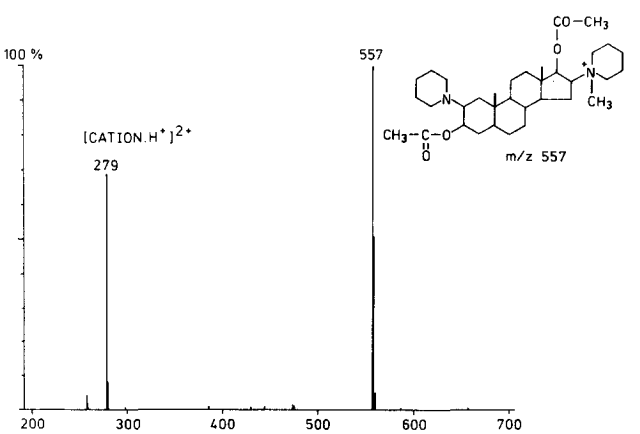

Fig. 3. Electrospray mass spectrum of 30 ng vecuronium bromide (ORG NC 45). Sample courtesy or Organon.

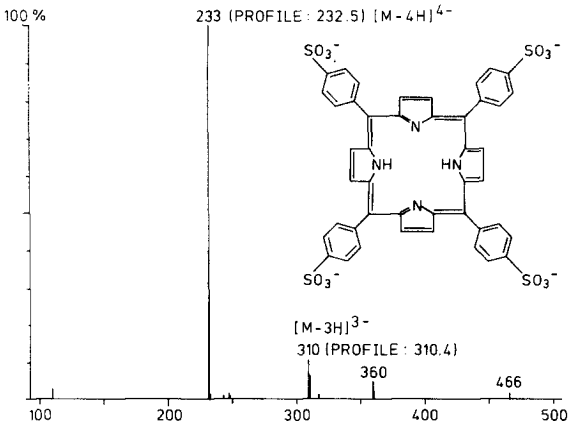

Fig. 4. Electrospray mass spectrum of 30 ng synthetic sulphonated *meso*-tetraphenylporphyrin (mol.wt. 934). Sample courtesy of J. Braams (University Centre for Pharmacy, Groningen, Netherlands) and R. J. B. J. Brouwers (Tjongerschans Hospital, Heerenveen, Netherlands).

drug synthesis. Continuous flow FAB of MPP^+ shows a SIMS sensitivity in the low nanogram range [26]. Electrospray gives low-nanogram full-spectrum sensitivity. Fig. 3 demonstrates the application of ionspray in the determination of multifunctional quaternary ammonium drugs. Vecuronium bromide undergoes extensive thermal demethylation during LC–MS with a moving belt [27]. Thermal dealkylation reactions are also a problem in thermospray [28]. The ion at *m/z* 279 is a doubly charged ion, corresponding to vecuronium protonated at the tertiary amino function. The mass spectrum in Fig. 3 shows the total absence of thermal degradation products. The ability of ionspray and electrospray to generate multiply charged ions from multifunctional molecules has already been mentioned in conjunction with the analysis of peptides [29]. On a more modest scale, the quadruply charged ion in the mass spec-

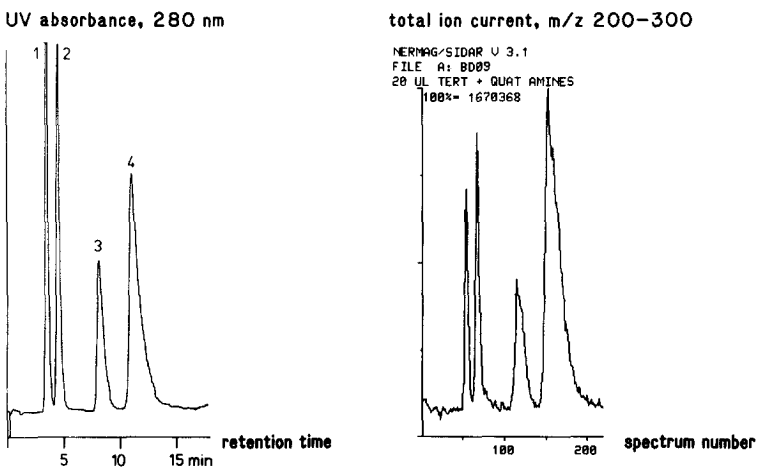

Fig. 5. HPLC separation with UV (left) and ionspray MS (right) detection of a mixture of dextrorphan (1), dextromethorphan (2), N-methyldextrorphan (3) and N-methyldextromethorphan (4). Injection: *ca.* 300 ng per component into HPLC system; *ca.* 3–30 ng into MS system. From ref. 30.

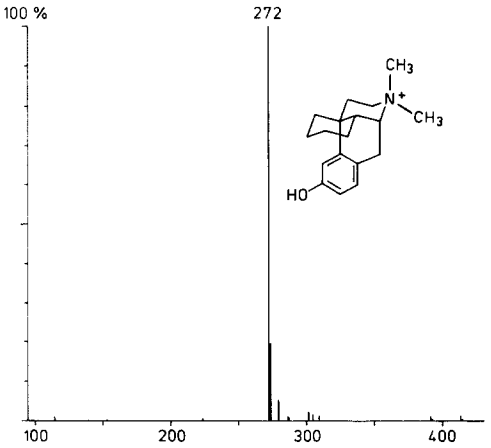

Fig. 6. Ionspray mass spectrum of N-methyldextrorphan. Sample courtesy of A. B. L. Lanting (University Centre for Pharmacy, Groningen, Netherlands).

trum of a synthetic tetrasulphonated porphyrin in Fig. 4 was one of the keys for the confirmation of the identity of this product.

Fig. 5 shows the on-line separation of a mixture of two morphinans (dextrorphan and dextromethorphan) and their *N*-methylated derivatives [30]. The mass spectrum of the third peak is presented in Fig. 6. A study of the metabolism of quaternary ammonium drugs in this department hinges on the ability of the ionspray LC–MS interface to produce clean spectra, without a trace of thermal dealkylation reactions. Details on metabolism, LC conditions and the LC–MS of samples obtained from biological materials will be published elsewhere.

CONCLUSIONS

Papers previously published and the examples shown here demonstrate that API with an electrospray or ionspray LC–MS interface is well suited for the support of biomedical research. The operation at room temperature (or up to 80°C at the most) is a significant advantage for thermolabile samples. The high mass capability and coupling with CZE make it a most promising new field.

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