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Liquid chromatography–mass spectrometry in bioanalysis: some applications and a new approach for non-volatile buffer systems*

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Abstract: Combined liquid chromatography mass spectrometry (LC/MS) is still a rapidly developing area. The advantages and limitations of the most important interfaces, i.e. the moving belt interface, the thermospray interface, and continuous-flow fast atom bombardment, are discussed from a bioanalytical point of view. The discussion is underlined by two bioanalytical applications of LC/MS using a moving belt interface. The first example is the identification of unknown compounds in alkaline extracts of post-mortem human urine and plasma. The second example is actually concerned with a new approach in LC/MS: phase-system switching using a short column between the LC chromatography and the moving belt interface. The perspectives of the latter are outlined.

Keywords: *Liquid chromatography mass spectrometry; bioanalysis; phase-system switching; moving belt interface; diuron and metabolites; mitomycin C.*

Introduction

The development of combined liquid chromatography–mass spectrometry (LC–MS) during the last decade has been spectacular [1]. Various new approaches have been tested and several commercial interface systems are available nowadays. Nevertheless, there is no LC–MS interface which is universally applicable and the choice of equipment strongly depends on the type of analysis.

In bioanalysis, a differentiation can be made between qualitative and quantitative LC–MS analysis since the demands of both approaches with respect to LC–MS instrumentation is often quite different. For target compound analysis soft ionisation conditions are to be preferred, however for identification purposes molecular weight information is insufficient and fragmentation also is required. Fragmentation can be induced via the ionisation process, for instance by means of a discharge in a thermospray source or in a later stage via collision induced experiments.

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Numerous interface types have been described, but only a few are commercially available. At the 35th Conference of the American Society of Mass Spectrometry held in Denver, USA, in May 1987, interest was approximately divided as follows: thermospray interface 65% of the contributions; continuous-flow FAB 13%; moving belt interface 6%; and other interfaces 16%. The three most popular interfaces will now be discussed briefly.

The moving belt is a flexible interface which allows both electron impact (EI) and chemical ionisation (CI) to be applied routinely, and in special cases fast atom bombardment (FAB), secondary ion mass spectrometry (SIMS) or laser desorption also can be used. The advantage of having electron impact spectra is often underestimated. In many cases the samples do not contain solely extremely polar or thermally labile substances. Also identification via EI spectra is straightforward because of the tremendous experience available in interpreting EI induced fragmentation.

The limitations of the moving belt are the incompatibility with highly aqueous mobile phases, although recently it was shown that using a thermally assisted spraying device [2] these limitations can be overcome to a certain extent. Applying FAB directly on the belt yields informative spectra, however detection limits are not very low. Nevertheless, identification of peptides [3] and metabolites of the drug almitrine [4] amongst others have been achieved successfully.

Thermospray (TSP) is applied on a routine basis in pharmaceutical and biomedical analysis. A fully automated system has been described [5]. The method is very selective which is an advantage for quantitative measurements provided the target compound is detected with sufficient sensitivity. On the other hand, general identification or profiling is difficult. By using a discharge or a filament in addition this can be compensated for, but, often fragments are formed which are not easily interpreted.

A more attractive approach seems to be TSP in combination with tandem mass spectrometry (MS/MS), not only because additional selectivity is obtained for quantitative analysis but also because useful structural information can be obtained in this way as shown for instance in a study of the identification of a catecholamine drug [6]. Also in this study the other important features of MS/MS, i.e. "constant neutral loss" and "parent ion" scanning, are applied in metabolite tracing with TSP.

Strong points of TSP are the compatibility with reversed-phase chromatography using aqueous mobile phases, the ruggedness of the system and its ease of operation. Detection limits obtained are very much compound dependent and in general are not very impressive.

A very recently introduced method is continuous flow fast atom bombardment (CF–FAB) [7–9] using the soft ionisation FAB method for the production of ions from high mass polar compounds eluting from a microbore HPLC system. The capillaries used in such experiments allow a flow of up to 15 μl min^{-1} which means that in the case of conventional columns (3–5 mm i.d.) some form of split has to be used.

In this paper a bioanalytical LC–MS problem that underlines the statements made here concerning the advantages in applying the moving belt interface is reported. Furthermore, a new approach based on phase-system switching (PSS) is presented which enables qualitative and quantitative analysis with different LC–MS interfaces using mobile phases containing non-volatile additives in high concentrations such as buffers. An example is given involving the use of phosphate buffers in the analysis of an anticancer drug with a moving belt interface.

Experimental

Moving belt LC–MS

LC–MS measurements with a moving belt interface were performed with a Finnigan MAT HSQ-30 hybrid MS/MS instrument (Bremen, FRG) operating in EI, or CI mode using ammonia as a reagent gas. The moving belt interface was operated with a solvent evaporator temperature of 150°C, a belt speed of 4 cm s^{-1} and the vaporiser at position 6.

The LC-system comprised of a model 2150 LKB high pressure pump (LKB, Bromma, Sweden), a 100 × 3.0 mm i.d. Chromsphere C18 cartridge column (Chrompack, Middelburg, The Netherlands), a type 7125 injection valve (Rheodyne, Berkeley, CA, USA) and a type 757 UV detector (Kratos, Manchester, UK). A mobile phase of 50:50 methanol–water (v/v) was used at a flow rate of 0.5 ml min^{-1}.

Phase system switching

The apparatus used for the phase system switching experiments consisted of an LKB type 2150 pump and two type 302 pumps (Gilson, Middleton, WI, USA), two MUST valve switching units (Spark, Emmen, The Netherlands) and a Kratos type 757 variable wavelength UV detector. A schematic diagram of the system is given in Fig. 1. The analytical column was a 100 × 3.0 mm i.d. Chromsphere C8 cartridge column (Chrompack). The short column (20 × 2 mm i.d.), the so-called trapping column, was hand-packed with 10 μm PRP1 particles (Hamilton, Bonaduz, Switzerland).

The phase system switching experiments were performed with a Finnigan-Mat HSQ-30 hybrid mass spectrometer equipped with a moving belt interface described above. It was operated in the CI mode using ammonia as a reagent gas.

Results and Discussion

Post-mortem case

Plasma and urine samples of a post-mortem case were analysed by high-performance liquid chromatography using UV-detection. Several unusual components were found after a basic extraction. A typical LC–MS chromatogram of a urine extract using a moving belt interface and the mass spectrometer operating in CI mode is shown in Fig. 2.

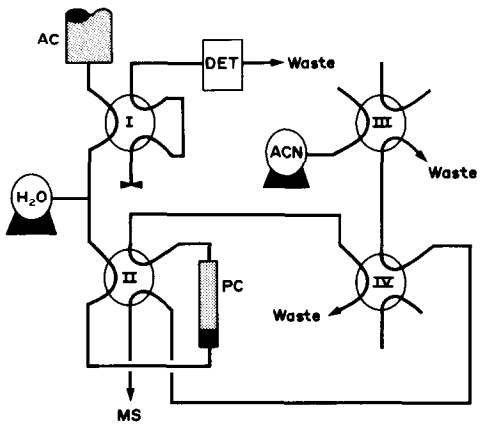

Figure 1
Schematic diagram of the apparatus used in phase system switching, with AC the analytical column, and PC the trapping column.

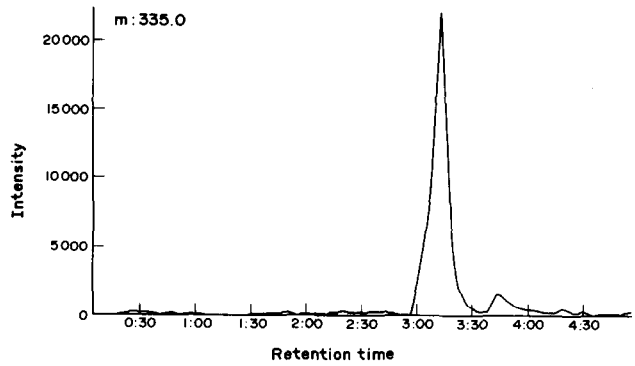

Figure 2
LC–MS chromatogram of the alkaline extracted urine sample of the post-mortem case. Conditions: see text.

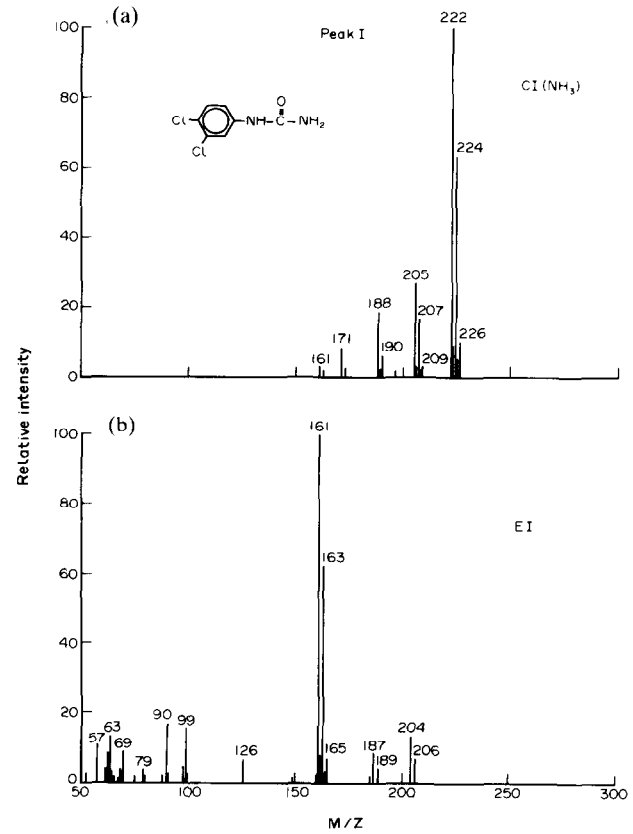

Figure 3
Mass spectra of peak 1 in the chromatogram of the alkaline extracted urine sample, with (a) electron impact; (b) chemical ionisation. Conditions: see text.

After analysing the same sample in the EI mode identification was possible. Peaks from compounds like theobromine, theophylline, nicotinamide, and a benzodiazepine were detected, together with five compounds which contained two chlorine atoms. In Fig. 3 the CI and EI spectra of the first compound (I) in the chromatogram are given. A molecular weight of 204 is indicated by the presence of protonated and ammoniated molecules at m/z 205 and 222. The isotope pattern reveals the presence of two chlorine atoms in the molecule. The peaks 34 mass units lower are somewhat confusing. They do not belong to a different compound with only one chlorine atom but are formed via ion–molecule reactions in the CI source as proved later by an investigation of authentic reference compounds. The EI spectrum confirms a molecular weight of 204, while the large apparently stable ions observed at m/z 161 and 163 suggest aromatic characteristics with two chlorine atoms present. Mass losses of 17 and 43 respectively can best be explained by the presence of an amide function which then leads to the proposed structure shown in Fig. 3. Other peaks with molecular weights of 218 and 232 (compounds 4 and 5) suggest the presence of one and two additional methyl groups, respectively. The assignments were confirmed by high resolution measurements and it was therefore concluded that the herbicide diuron (compound 5) and two of its major metabolites (compounds 1 and 4) were present. Metabolites formed after hydroxylation and partial demethylation also were detected (compounds 2 and 3).

Phase system switching

In LC–MS applications it is still impossible to have a free choice of the chromatographic system. Especially non-volatile additives such as buffers, complexing and/or ion-pairing agents generally cannot be used. Although solvent extraction systems have been described for a moving belt [10] and a direct liquid introduction interface [11], there is a clear need to further widen this difficult area.

Column switching techniques are used routinely nowadays in liquid chromatography and allow not only the tuning of the selectivity, but also the switching from one mobile phase system to another. This prompted an investigation as to whether additional degrees of freedom in the selection of the mobile phase compositions applicable to LC–MS systems in general can be generated by the use of column switching techniques. In the system developed the analytical column is followed by a second column acting as a trapping column, which is much smaller, typical of the type of column normally used as a pre-column.

As an illustrative example the analysis of Mitomycin C (MMC) was chosen, the liquid chromatography of which normally is carried out using a reversed-phase system involving a mobile phase consisting of a 0.05 mol l^{-1} phosphate buffer at pH = 7.0 and a low modifier content (<20% methanol or acetonitrile [12]). LC–MS analysis of MMC with the use of a moving belt interface is hampered by the high water content and the presence of a non-volatile buffer in the mobile phase. In this particular example the valve switching techniques are applied for heart-cutting the peak of interest from the chromatogram developed on the analytical column, for performing an on-line washing step to remove the residual buffer components, and for the desorption of the component with a solvent which can easily be handled by the moving belt interface, pure acetonitrile in this case. In Fig. 4 the UV-trace after separation on the analytical column and the MS-trace obtained after applying the phase system switching technique by monitoring the $[M + H]^+$ peak of MMC at m/z = 335 in CI mode using NH_3 as the reagent gas. Both optimum LC and MS conditions are realised by this method. As a result of the phase

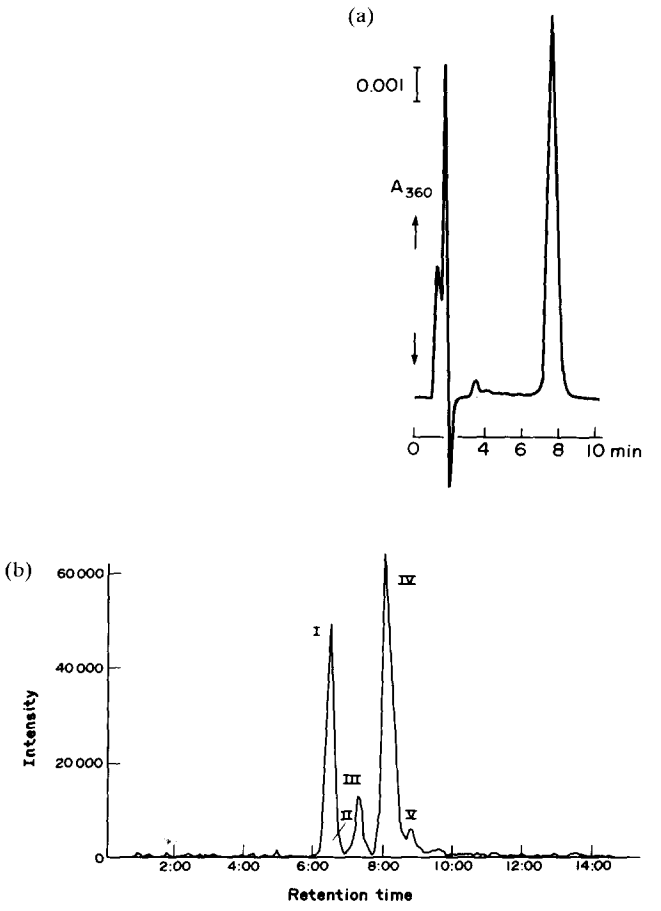

Figure 4
Demonstration of phase system switching with mitomycine C, with (a) UV chromatogram (360 nm) of mitomycine C from the analytical column; (b) MS chromatogram monitoring the protonated molecular ion at m/z = 335 in CI (NH_3). Conditions: see text.

system switching a peak compression of roughly one order of magnitude in concentration was obtained, whilst the flow rate was decreased from 1 to 0.2 ml min^{-1}.

In this example three advantages of the phase system switching approach are demonstrated. First, it has been shown, that it is possible to use a mobile phase containing non-volatile buffer components in LC–MS analysis using the moving belt interface. Similar applications with other non-volatile mobile phase additives, such as ion-pairing or complexing agents, can be envisaged. Next, it has been shown, that it is possible to obtain considerable peak compression. This is an interesting aspect as it results in better detection limits in LC–MS. Thirdly, it has been shown, that it is possible to change the solvent flow rate to be delivered to the LC–MS interface. Of course, there are some limitations to the use of the phase system switching. The approach depends on the retention characteristics of the compound of interest in a particular phase system of a stationary and a mobile phase. Although the possibilities for selecting appropriate phase

combinations is quite extensive, post-column addition of a solvent to increase the retention of the compound of interest on the second column may be necessary. In principle, it should be possible to switch from reversed-phase to normal-phase systems, or vice versa, as well.

At present the application of the phase system switching approach results in an almost independent operation of the liquid chromatograph and the mass spectrometer. As it is possible to select the most favourable solvent and flow rate, the phase system switching approach is compatible with all current types of LC–MS interfaces, in particular with the thermospray, the moving belt and the continuous-flow FAB interface. The possibility of obtaining peak compression and changing the flow rate can improve the detection limits of the LC–MS analysis. Although a mass spectrometer is a mass-flow sensitive detector and consequently higher analyte mass-flows will improve the detection limit, the gain in detection limit is limited by the maximum allowable flow rate of the interface and/or MS vacuum system. Therefore, an increase of the analyte mass-flow by the use of the phase system switching technique while a reasonable effluent flow rate is maintained is preferred. Tuning the flow rate for a particular interface is also of interest, because the flow rate is an important parameter in instrument performance, e.g. flow rates up to 1–1.5 ml min^{-1} of pure organic solvents, such as acetonitrile or methanol, can easily be handled by a moving belt interface. On the other hand with continuous-flow FAB applications it is necessary to minimize the flow rate as much as possible. The various possibilities and several applications of the phase system switching approach are currently under investigation in the authors' laboratory.

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