Journal of Chromatography, 394 (1987) 239–251
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 379

EXPERIENCE WITH ROUTINE APPLICATIONS OF LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY IN THE PHARMACEUTICAL INDUSTRY

K. H. SCHELLENBERG*, M. LINDER, A. GROEPPELIN and F. ERNI
Analytical Research and Development, Pharma Division, Sandoz Ltd., CH-4002 Basle (Switzerland)

SUMMARY

The combination of liquid chromatography and mass spectrometry (LC–MS) has been established to complement gas chromatography (GC)–MS in the analysis of non-volatile and labile drugs in complex materials. The possibilities of LC–MS in the pharmaceutical industry for the analysis of drug substances and dosage forms, metabolism studies and the elucidation of the structures of materials of biological origin are discussed. Instrumental requirements, limitations and applications of LC–MS are considered and experiences with LC–MS in routine applications are reported. Preliminary results obtained with thermospray LC–MS are compared with those using a direct liquid inlet interface.

INTRODUCTION

Many modern drug substances are non-volatile and also often thermally or chemically labile. In addition, newly developed drugs are generally more potent and specific, and therefore very low dosage forms are increasingly being used. Also, with the advent of biotechnological methods, biologically active components such as peptides and proteins of higher molecular mass are gaining increasing importance. The availability of liquid chromatography–mass spectrometry (LC–MS) as a sensitive and highly selective method for the analysis of non-volatile and labile compounds in complex matrices is therefore of great interest to the pharmaceutical industry.

In our department, LC–MS has two major areas of application: the identification of byproducts and degradation products in drug substances and dosage forms, and the selective identification and quantitation of drugs and their metabolites in biological material. Other fields of activity are contributions to the elucidation of the structures of active compounds in biological materials, *e.g.*, plant extracts or animal tissues, the validation of methods such as radioimmunoassay and analytical "troubleshooting".

EXPERIMENTAL

Fig. 1 shows schematically the instrumental setup. A ternary gradient liquid chromatograph is coupled to the direct liquid inlet (DLI) LC–MS interface, which in turn is connected via a UV detector to the back-pressure regulating device. This

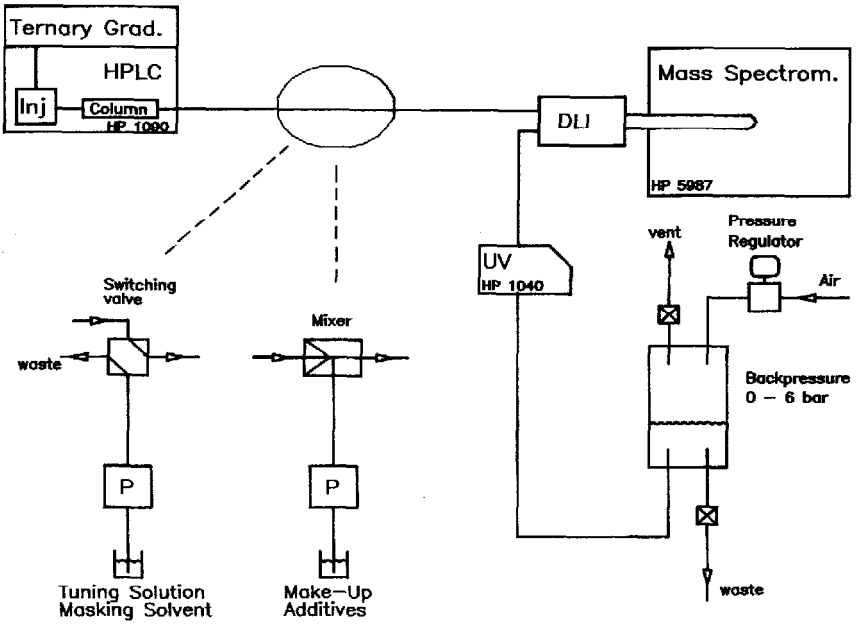


Fig. 1. Schematic diagram of the experimental setup.

device replaces the commercially provided needle valve and consists of a container pressurized by air through a pressure regulator. If the hydrodynamic resistance of the connection between the DLI and the container is low, *i.e.*, made with short and relatively wide capillaries, the back-pressure becomes virtually independent of the viscosity, composition and flow-rate of the eluent. In addition, sharp pressure pulses are strongly damped, which allows solvent switching, for example to cut off undesired parts of the chromatogram. It is even possible to change columns quickly without losing the jet of the DLI.

The source pressure was monitored with the CI–GC pressure gauge of a Model 5987 mass spectrometer and adjusted to the optimal value by changing the back-pressure (typically 3–5 bar) and varying the distance of the DLI probe to the desolvation chamber. A micrometer screw made it possible to adjust and maintain the exact position of the DLI probe. The pressure in the TSP source was measured with a gauge mounted directly at the vacuum flange between source and isolation valve and adjusted to the optimal value (typically 7–9 Torr) by partly closing the valve.

RESULTS AND DISCUSSION

Identifications with LC–MS

Fig. 2 shows an example of the identification of byproducts in bromocriptine, an ergotalkaloid drug substance. The signals A–F could be identified as brominated ergocriptine, ergocristine and ergocornine and their isomers. Structure fragments could be assigned to the signals X, Y and Z.

The mass spectrum of compound Y is shown in Fig. 3. The isotope pattern of

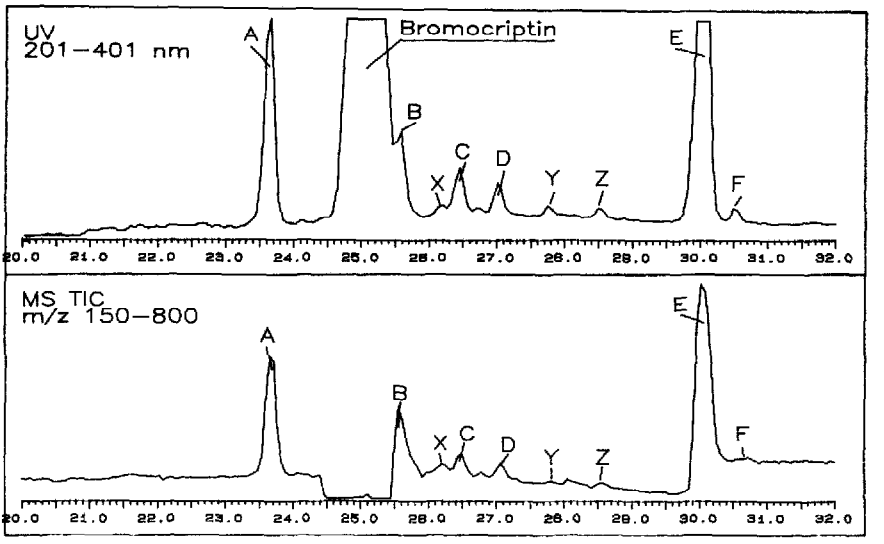


Fig. 2. Byproducts in bromocriptine. The signal of the drug itself was switched away to avoid overloading the multiplier and source contamination. The UV chromatogram was recorded separately. Column, 125 × 4.6 mm I.D., RP-18; mobile phase, 0.01 *M* ammonium carbonate–acetonitrile, gradient 10–60% acetonitrile in 30 min, flow-rate 2 ml/min; source temperature, 250°C; source pressure, 0.9 Torr; negative ions.

the molecular ion of *m/z* 733 ($M - H^-$) suggests the presence of two bromine atoms, while the fragments of *m/z* 443 and 386 contain only one bromine and are typical of bromoergocriptine. This indicates that the additional bromine is located in the peptide part of the ergotalkaloid, although the exact position cannot be determined.

Fig. 4 shows the identification of a metabolite in rat bile. Bile was injected directly onto the column without sample cleanup, and the early eluting strongly polar components were cut off with solvent switching. In addition to being simple, direct

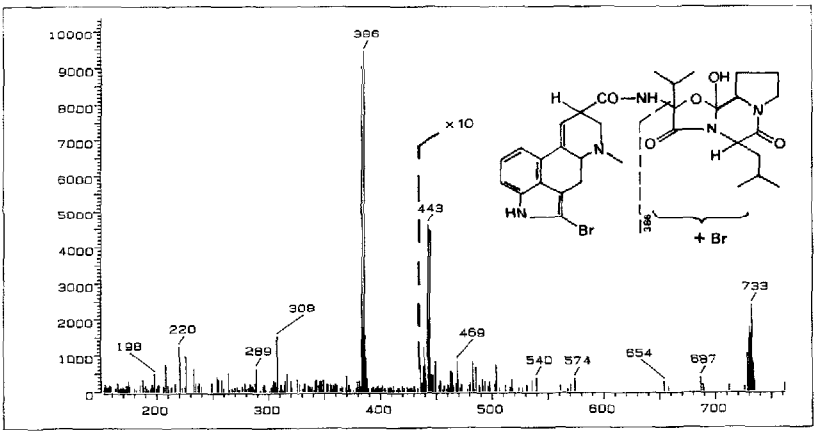


Fig. 3. Mass spectrum (negative ions) of compound Y (Fig. 2). Experimental conditions as in Fig. 2.

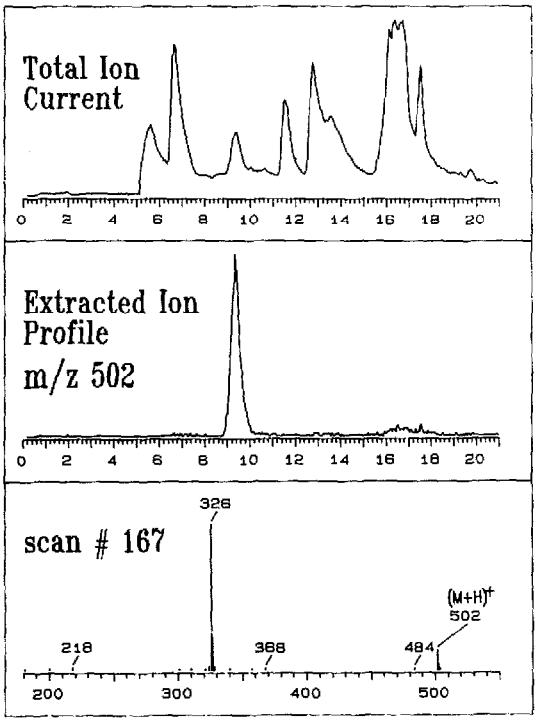


Fig. 4. Chromatography of metabolites of fluperlapine in rat bile and mass spectrum (positive ions) of a glucuronide. A volume of 200 μl of bile was injected directly. The first 5 min of the eluting chromatogram were switched away. Column, 100 × 2.1 mm I.D., RP-18; mobile phases, (A) 0.005 *M* ammonium hydrogen carbonate, (B) acetonitrile; linear gradient, 0 to 5 min from 0 to 25% B, 15 to 20 min from 25 to 80% B; flow-rate, 1 ml/min; source temperature, 200°C; source pressure, *ca.* 1.4 Torr; positive ions.

sample injection maximizes the sensitivity and polar or labile metabolites are not lost.

The expected metabolite, a glucuronide, is found with high selectivity in the extracted ion profile for *m/z* 502, the protonated molecular ion. The corresponding mass spectrum shows a relatively weak molecular ion of *m/z* 502 ($M + H^+$) and the intense base peak of the aglycone fragment of *m/z* 326.

These examples illustrate that the spectra obtained with the chemical ionization gas of the mobile phase, *i.e.*, methanol, acetonitrile, etc., usually contain only a few but nevertheless characteristic fragments, apart from the molecular ion. As the range of possible reaction byproducts, degradation products or metabolites is limited, the structural information obtained is very often sufficient for identification. However, it is clear that without some rough knowledge of what to expect, *ab initio* structure elucidation with LC(DLI)–MS will be the exception because of the limited structural information contained in chemical ionization (CI) mass spectra.

Quantitation with LC–MS

To quantitate drug substances and metabolites in biological material, the method has to be highly sensitive, very selective, reproducible and stable. As the

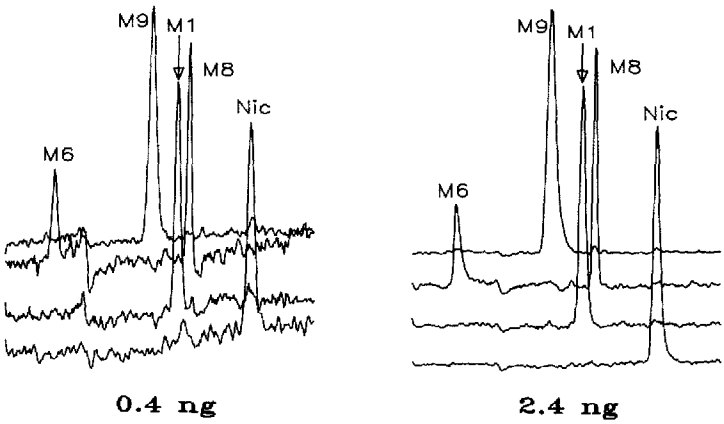
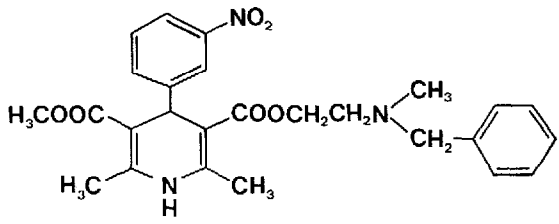


Fig. 5. Calibration standards of nicardipine and metabolites. Column, 100 × 2.1 mm I.D., RP-18; 50 μl injected; mobile phases, (A) water, (B) acetonitrile–tetrahydrofuran (80:14, v/v), (C) 0.02 *M* tetramethylammonium formate (pH 2.3); step gradient from 50:30:20 to 30:50:20 (A:B:C) at 1.6 min; flow-rate, 0.36 ml/min; source temperature, 350°C; source pressure, 1.2 Torr; negative ions; selected ion monitoring (SIM).

amount of sample, *e.g.*, blood plasma, is usually limited, the detection limit should be typically 100–500 pg per injection or lower, and low noise and background are therefore essential. Also, the selectivity must be high in order to avoid interferences from endogenous substances. In our experience, this leads to a lower limit of about *m/z* 250 for the useful mass range for quantitation in LC(DLI)–MS.

Equally important, even when using internal standards, is the reproducibility and stability of the method. A standard deviation of 5% or better and *ca.* 10% near the detection limit is reasonable for pharmacokinetic studies. This calls for stable and reproducible ionization conditions, which means that the source pressure, temperature, sorptive surfaces, tuning parameters, etc., need to be controlled reproducibly. As the jet of the DLI can vary slightly in length and direction with time and especially from day to day, and the design of the desolvation chamber is not necessarily optimal, the evaporation conditions cannot be controlled very accurately. Therefore, the ionization yields of different ions can vary greatly, which makes the use of an internal standard, preferably a deuterated analogue of the substance to be quantitated, almost mandatory. Fig. 5 shows the selected ion profiles of calibration standards of nicardipine (I) and four of its metabolites.



(I)

The detection limit is below 50 pg per injection and only slightly higher for plasma extracts. Owing to the extreme range of polarity and the interferences between

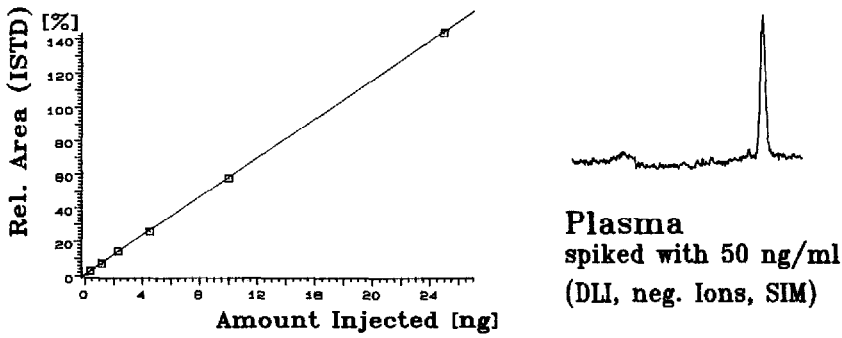


Fig. 6. Calibration graph for the quantitation of nicardipine and chromatogram of an extract of spiked plasma (SIM, *m/z* 479). Conditions as in Fig. 5.

M6 and M8, and M1 and nicardipine, a high-performance liquid chromatographic (HPLC) separation with a step gradient had to be used.

The selected ion profile for nicardipine in a plasma extract is shown in Fig. 6. The calibration graph, also shown in Fig. 6, is linear with a standard deviation of about 7%.

Nicardipine, with its excellent ionization yield of negative ions, represents an ideal case for quantitation. Nevertheless, in our experience, for many substances having average ionization yields for positive or negative ions with *m/z* greater than 250, detection limits of 200–500 pg per injection can easily be achieved with LC(DLI)–MS.

Metabolism studies with LC–MS

Applications of LC–MS in metabolism studies involve the investigation of metabolic pathways, especially contributions to the elucidation of the structures of polar and labile metabolites, the validation of other methods of quantitation or identification, pharmacokinetic measurements, comparison of species or the correlation of *in vitro* with *in vivo* metabolism.

Typically, urine or bile samples contain 5–20 different metabolites with a total amount of 10–50 μg/ml. Plasma samples normally contain even lower concentrations. The high sensitivity needed for distinct mass spectra can be achieved by increasing the injection volume, by pre-concentration of the sample or by reducing the dilution of the sample by the use of shorter columns with smaller diameters and higher resolving power together with shorter retention times.

However, interferences and overloading effects set a limit to the amount of sample injected. Also, conjugates are often labile, so that pre-concentration and extraction of the sample can lead to decomposition.

As mentioned above, some initial information is necessary for structure elucidation. In metabolism studies, the expected metabolites can be predicted by experience and with the help of a computer program (CAMP, computer-assisted metabolite prediction[1]). Fig. 7 shows metabolites of fluperlapine that were predicted and actually found and identified[2]. Bile from rats fed with fluperlapine was chromato-

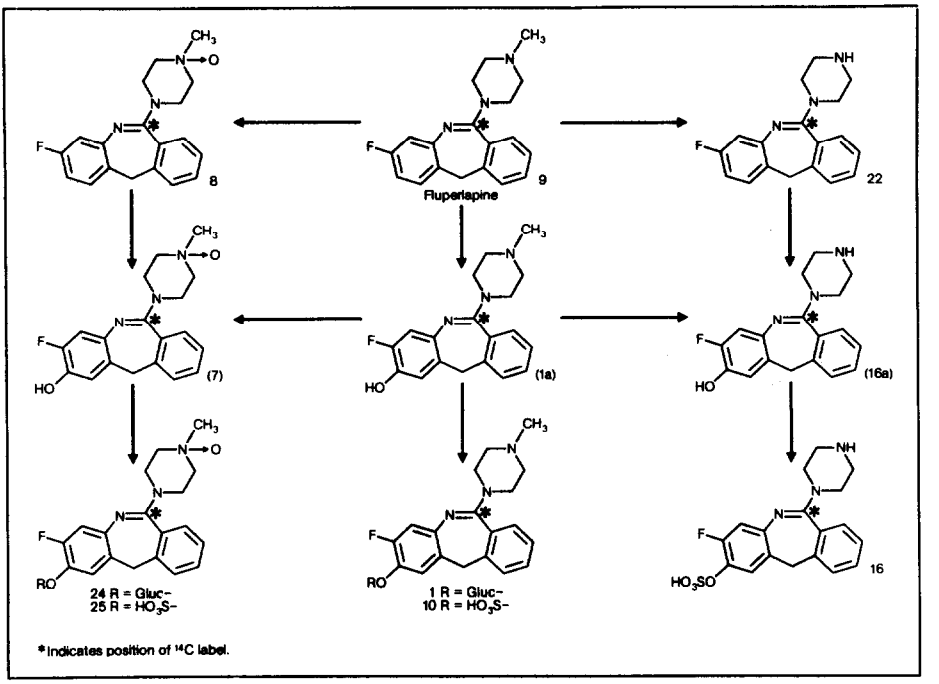


Fig. 7. Proposed pathways of biotransformation of fluperlapine.

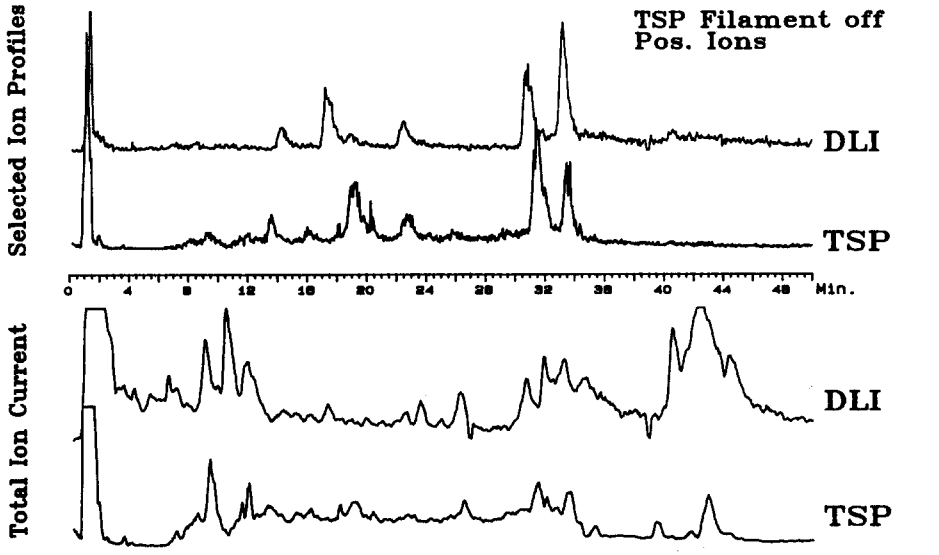


Fig. 8. Chromatograms of rat bile containing *ca.* 250 μg equiv./ml of metabolites of fluperlapine. Column, 100 × 2.1 mm I.D., RP-18; 200 μl injected; mobile phase, acetonitrile–water, linear gradient from 10 to 50% acetonitrile in 50 min, from 50 to 80% from 50 to 70 min; flow-rate, 0.5 ml/min; post-column addition, DLI, from 0 to 50 min 0.2 ml/min acetonitrile; TSP, 1.0 ml/min 0.1 *M* ammonium acetate during the whole separation. MS: positive ions; DLI source temperature, 250°C; source pressure, 0.8 Torr. MS with TSP: tip temperature *ca.* 210°C; source temperature, 270°C; source pressure, 8 Torr; filament off.

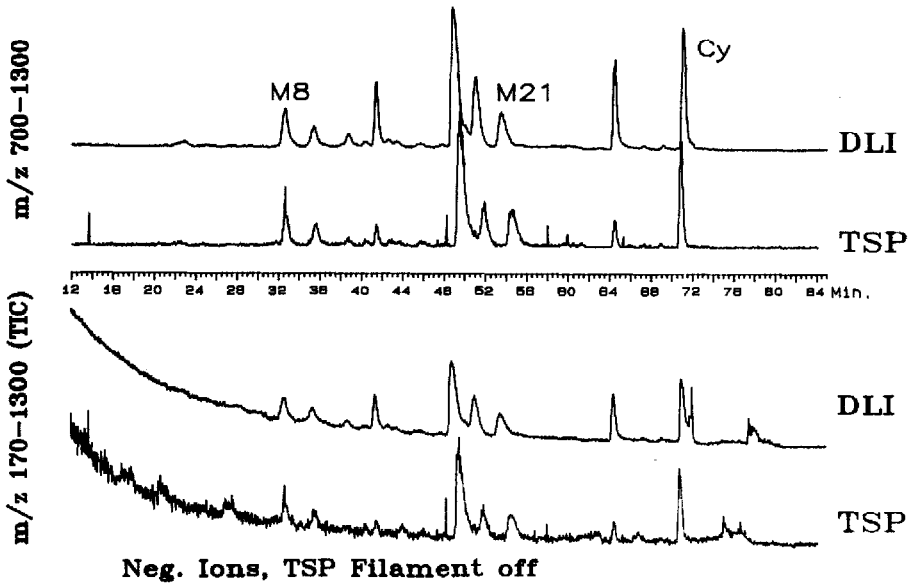


Fig. 9. TIC and extracted ion profile chromatograms of an extract of human urine containing *ca.* 420 μg equiv./ml of metabolites of cyclosporin. Column: 200 × 2.1 mm I.D., RP-18; 200 μl injected; mobile phases, (A) water, (B) acetonitrile, (C) methanol, linear gradients from 0 to 40 min 30% to 47.5% B, 40 to 55 min 47.5% B, 55 to 70 min 47.5 to 65% B, 70 to 75 min 65 to 90% B, C constant at 10%; flow-rate, 0.3 ml/min. MS: DLI source temperature, 300°C; source pressure, 1 Torr. TSP: tip temperature, *ca.* 180°C; source temperature, 270°C; source pressure, 8 Torr; filament off; negative ions.

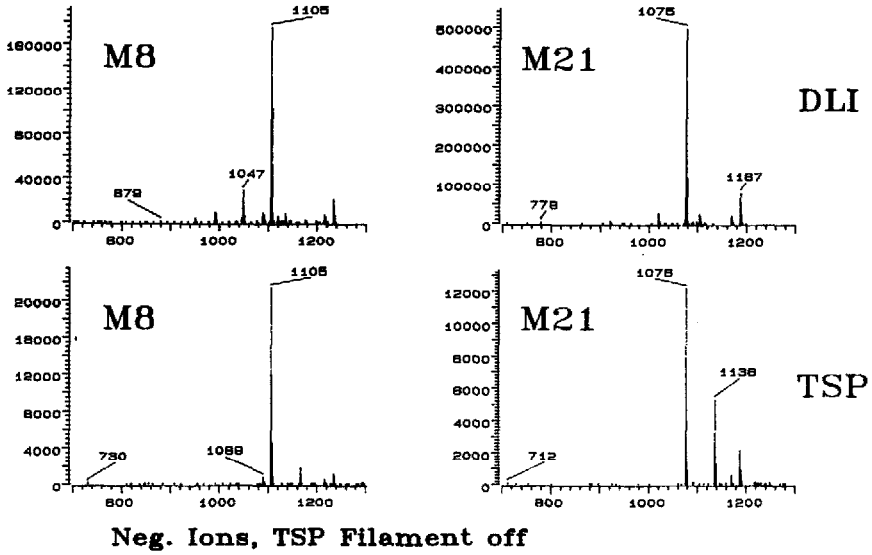


Fig. 10. Mass spectra (negative ions) of two metabolites of cyclosporin. Metabolite M8 contains two additional hydroxy groups, metabolite M21 is cyclosporin less a methyl group. Conditions as in Fig. 9.

graphed and Fig. 8 shows the results obtained with both DLI and thermospray LC–MS. Despite the strong interferences found in the total ion current (TIC) chromatograms, the summed selected ion profiles extracted for characteristic ions of the expected metabolites reveal clearly and specifically the signals of some metabolites. Four of these compounds could be identified and correlated with known metabolites.

A metabolite profile of cyclosporin A in an extract of human urine is depicted in Fig. 9. In this instance, the TIC chromatograms show much less interference. Owing to the low polarity of cyclosporin and most of its metabolites, they can be easily separated from the more hydrophilic constituents of the urine. The mass spectra of two metabolites of cyclosporin A are shown in Fig. 10. Again, the spectra show very few details. The molecular ion ($M - H^-$) has about 10–20% of the base peak intensity, the main fragment is generated by the loss of the side-chain of amino acid No. 1 and only a few minor fragments of lower mass are observed.

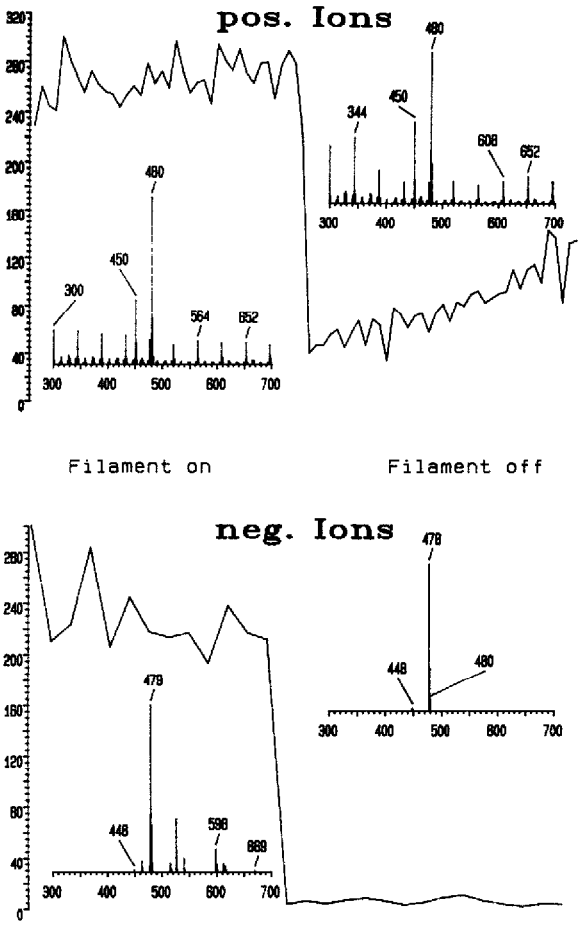


Fig. 11. TIC trace and mass spectra of a calibration solution of polyethylene glycol (PEG 1200, 40 μg/ml) and nicardipine (4 μg/ml) in 0.02 *M* ammonium acetate–acetonitrile (50:50, v/v). TSP: tip temperature, 208°C; source temperature, 250°C; source pressure, 8 Torr; flow-rate, 1.5 ml/min.

Direct liquid inlet (DLI) compared with thermospray (TSP)

It is well known that the ionization mechanisms of TSP LC–MS differ from those of chemical ionization observed in DLI LC–MS. Hence the specificity and sensitivity of the two methods may not be the same for a given compound.

Fig. 11 shows thermospray total ion current traces and mass spectra with the filament on or off for a mixture of polyethylene glycol (PEG) and nicardipine. For positive ions, the TSP mass spectra with the filament on or off show no significant differences. This indicates that obviously in both modes the same ionization processes dominate. With the filament on, the efficiency of ionization is 5–10 times higher for both substances. Compared with DLI chemical ionization, both nicardipine and PEG show about two orders of magnitude higher ionization yields for positive ions in TSP with the filament on.

For negative ions, the TSP mass spectra of PEG show only extremely weak signals. In DLI chemical ionization with acetonitrile–water, no negative ions of PEG are observed. This is as expected, as the gas-phase acidity of the glycol OH groups is not very high[3]. The TSP negative ion mass spectra of nicardipine exhibit an interesting difference between the filament on and off modes. With the filament on, nicardipine is ionized by an electron-capture mechanism, producing a molecular ion M^- (*m/z* 479), whereas with the filament off, ionization through proton abstraction becomes predominant, resulting in a molecular ion ($M - H^-$) (*m/z* 478).

For TSP in both modes, the yield of positive ions from nicardipine is 2–3 times higher than for negative ions. In DLI LC–MS however, the ionization efficiency is several orders of magnitude higher for negative ions (molecular ion M^-, *m/z* 479) than for positive ions [molecular ion ($M + H^+$), *m/z* 480].

Overall, for nicardipine, DLI LC–MS (negative ions) provides a 2–5 times higher sensitivity than TSP LC–MS (positive ions, filament on). It must be emphasized that this result cannot be generalized, as depending on the conditions and the substance involved, the ionization mechanism may be different for the two methods. For example, TSP and DLI show approximately the same sensitivity in the analysis of metabolites of fluperlapine (Fig. 8), and whereas the sensitivity of TSP for cyclosporine and its metabolites (Fig. 9) is 10 times less than with DLI, other examples in which TSP has a superior sensitivity have been reported[4].

A chromatographer tends to consider the mass spectrometer simply as a sophisticated detector. The chromatographic properties of the DLI and TSP interfaces were also investigated from this viewpoint.

Fig. 12 shows the influence of column diameter on the chromatography of a test mixture. The flow velocity and the injection volume were kept constant. As the tip temperature of the TSP interface and in parallel the response are very flow sensitive, a makeup flow was added through a post-column mixer[5] to keep the flow-rate constant at 1.5 ml/min. It can be seen that the response of both interfaces is concentration dependent. Decreasing the column diameter while keeping the linear velocity and injection volume constant leads to lower peak volumes and therefore less dilution of the sample. With the DLI the eluent is split and a decrease in column diameter leads to higher signals, *i.e.*, higher sensitivity. As the TSP interface needs higher flow-rates, a makeup flow becomes necessary for narrow-bore columns. This causes additional dilution of the sample and band broadening and consequently a decrease in sensitivity.

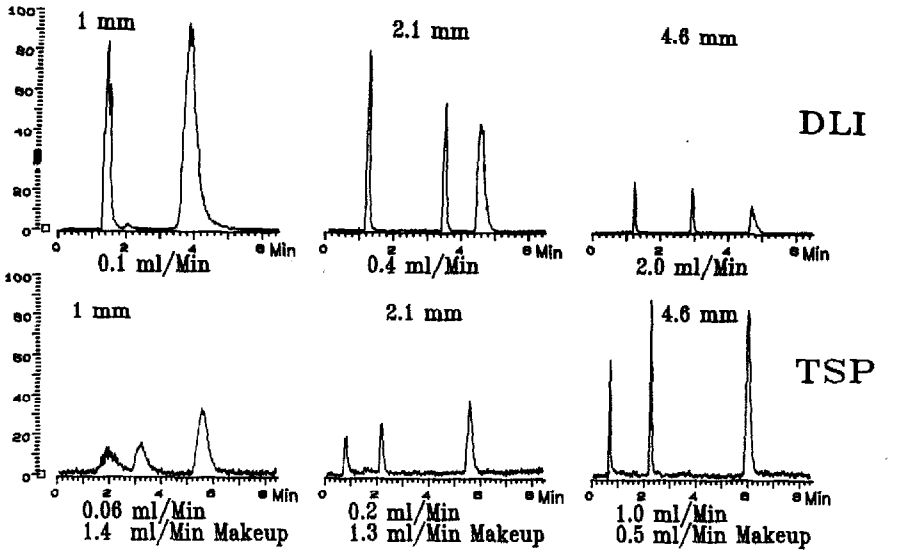


Fig. 12. Influence of column diameter on peak height. Column length, 100 mm, RP-18 ODS-1 (2.1 and 4.6 mm I.D.) and ODS-2 (1 mm I.D.). The test mixture was not the same for TSP and DLI. Mobile phase: water–acetonitrile (50:50, v/v); for DLI, for TSP both eluent and makeup were 0.1 *M* ammonium acetate–acetonitrile (50:50, v/v).

The chromatograms obtained with a column of 1 mm I.D. illustrate the limits of the experiment. Apart from the fact that the chromatographic selectivity differs owing to the different stationary phase used, the peaks are much broader, indicating overloading of the column or too large an injection volume.

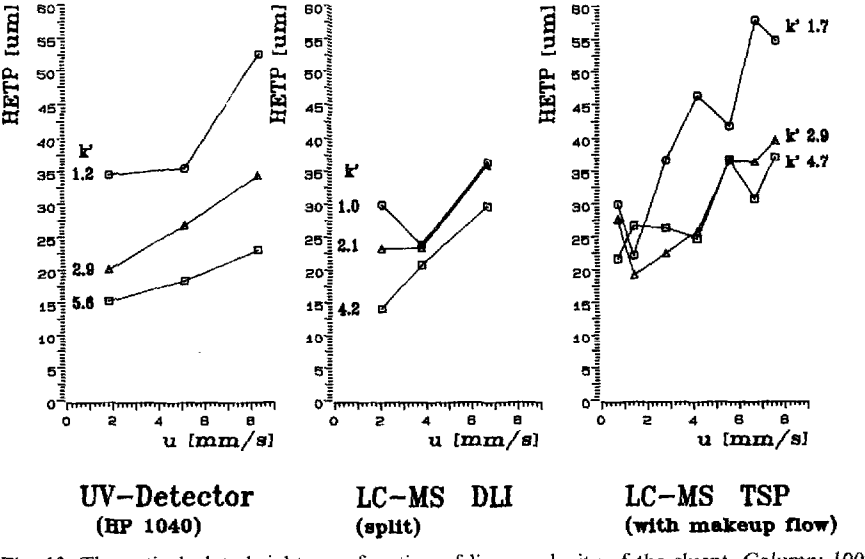


Fig. 13. Theoretical plate height as a function of linear velocity of the eluent. Column: 100 × 2.1 mm I.D., RP-18, 5 μm particles. Other conditions as in Fig. 12. Conditions for the experiment with the UV detector were the same as for LC–MS with DLI.

The peak broadening caused by the detector is another important factor in HPLC. Fig. 13 shows the dependence of theoretical plate height on linear velocity (H/u) for the same column with different detectors (UV, TSP and DLI). With a well optimized HPLC system, reduced plate heights of 2–5 d_p should be observed. This occurs with the DLI interface, where the only volumes causing band broadening are the long capillary connections and the mandatory in-line filter. The UV detector shows slightly more band broadening, caused by the cell volume, which, dynamically measured, was about 15 μl. With the TSP interface, a makeup flow had to be added to keep the flow-rate in the ideal range needed. The mixer and the several unions necessary cause a considerable decrease in separation efficiency at higher flow-rates.

To summarize, the TSP interface in its present technical state is combined best with conventional 4.6 mm I.D. columns. There are no restrictions on the composition of the mobile phase except that all components must be volatile. However, maximum sensitivity is strongly sample dependent and very sensitive to the mobile phase composition and instrument parameters (source and tip temperature, pressure, etc.). Quantitation at trace levels with TSP seems to be limited owing to the high noise and frequent spikes (single scans with extremely high intensity). However, the interface is simple and easy to operate and therefore also suitable for automated analysis.

In contrast, the DLI interface requires considerable technical skill of the operator and cannot be used in unattended automated analysis. The sensitivity is also sample dependent, but the influence of instrumental parameters is smaller. Obviously, additives to the mobile phase also must be volatile. In addition, however, extremely low (*ca.* $<15\%$) or high (*ca.* $>80\%$) water contents of the eluent in reversed-phase chromatography can lead to an unstable jet. Quantitation with DLI is also feasible at very low concentrations owing to the generally low noise level.

A serious drawback of the DLI, in our opinion, is that it is still only an accessory, and has remained virtually unimproved for the last few years. In contrast, dedicated ion sources have been developed for the thermospray technique, and both interfaces and sources have been continuously updated by several manufacturers.

CONCLUSIONS

Our experience with more than 2 years of routine application of LC(DLI)–MS has shown that the method is suitable for everyday analysis in an analytical laboratory in the pharmaceutical industry. The DLI interface shows good stability and, after acquisition of the necessary skills, a very high system availability. The excellent selectivity of the method is ideal for the qualitative and quantitative analysis of biological samples with complex matrices. The sensitivity is adequate although, especially with biological samples, where amounts and concentrations are often low, the limit to obtaining good mass spectra is reached quickly. The consequent use of short columns with smaller diameters solves this problem in many instances. The good reproducibility and stability of DLI LC–MS allows quantitative work down to very low concentration levels in the range 50–200 pg per injection.

ACKNOWLEDGEMENTS

We thank Dr. V. Fischer, Dr. P. Koch and Dr. G. Maurer for the urine, bile

and plasma extracts of the metabolites of fluperlapine and cyclosporin, and Dr. M. Guerret, Sandoz, Rueil, France, for the plasma extracts of nicardipine and its metabolites. The Vestec TSP interface was a generous loan from Dr. D. Dixon, Hewlett-Packard, Geneva, Switzerland.

REFERENCES

1 B. R. von Wartburg, W. Sieber and J. P. Gourraud, paper presented at the *8th European Workshop on Drug Metabolism, Sart Tilman, Liege, Belgium, 1982.*
2 A. J. Paine, G. Maurer and B. R. von Wartburg, *Biochem. Pharmacol.*, 33 (1984) 3111.
3 J. E. Bartmess and R. T. McIver, Jr., in M. T. Bowers (Editor), *Gas Phase Ion Chemistry*, Vol. 2, Academic Press, New York, 1979, Ch. 11.
4 R. D. Voyksner, J. T. Bursey, J. W. Hines and E. D. Pellizzari, *Biomed. Mass Spectrom.*, 11 (1984) 616.
5 B. Lillig, *Dissertation*, Universität des Saarländes, Saarbrücken, F.R.G., 1984.