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DIRECT LIQUID INTRODUCTION MICRO-LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY COUPLING

OPTIMIZATION OF DROPLET DESOLVATION AND INSTRUMENTAL PARAMETERS FOR HIGH SENSITIVITY*

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SUMMARY

Direct coupling of microbore liquid chromatography to mass spectrometry via the Direct Liquid Introduction interface allows the mass spectrometer to be adapted to the eluent, rather than the other (usual) way that uses an external splitter to limit the liquid chromatograph eluent flow entering the mass spectrometer. We have examined a new design of desolvation chamber that achieves internal splitting with solute enrichment. This device, combined with the micro-bore column, permits utilization of the entire injected sample and therefore provides high-sensitivity liquid chromatography–mass spectrometry.

The greater ease and reproducibility in the tuning of the mass spectrometer, together with the stability of the chemical ionization conditions over a period of 1 day, permits quantitative measurements. Examples of the analysis of corticosteroids at the low-nanogram level in equine plasma and urine are presented.

INTRODUCTION

As microbore liquid chromatography (μLC) columns (1 mm I.D.) become more readily available and more efficient, they may become more widely used to monitor low levels of drugs in biological fluids. Certain potent drugs have lower effective plasma levels and often the available sample volume is necessarily limited. The advantage of microbore columns over conventional 4.6 mm I.D. columns is that the samples can be concentrated to a suitable injection volume and, after separation,

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eventually enter the detector in a much more concentrated solution than after separation on a larger bore column.

Although the conventional liquid chromatographic (LC) detectors (UV, fluorescence and electrochemical) offer detection limits sufficient for the pharmacological studies of a wide range of drugs, they lack sufficient specificity. An extensive sample preparation before analysis is required to eliminate the interference of endogenous biological compounds. This often results in a low recovery of drug-related substances as very low levels of drugs are involved. Screening of biological fluids containing trace levels of unknown substances necessitates minimal sample preparation and a universal, though specific, detector.

The mass spectrometer coupled to a microbore column through a direct liquid introduction (DLI) interface[1] meets most of these requirements. In particular, it takes advantage of the transfer of the entire μLC column effluent to the mass spectrometer, thus promoting high sensitivity.

The DLI interface sprays the LC effluent into droplets through a pinhole diaphragm[2]. One of the most effective ways of controlling the desolvation of the solutes from the droplets is to use a heated zone, usually called a *de*solvation *c*hamber (DSC), in which the droplets acquire a high speed before entering the chemical ionization (CI) source[3,4]. The commercial version of the DSC installed on our instrument was designed for flow-rates in the neighborhood of 10 μl/min. It did not allow operation of μLC at its optimum flow-rate (40 μl/min) without critical, delicate adjustment of the DLI interface tip position relative to the DSC.

This paper describes a new design of the DSC that allows tuning of the desolvation and the CI conditions with a wider margin of reproducible operation. In addition, this DSC provides a higher sensitivity by a higher effective use of the separated solutes.

EXPERIMENTAL

Chemicals

Methanol was distilled-in-glass grade (Burdick & Jackson, Muskegon, MI, U.S.A.) and water was Baker Analyzed HPLC Reagent (J. T. Baker, Phillipsburg, NJ, U.S.A.). The premixed solvents were filtered through a 0.2-μm pore size filter (Millipore, Bedford, MA, U.S.A.) to eliminate particulate matter, degassed in an ultrasonic bath and then continuously purged with helium.

The drug standards, dexamethasone, betamethasone, 6-methylprednisolone and hydrocortisone, were obtained from Steraloids (Wilton, NH, U.S.A.). A sample of 6-hydroxybetamethasone was kindly donated by Dr. R. Draper, Schering Corp.

Micro liquid chromatography

The micro-high-performance liquid chromatographic system used consisted of a Waters M-6000A pump (Waters Assoc., Milford, MA, U.S.A.) controlled by a Waters M-660 solvent programmer modified for lower flow-rates[5], a 0.5-μm solvent line filter (Rheodyne, Cotati, CA, U.S.A.), a Rheodyne Model 7410 micro loop injector equipped with a 0.5-μl loop, a Whatman glass-lined μLC column (ODS-3, 7-μm particle size, 25 cm × 1 mm I.D., Whatman No. 4240-128, Clifton, NJ, U.S.A.) and a 1/16-in. low-dead-volume union equipped with a 0.5-μm stainless-steel frit

(Valco, No. ZUVF-0620.5, Houston, TX, U.S.A.) lined with two layers of 0.2-μm Millipore filter. Stainless-steel tubing with 1/16 in. O.D. and 0.1 mm (0.004 in.) I.D. connected the components of the system; before installing the short sections of tubing, their bores were opened with the electropolishing technique previously described[6].

Modified DLI micro-liquid chromatography–mass spectrometry interface

The μLC-mass spectrometry (MS) interface has been described previously[1]. In addition, three different designs of a DSC were successively used in this study: the standard HP DSC (Part No. 05985-20587, Hewlett-Packard, Palo Alto, CA, U.S.A.), a heated "extended" DSC and a heated "solvent stripping" DSC (*cf.* Fig. 3). The two last designs involve essentially the same hardware: a 304 stainless-steel rod (40 mm × 16 mm O.D.; 1.600 × 5/8 in.) machined to accomodate a cartridge heater (Hewlett-Packard cartridge, Part No. 05985-60171) in a separately machined stainless-steel holder, a probe guide and seal, and a droplet transfer line (Fig. 1).

The DSC was threaded to the source block in the usual manner. The DSC heater was supplied with current from the same computer-controlled supply as the source block heaters. In addition to these features, the "solvent-stripping" DSC includes a 0.5-mm gap between the tip of the DSC and the CI source aperture that is pumped with the high vacuum of the mass spectrometer through the threaded hole of the removed electron-controller-mask holding screw (6-32 machine screw). The aperture of the HP CI source plunger was also enlarged to 3.2 mm (1/8 in.) to maintain a constant section of the transfer line matching the I.D. of the DSC (see Fig. 1).

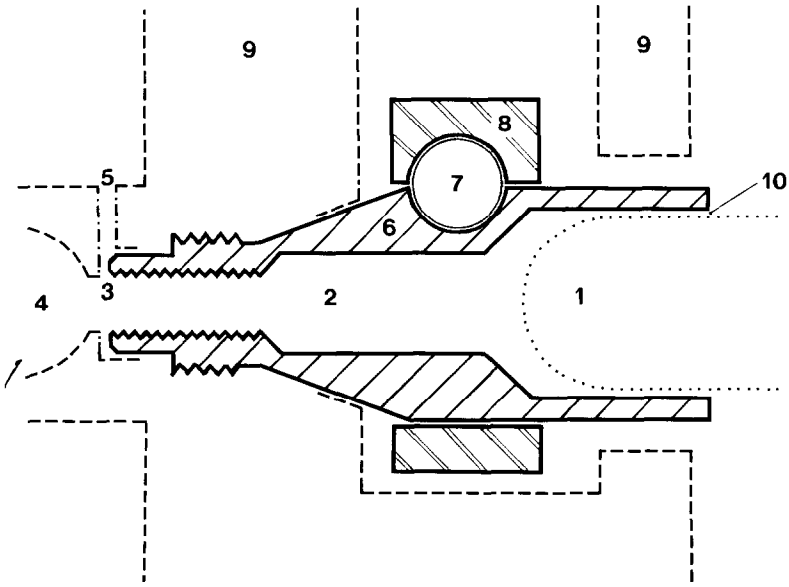


Fig. 1. Solvent-stripping desolvation chamber (longitudinal section). 1 = Tip of DLI LC–MS probe; 2 = heated droplet desolvation chamber and transfer line; 3 = solvent-stripping annular pumping gap (not present in extended DSC); 4 = CI ion source; 5 = solvent-stripping pumping line (screw hole through source body); 6 = desolvation chamber walls; 7 = heater cartridge; 8 = cartridge holder ring; 9 = source body and magnet; 10 = adjustable internal splitting gap.

The principle of operation for the different interfaces in the μLC–MS mode is described in the Results section.

Mass spectrometry

Mass spectra were recorded on a Hewlett-Packard Model 5985B gas chromatography (GC)–MS instrument equipped with option 04 for LC–MS operation. The liquid nitrogen-cooled cryopump was used in all experiments. Primary ionization of the solvent vapours was accomplished by a 140-eV beam of electrons from a heated rhenium wire. Improved pumping around the filament was obtained by replacing the standard electron shield by a stainless-steel mesh (mesh size 40, 50% transmission) of the same geometry.

The typical CI operating parameters of the mass spectrometer for the μLC–MS experiments were as follows: emission current, 200 μA; ion source temperature, 200°C; ion source pressure, 0.7 Torr (as measured at the CI GC–MS interface thermocouple); repeller, 8 V. The μLC eluent and CI reagent gas was methanol–water (65:35) and the detection was in the negative ion mode with a 2400 V electron multiplier voltage.

Sample clean-up

Urine and plasma samples were collected at 2-h intervals after intramuscular administration of the corticosteroids to standard-bred horses. Urine saturated with sodium sulphate and plasma were extracted at pH 4 with 1 volume of diethyl ether–dichloromethane–isopropanol (2:1:1). The organic phase was washed with 1 volume of 15% sodium sulphate in 2 *N* sodium hydroxide and the extract was dried under a stream of nitrogen. The residue was redissolved in ethyl acetate and applied onto an analytical silica gel thin-layer chromatography plate. The sample was developed to 5 cm with chloroform–ethyl acetate–light petroleum (b.p. 65–95°C) (5:3:2). The areas corresponding to corticosteroids and metabolites were located with 254-nm UV light, scraped, and eluted with ethyl acetate. The residual urine or plasma material was then analysed by μLC–MS.

RESULTS

After development of a satisfactory μLC separation of a particular sample mixture utilizing a micro flow cell UV detector[1], at least two problems face the analyst using MS as a detection method: first, how to tune the mass spectrometer in order to ensure steady and sensitive levels of detection; secondly, how to document these conditions in order to reproduce them in later experiments.

μLC solvent composition

The solvent composition which gave a satisfactory separation of the corticosteroids of interest in this study (in particular hydrocortisone, betamethasone, dexamethasone and 6-methyl prednisolone) was methanol–water (65:35). Since the MS detection limits for these solutes were sufficient in the negative CI mode for our study, no attempt was made to modify the pH or the water–methanol solvent ratio. Furthermore, methanol–water at CI pressures forms mainly negative and positive methanol cluster ions. Other studies with methanol–water mixtures have shown that the

intensity of the protonated solvent ions is independent of the amount of water[7] and that the absolute sensitivity for a protonated solute remains constant over the entire solvent composition range[8].

μLC solvent flow-rate

The optimum chromatographic efficiency of the μLC column was observed at a flow-rate of 20 μl/min, but a compromise flow-rate of 40 μl/min was utilized to expedite the separations within 20 min. At this flow-rate the column head pressure was 900 p.s.i. (6.4 MPa).

Because the mass spectrometer diffusion pump above could not handle this flow-rate, a liquid nitrogen cryopump was used to complement the standard CI pumping system. The source envelope pressure was then $2.4 \cdot 10^{-4}$ Torr, which was one quarter of the maximum operating pressure. This was independent of the dimensions or supplier of a particular diaphragm.

Ion source pressure

Fig. 2 shows the typical source pressures that can be obtained by changing the distance between the DLI probe tip and each one of the three different DSCs (see Fig. 3). The source block and DSC temperatures were maintained constant at 200°C throughout this experiment. With the standard Hewlett-Packard DSC and the extended DSC, the same high pressure (2.2 Torr) was observed when the probe butted against the DSC, keeping the chamber tightly closed. When the probe was withdrawn however, the pressure dropped much more rapidly in the standard DSC, within the first millimeter from the DSC seat. The pressure was therefore much more difficult to regulate in this chamber than in the extended DSC. The proposed flow of droplets

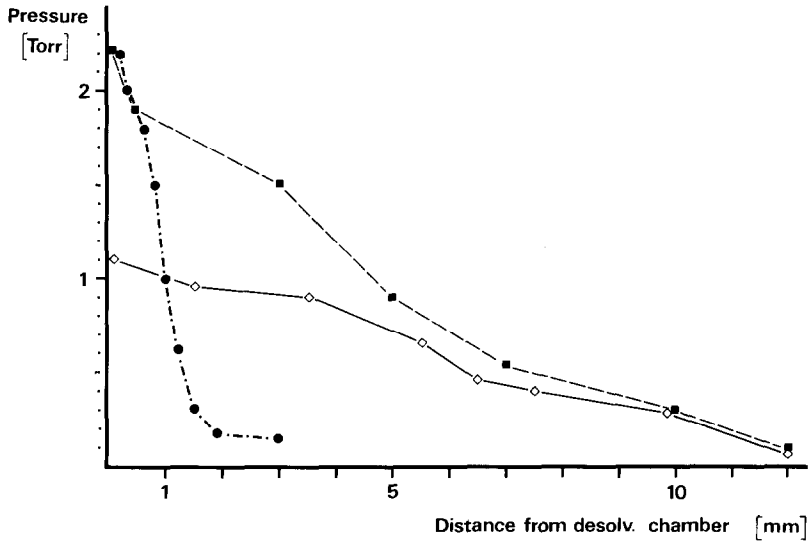


Fig. 2. Plots of ion source pressure *versus* distance between DLI probe tip and seat of desolvation chamber (DSC) for three different DSCs: ●- · - · -● = standard Hewlett-Packard DSC; ■ - - ■ = extended DSC; ◇——◇ = solvent-stripping DSC. Solvent methanol–water (65:35); source and DSC temperature, 200°C.

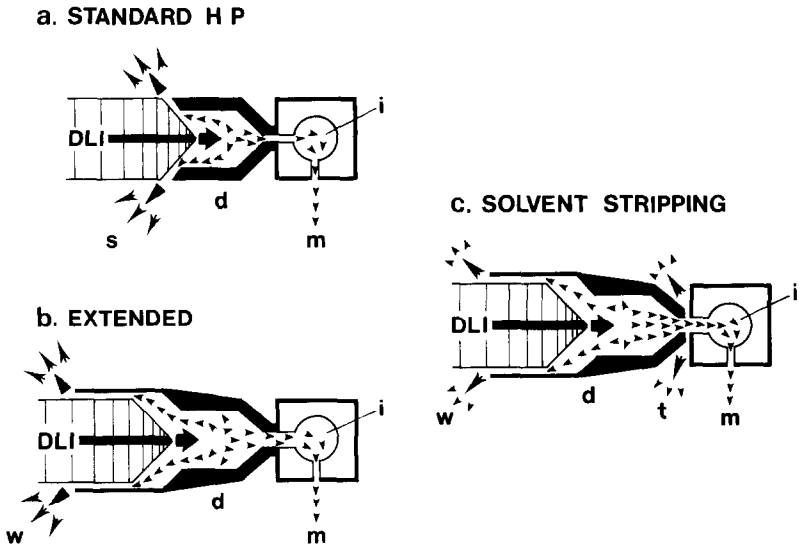


Fig. 3. Schematic diagram showing functioning of the three different DSCs. DLI = μLC probe producing a stream of droplets; d = desolvation chamber; i = ion source; m = ionized stream of solute and solvent molecules focused towards *mass* filter; s = flow pumped between DSC *seat* and μDLI probe tip; w = flow pumped through the gap between DSC *walls* and μDLI probe; t = flow pumped through the gap between DSC *tip* and ion source body.

and gases in these DSCs is schematically depicted in Fig. 3a and b. It consists basically in the splitting, outside of the DSC (*i.e.*, external to the ionization chamber), of the solvent *and solute* vapours that are in excess of the optimum CI working pressure. The differences between the standard and the extended DSC are that the latter features a more progressive splitter, a separate heater, a larger source inlet diameter and a possible minor extension of the jet length.

The third DSC design, schematically shown in Fig. 3c, may be called a "solvent-stripping desolvation chamber" and its basic pressure control is the same as that of the extended DSC. In addition, a gap of adjustable width pumped to a high vacuum is introduced between the DSC and the CI ion source. Thus, typically, pressures reached a maximum of 1.1 Torr with the probe tightly fitting the DSC, and the solvent vapours were removed from the *inside* of the DSC at a point where the droplets and gases have reached their highest temperature and velocity immediately before entering the ion source. Lower pressures could be obtained by an additional external splitting, controlled by the probe-to-DSC distance.

The source pressure provided useful information about the CI operating conditions but since its reading with a thermocouple gauge was not very precise and the optimal pressure varied with the different diaphragms, a pressure of 0.6–0.7 Torr at 200°C can only be given as an indication of average CI conditions. The tuning and recording of the CI conditions was actually carried out with the solvent cluster ions and the repetitive injection of a reference solute (see below).

Temperature of source and DSC

The effects of source and DSC temperature on the chromatographic peak

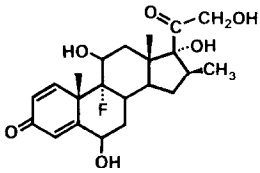
shapes and fragmentation were studied for the model compound 6-hydroxybetamethasone (6-OH-β-METH), the major urinary metabolite of betamethasone in horses[9]. The negative CI fragmentation of 6-OH-β-METH can be rationalized by comparison with the negative CI mass spectra of corticosteroids with similar structures[10]; the principal fragments are reported in Table I. Of particular interest is the ion of *m/z* 313, corresponding to the loss of neutral hydrogen fluoride from the *m/z* 333 ion.

These experiments were carried out with the source and DSC at the same temperature, between 150 and 300°C. Below 185°C, the peaks were hatched and tailing occurred, showing that too little heat was being transferred to the droplets in order to achieve an extensive desolvation of the solute. However, these low temperatures gave mild ionization conditions and a relationship was observed between fragment ion intensity and temperature: the loss of hydrogen fluoride becomes more extensive with higher temperatures. With the extended DSC, the *m/z* 313:*m/z* 333 ratio varied from 0.3 (at 150°C) to 1.1 (at 185°C). At DSC temperatures greater than 190°C, the peaks exhibited smooth shapes, showing that enough heat was being evenly transferred to the droplets during their drift along the DSC. In the extended DSC, higher temperatures produced greater fragmentation, the *m/z* 313 peak being 1.4 times more intense than the *m/z* 333 one at 200°C and 5 times more at 250°C. Although the fragmentation increased with temperature, the absolute intensity of the *m/z* 333 ion measured at the apex of the chromatographic peaks doubled from 150 to 200°C and decreased at higher temperatures.

The performance and sensitivity of the micro-DLI interface could be improved further by using the solvent-stripping DSC (Fig. 3c): at 200°C, the fragmentation of

TABLE I

FRAGMENT IONS DERIVED FROM 6-HYDROXYBETAMETHASONE UNDER NEGATIVE ION CI MASS SPECTROMETRIC CONDITIONS



6-Hydroxybetamethasone
Mol. wt. 408

CI reagent gas, methanol–water (65:35); 200°C; extended DSC. Solvent tuning ions ratios: *m/z* 63:81:95, 55:26:100% relative intensity.

Fragment(s) lost	*m/z*	*Relative intensity (%)*
Hydrogen	406	2
Water	390	10
Water and a methyl radical	375	25
Water, a methyl radical and hydrogen	373	46
Ketene and hydrogen	364	20
Water, a methyl radical and HF	355	36
Methanol, water and a methyl radical	343	52
Ketene, water and a methyl radical	333	88
Ketene, water, a methyl radical and HF	313	100

6-OH-β-METH was substantially reduced, with a ratio of *m/z* 313 to *m/z* 333 of 0.2, and the total ion current intensity of the smooth chromatographic peaks was doubled as compared with the extended DSC. The overall gain in sensitivity was eight-fold for the *m/z* 333 ion.

Tuning for optimal CI conditions

Most experimental procedures for optimizing jet length and source CI parameters use a continuous introduction of the tuning solution[8,11,12]. However, this method has the following limitations in the case of μLC–MS when it is used as a daily start-up procedure.

(1) The column and the connecting tubing of the entire chromatographic system must be filled with the tuning solution and then rinsed with pure solvent. It takes considerable time to return the column to its original condition after being "saturated" with the test solution.

(2) Continuous introduction of the test solution into the mass spectrometer unnecessarily contaminates the ion source.

(3) If the DLI probe is directly connected to the tuning solution pump during optimization, the jet must be interrupted to hook up the column and the injector. Furthermore, the diaphragm may clog after a column change.

(4) Column and injector switching with three-way valves would be effective with 4 mm I.D. columns, but the currently available hardware introduces an excessive dead volume when μLC columns are used.

(5) The maximum sensitivity does not mean that tuning is best for the stability and the shape of the chromatographic peaks. For example, Fig. 4 shows that the peak shape is better at a lower-than-maximum sensitivity.

The repetitive injection of a tuning solute is therefore the method of choice to determine the best jet conditions. Once these conditions have been established, the recording of the solvent cluster ion ratios gives a rapid solute-independent means of documenting the exact CI plasma conditions.

The intensity of solvent ions is thirty times larger than that of the solute ions. Unfortunately, the standard software package of the mass spectrometer (Hewlett-Packard, No. 05985-1004) does not allow simultaneous changes of scanning mass range and electron multiplier (EM) high voltage during an acquisition run. The solvent cluster ion ratio must be obtained with a low EM voltage, after interruption of continuous scanning of the mass range of the solute ions.

Typical solvent cluster ion patterns as a function of the source pressure are shown in Fig. 5. The ions selected as "solvent tuning ions" in the negative CI mode using methanol–water were those of *m/z* 63, 81 and 95. These ions correspond to $[(CH_3OH)_2 - H]^-$, $[(CH_3OH)_2 \cdot H_2O - H]^-$ and $[(CH_3OH)_3 - H]^-$, respectively, although we have no direct evidence for these compositions. As observed for positive CI solvent cluster ions[7,8], in negative CI the extent of solvent clustering and the relative intensity of the clusters depend strongly on the source pressure. As expected, the higher the pressure, the larger the degree of aggregation. However, ion quenching occurs above 1 Torr and decreases the intensity of the solute ions and the solvent cluster ions. A 2:1 intensity ratio of methanol trimer (*m/z* 95) to methanol dimer (*m/z* 63) was found to give the best conditions for obtaining a high intensity and a good chromatographic peak shape for the model solute 6-OH-β-METH. The intensity of

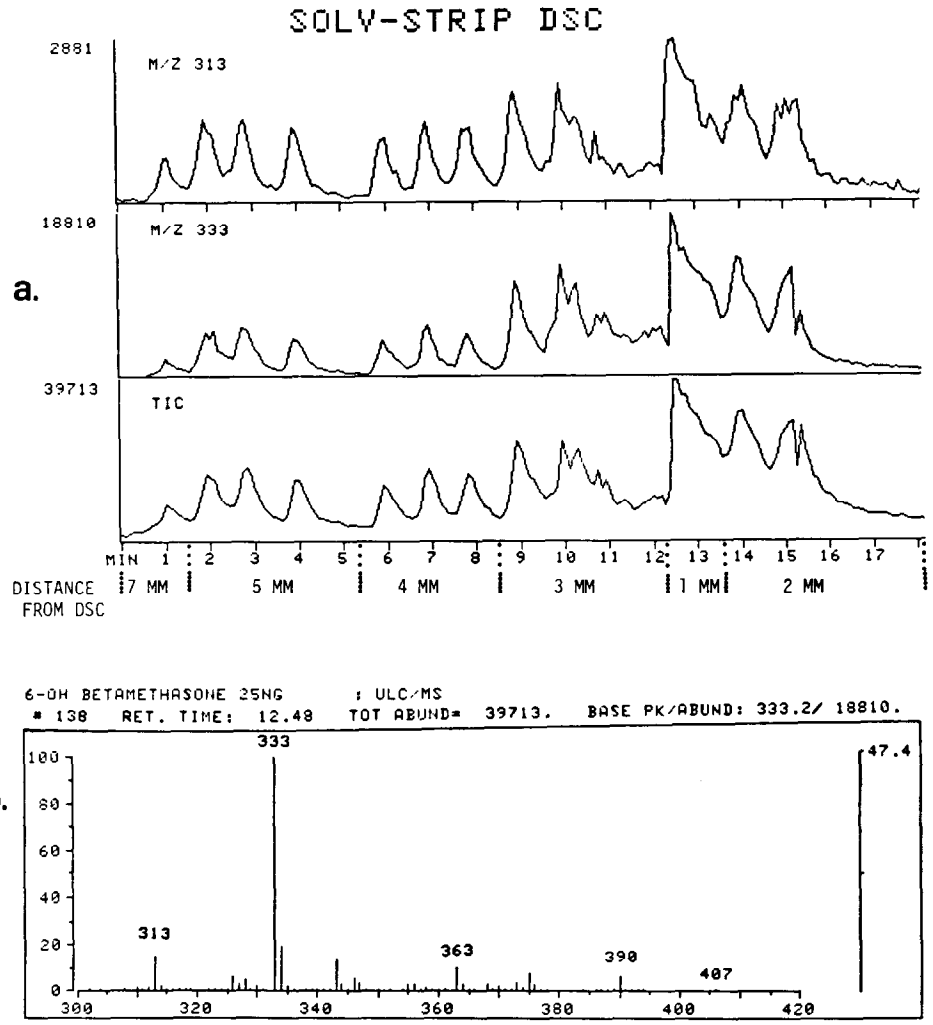


Fig. 4. μLC–MS separation of 6-hydroxybetamethasone (6-OH-β-METH) after repeated 25 ng injections; solvent-stripping DSC at 200°C (other standard parameters described in Experimental section). (a) Reconstructed ion chromatograms at different distances of DLI probe in DSC. (b) Mass spectrum recorded at the top of the largest peak (t_R = 12.5 min); solvent tuning ion ratios: m/z 63:81:95, 36:38:100% relative intensity.

the solvent cluster ions also depends strongly on the temperature of the DSC and ion source. Typical solvent tuning ion ratios at different temperatures of the solvent-stripping DSC and source are shown in Fig. 6. At 200°C, the trimer-to-dimer methanol cluster ion intensity ratio could be easily adjusted, using the variable splitter of the DSC, to stay at or slightly above the desired 2:1 ratio value.

With the extended DSC, the same procedure and a solvent tuning ion ratio of at least 1:1 at 200°C could be used. However, a 50% decrease of the relative intensity of the hydrated methanol dimer cluster ion (m/z 81) was observed, as compared with the solvent-stripping DSC (see Fig. 5).

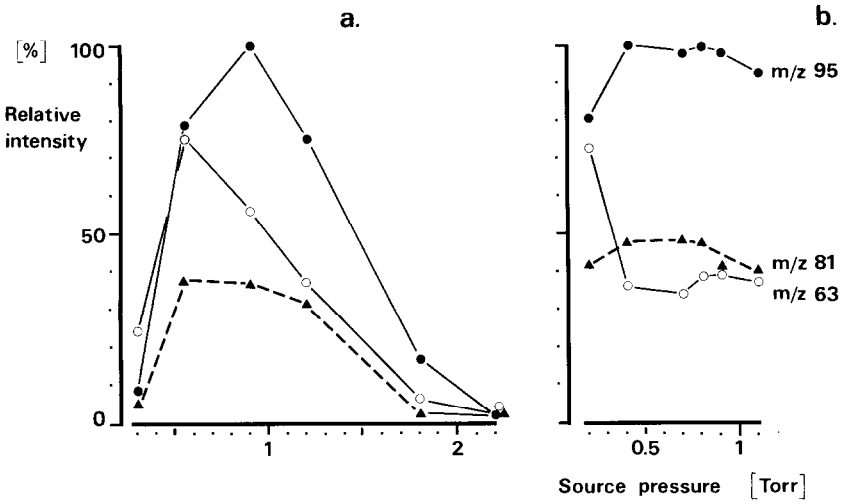


Fig. 5. Plot of the intensity of the three solvent cluster tuning ions *versus* source pressure, in negative CI at 200°C source and DSC temperature. (a) Extended DSC; (b) solvent-stripping DSC. Intensity normalized according to the most intense value in each DSC; solvent, methanol–water (65:35); flow-rate, 40 μl/min.

High sensitivity detection

An evaluation of the sensitivity provided by the solvent stripping DSC interface was made with the model compound 6-OH-β-METH. On-column 0.5-μl repetitive injections of 250 and 500 pg gave the chromatogram shown in Fig. 7. With full scan mode detection starting at *m/z* 240 to avoid continuous background ions originating from solvent impurities, the total ion current trace had a signal-to-noise ratio of 20. Similar values were observed for the reconstructed ion chromatograms at *m/z*

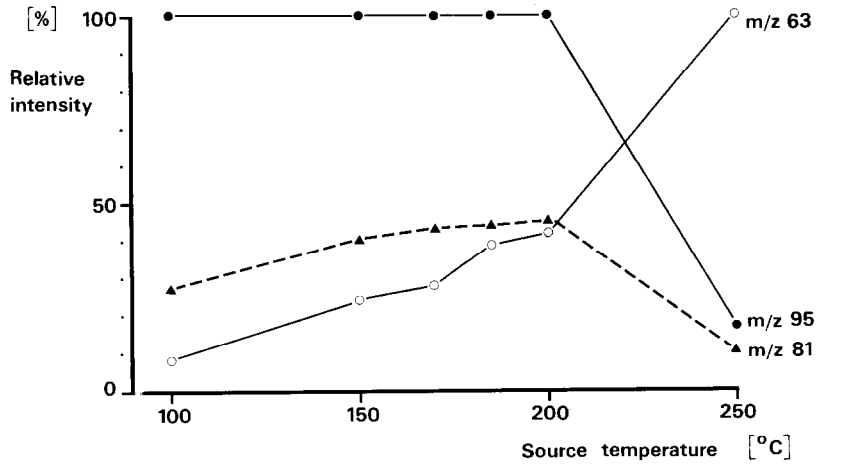


Fig. 6. Plot of the intensity of the three solvent cluster tuning ions *versus* source temperature, in negative CI at the pressure giving the highest intensity ratio of methanol trimer ion (*m/z* 95) to methanol dimer ion (*m/z* 63) with the solvent-stripping DSC. Intensity normalized according to the most intense ion in each measurement; solvent, methanol–water (65:35); flow-rate, 40 μl/min.

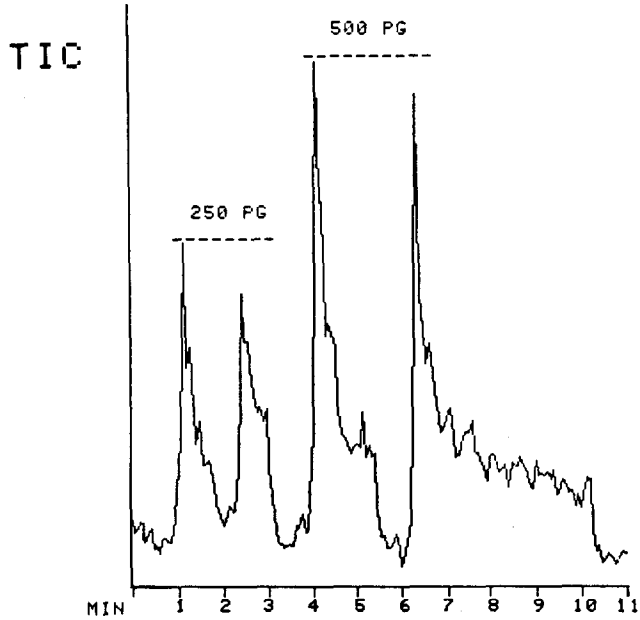


Fig. 7. Negative ion CI μLC–MS total ion current (TIC) profile for repeated injections of 250 and 500 pg of 6-OH-β-METH. The μLC–MS eluent/CI reagent gas was methanol–water (65:35) maintained at 40 μl/min through a C_{18} 250 mm × 1 mm I.D. microbore column; solvent-stripping DSC; MS conditions described in Experimental section.

313 and 333. One could therefore expect that detection limits below 1 pg could be obtained in the selected ion monitoring mode.

Analysis of biological samples

Figs. 8 and 9 show the μLC–MS separation of equine urine extracts and Fig. 10 shows the analysis of crude extracts of equine plasma.

The urine for Fig. 8 was collected during the 6 h after administration of 6-methylprednisolone (6-Me-PRED). On the total ion current trace (Fig. 8b), no peak could be observed at the retention time expected for 6-Me-PRED. However, the mass spectrum recorded at this retention time (Fig. 8c) did reveal the presence of the drug, together with endogenous compounds. Fig. 8a, the reconstructed ion chromatogram for m/z 356, showed the peak corresponding to the loss of one molecule of water from the molecular ion of 6-Me-PRED to be present, albeit at a level of only *ca.* 10 ng in a biological matrix of 20 μg.

The urine for which data are shown in Fig. 9 was collected during the 6 h after administration of betamethasone (β-METH). In Fig. 9a, the resolution of the different overlapping polar compounds constituting the major peaks is seen to be better than in Fig. 8 because of the lower column load. This could be achieved because the ratio of the urinary level of β-METH to that of the biological matrix was more favourable than for 6-Me-PRED. The mass spectrum of the largest peak in Fig. 9a, (peak b) shown in Fig. 9b, and its retention time corresponded to those of 6-OH-β-METH. The unmetabolized drug, β-METH, could also be detected in this urine extract (peak c) and characterized, albeit at a much lower level (Fig. 9c).

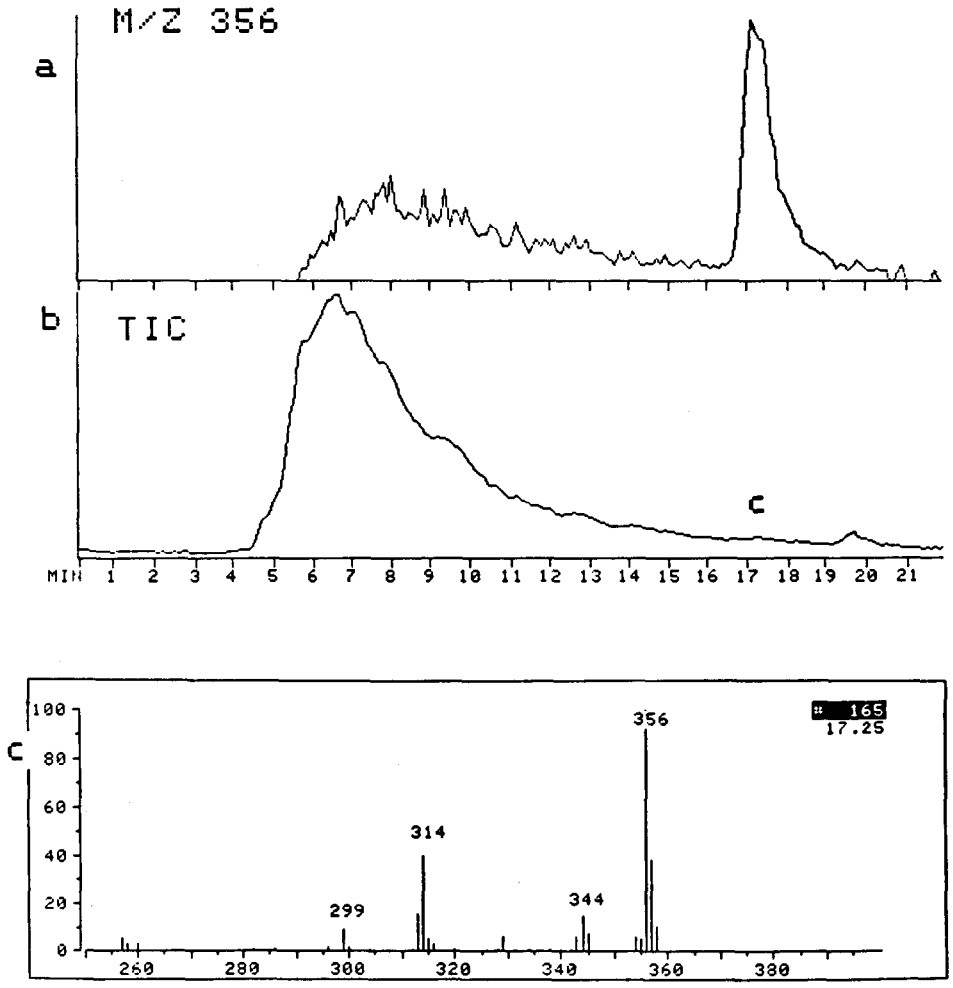


Fig. 8. Negative ion CI μLC–MS analysis of the extract of equine urine collected for 6 h post i.m. administration of 6-Me-PRED. Injection volume, 0.5 μl. μLC–MS conditions as in Fig. 7. (a) Reconstructed m/z 356 ion and (b) total ion current profiles; (c) negative CI mass spectrum of 6-Me-PRED, taken at the maximum of the m/z 356 peak in Fig. 8a. Solvent tuning ions ratios: m/z 63:81:95, 47:21:100% relative intensity.

For the same administration of β-METH to horses, the plasma levels were also measured. Fig. 10 shows an example of a chromatogram of a plasma extract obtained after μLC separation and selected ion monitoring (SIM) MS detection of β-METH, 6-OH-β-METH and hydrocortisone. Quantification of the compounds selectively detected in the samples was performed by comparing their peak heights with those of an external standard, a mixture of pure compounds injected before and after the samples. For the example shown in Fig. 10, 6 ng of β-METH and 0.6 ng of 6-OH-β-METH were determined in the plasma extract 1 h after administration, together with 21 ng of the endogenous corticosteroid hydrocortisone.

DISCUSSION

An ideal detector for liquid chromatography has not yet been developed; however, MS is a highly sensitive detection method that fulfills most of the needs for a detector that is at the same time both universal and specific. Commercially available mass spectrometers that can be interfaced to LC were not specifically designed for this use; without modification of the mass spectrometer, a DLI interface has provided good results[13], but 99% of the LC flow had to be split while only 10 μl/min could be introduced into the mass spectrometer. To handle LC effluent flow-rates in excess of 10 μl/min, the conventional vacuum pumping system had to be modified by supplementing a cryopump[14,15] or a vacuum-lock system[16]. This improvement was still not sufficient for pumping the entire flow from a conventional 4.6 mm I.D.

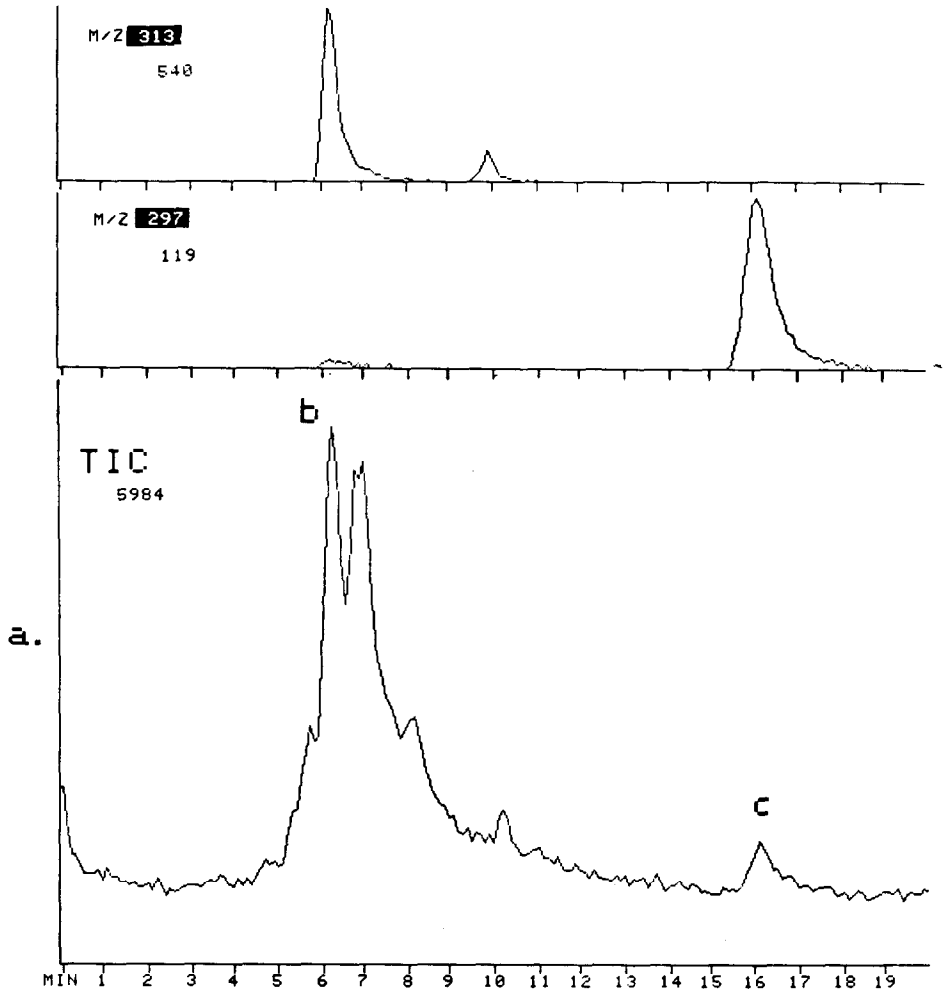


Fig. 9

(*Continued on p. 370*)

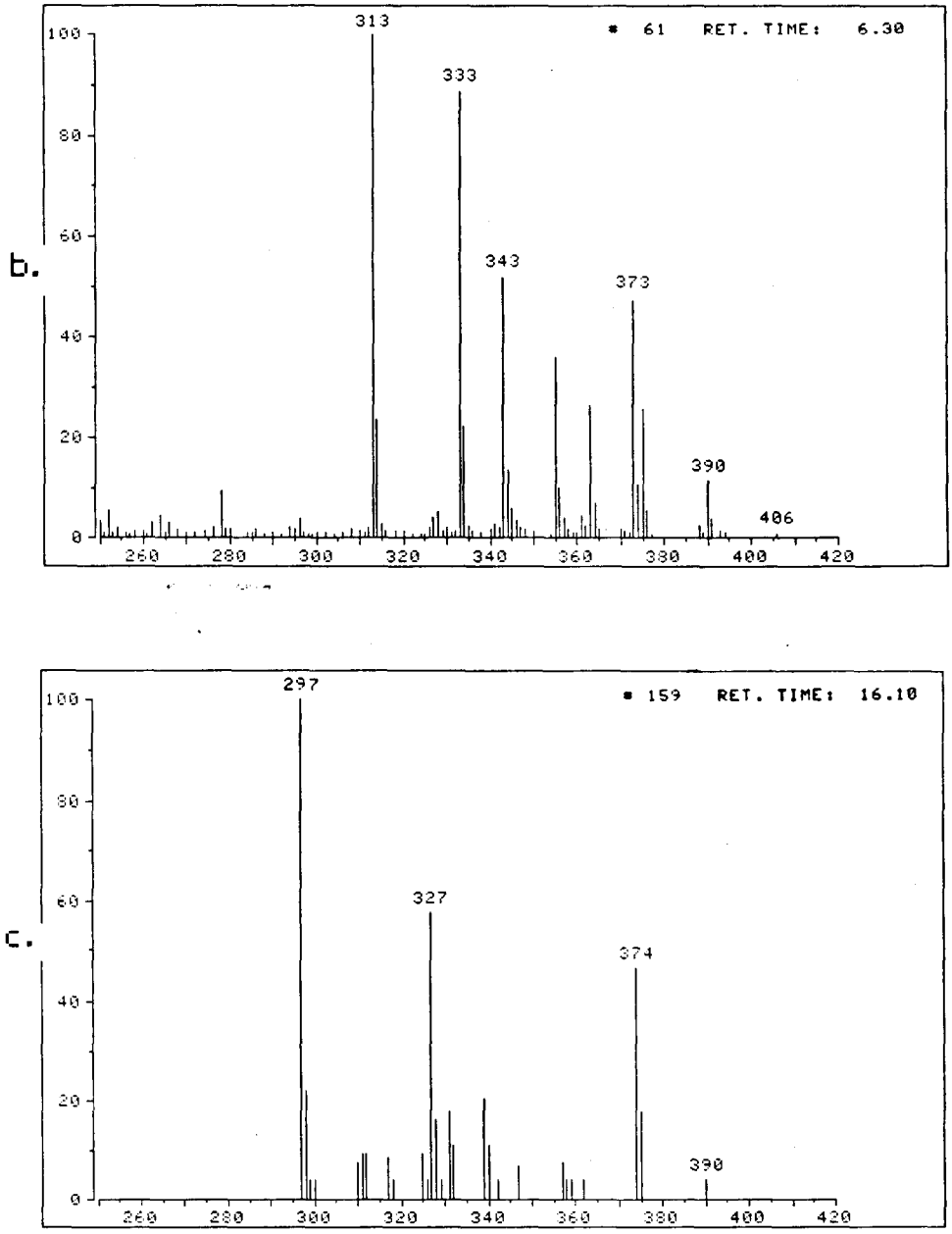


Fig. 9. Negative ion CI μLC–MS analysis of the extract of equine urine collected for 6 h post i.m. administration of β-METH. Injection volume, 0.5 μl; extended DSC; solvent tuning ion ratios as ion Fig. 8, other μLC–MS conditions as in Fig. 7. (a) Negative CI LC–MS total ionization chromatogram; (b) negative CI mass spectrum of 6-OH-β-METH (MW 408), obtained at 6.3 min at the apex of the most intense peak; (c) negative CI mass spectrum of β-METH (MW 392), obtained at 16.1 min.

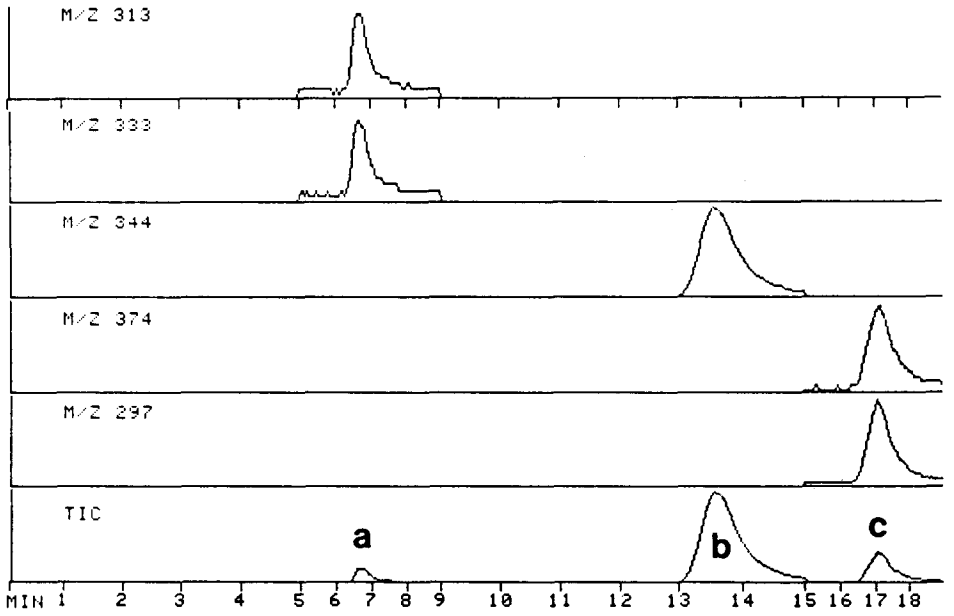


Fig. 10. SIM negative ion CI μLC–MS analysis of the extract of equine plasma collected 1 h post i.m. administration of β-METH. Injection volume, 5 μl; extended DSC; LC conditions as in Fig. 7. TIC = total selected ion current profile. The same dwell-time (100 msec) was used for each selected ion, but the frequency of data acquisition was two times slower in the 5–9-min and 15–19-min periods, where there was a pair of ions. Peaks: a = 6-OH-β-METH; b = hydrocortisone; c = β-METH.

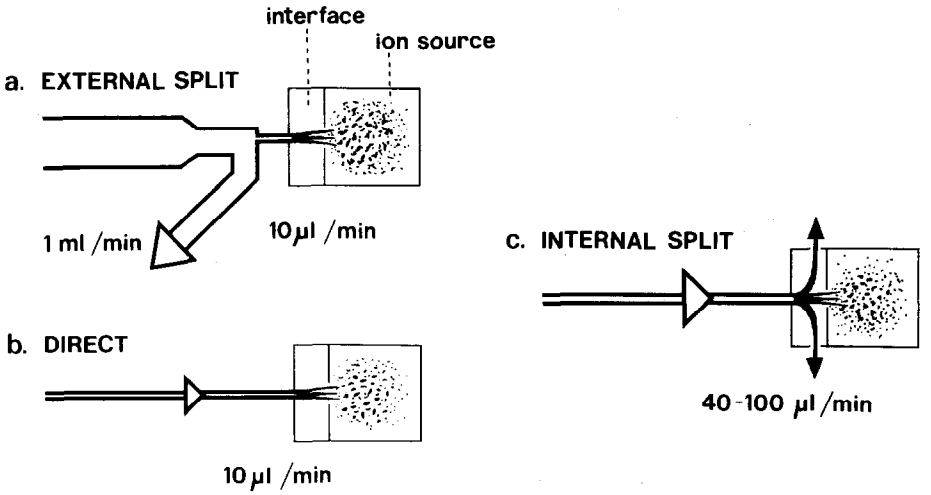


Fig. 11. Schematic diagram of different possible splitting strategies in DLI LC–MS, with typical values of LC flow-rates. (a) Splitting external to the mass spectrometer of a major portion of the LC flow from a conventional 4 mm I.D. LC column; (b) unsplit introduction of the entire flow from a μLC column; (c) introduction of the entire flow from a μLC column (up to 2 mm I.D.) and selective splitting (solute enrichment) internally to the mass spectrometer.

column. By allowing only *ca.* 5% of the eluent into the mass spectrometer through a splitter, DLI LC–MS interfacing could be achieved, but at the cost of a loss in sensitivity (Fig. 11a).

Interfacing μLC columns with a mass spectrometer has closed the gap between the solvent flow-rate which gives optimal LC separations and the flow-rate pumping capacity of the mass spectrometer with the transfer of the complete eluent flow into the spectrometer. This represented a radical change for the chromatographer: instead of having to adapt chromatography to the predominant needs of the mass spectrometer, he could choose the optimal μLC conditions and expect the flexibility from the mass spectrometer within its pumping range. The transport-type LC–MS interface allows one to take advantage of μLC by putting the entire effluent of a μLC column on the belt[17], but the full potential of the DLI-type interface has been limited by the high CI pressure generated by the LC solvent in the ion source. Unfortunately, the commercially available CI ion sources used to date with DLI have a fixed geometry; they generally can accept, without splitting, at the most 10 μl/min of LC solvent. This situation is illustrated in Fig. 11b. The major drawback of 1 mm I.D. microbore columns eluted at 10 μl/min is that elution times are extremely long; in addition their efficiency is hampered by diffusion at this low flow-rate.

An attempt to shorten retention times by using a higher flow-rate combined with splitting external to the mass spectrometer resulted in a decrease in sensitivity[18]. This is in essence similar to the situation shown in Fig. 11a, but with a more favorable splitting ratio.

Another approach for increasing the sensitivity is to use a heated desolvation chamber[3,4]. This device promotes uniform vaporization of the droplets with a range of solvents, although one can avoid the use of this accessory if the diaphragms have a pinhole size which ideally matches the LC solvent and flow-rate[13,18]. In the absence of a splitter, the desolvation chamber transmits the entire LC flow to the source. Subsequently the CI pressure limits the LC flow-rate and the sensitivity is, at best, the maximum that can be obtained from the unsplit direct coupling shown schematically in Fig. 11b.

To allow a higher throughput of solute to enter into the mass spectrometer, the ion-source side of the LC–MS instrument was modified. A variable gas conductance of the ion source was set by retracting the GC interface tip which seals the source; this reduced the extent of clustering by solvent tuning ions but did not increase the sensitivity for solutes. The pressure was also reduced, although a direct measure of the source pressure through the GC interface was not possible under these circumstances. More sophisticated modifications of source conductance would have required redesigning of the source, a task beyond the scope of this research.

The principle of the solution that has been shown in the present paper is illustrated in Fig. 11c. It consists of introducing the entire LC flow into the mass spectrometer (between 10 and 100 μl/min) and then using an interface designed to match the flow with the needs of the CI source. In this way all the adjustments occur within the mass spectrometer. Therefore, the parameters of the interface can be fully documented for the understanding of its operation and for reproducing its optimal conditions.

A practical way to deal with the excess of pressure and solvent reaching the ion source was to equip the interface with an adjustable splitter inside the mass

spectrometer; the standard Hewlett-Packard DSC with a narrow gap in front of the DLI probe (*cf.* Fig. 3a) answered the purpose, but the gap (*ca.* 0.8 mm) was very difficult to adjust reproducibly. The controlled leak of the "extended" DSC, adjustable over 10 mm (*cf.* Fig. 3b), solved this problem.

Another advantage gained with LC flow-rates of 40 μl/min and higher was that they permitted the use of diaphragms with relatively large pinholes, typically 4–6 μm I.D. (larger than the 2–3 μm pinholes used at 10 μl/min). These diaphragms have the advantage of being easier to manufacture in reproducible dimensions and are much less likely to be plugged by deposits.

The above solved two pertiment problems: first, how to have a LC–MS ion source with an adjustable CI pressure and secondly, how to improve the lifetime and the uniformity of the DLI diaphragms. However, the two splitting DLI interfaces described previously removed, with little discrimination, a large part of the solute vapors while little sensitivity was gained as compared with a directly coupled μLC interface.

This problem was solved by adding to the "extended" DSC a pumping slit that selectively removed the more volatile and diffusible vapors from the mist of droplets. This "solvent stripping" version of a DSC (*cf.* Fig. 3c) contributed to transfer of the less volatile droplets, containing the solutes, into the ion source. These have a high momentum and reach preferentially the source while the light, diffusible vapors (mostly solvent molecules) are pumped away by the solvent-stripping annular pumping gap (see Fig. 1). This allowed a higher flow of solute to reach the ion source and increased the sensitivity, but at the same time the solvent vapors were not in such excess so as to cause ion quenching; the water-to-methanol ratio in the solvent cluster ions also increased, showing the greater ease of the "solvent stripping" DSC to remove the more volatile molecules.

The final step in this development was to adjust the variable DSC for the highest sensitivity. In this simple "solvent stripping" DSC version, the area of the gap between the chamber and the ion source could be adjusted only when the DSC was threaded onto the source block, and not during routine high-vacuum operation of the mass spectrometer. Thus, only one control is needed, the distance between the DSC and the tip of the DLI, in order to determine the source pressure which gives the highest sensitivity.

When the LC–MS experiment is entirely new, the optimal temperature and pressure conditions can be determined by repeated injections of a solution of a model compound. As compared with a continuous infusion, this procedure provides optimum tuning because it checks the whole instrument, offers a lower contamination of the system and permits optimization of the sensitivity while maintaining the shape integrity of the chromatographic peaks. Source temperature and pressure are intimately interrelated, as both control the vaporization of the LC eluent. Vacuum alone is insufficient since it would freeze the solvent[2]; therefore, a minimum of heat must be transferred to the droplets.

A universal recipe for the selection of the optimum ion source temperature and pressure could not be devised, but general guidelines with adaptations depending on the thermal stability of the compounds analysed can be given as follows. First, rough values of 150°C and 0.7 Torr in negative CI or 0.3 Torr in positive CI are established. The temperature of the ion source and the DSC is increased to a value at which the

constancy of pressure and total ion current indicate that even vaporization conditions are achieved. Then, the source pressure is adjusted by moving the DLI probe to a position where the intensity of the repetitively injected solute is peaking and a good chromatographic peak shape is maintained. If the mass spectrum of the model compound used for tuning shows an acceptable intensity ratio of molecular ion to fragments, the temperature can be raised to produce less critical vaporization conditions in the DSC; conversely, for labile compounds, the temperature can be decreased to a value at which just enough heat can vaporize the droplets.

The results obtained with the solvent and solute ions have shown that changes of temperature have more dramatic effects than changes of pressure: temperature modifies mostly the ratio of the ions, while pressure has more influence on their overall intensities.

In the particular case of 17-hydroxy corticosteroids and our model compound 6-OH-β-METH, abundant $(M + 1)^+$ ions have been obtained in positive CI, as previously described for some of these compounds[14]. However, when compared with negative CI, positive CI has been less sensitive (see also ref. 10) and has given a higher background from the impurities of the solvent. Negative CI was therefore preferred for high-sensitivity measurements, but it had the drawback of not giving direct molecular weight information. Except for very weak $(M - 2)^-$ ions, 17-OH corticosteroids lose one molecule of water after electron capture to give a more stable $(M - 18)^-$ ion. Further losses of methanol, ketene or hydrogen fluoride are due in part to thermal degradation which could be prevented with the solvent-stripping DSC. Betamethasone, for example, yields a relative intensity of the $(M - H_2O)^-$ ion (m/z 374) of 4% for vaporization from a heated-belt interface[10] and 41% for the solvent-stripping DSC. Another typical thermal fragmentation process, the loss of hydrogen fluoride, could be reduced in 6-OH-β-METH from 300% to 15% of the m/z 333 peak by using the solvent-stripping DSC at a setting close to that which produces the minimum of energy required for the desolvation (see Fig. 4).

Once the optimal pressure and temperature conditions have been established, they must be documented so as to be readily reproducible later either with the same, or a different instrument. The *intensity ratios* of the solvent cluster ions give an accurate description of the CI conditions obtained in the source which is independent of any particular solute. For our methanol–water eluent, we observed that a 1:2 ratio of m/z 63 to m/z 95 ions, along with a 1:1 or slightly higher ratio of m/z 63 to m/z 81 ions, gave optimal CI-LC–MS conditions. Simultaneously, the m/z 95 ion reached an absolute intensity maximum. These conditions could be obtained over a range of pressures with the solvent-stripping DSC at a temperature of 200°C (see Fig. 5b). For the solute, a higher sensitivity was obtained on the high pressure side of this range (*ca.* 0.8 Torr).

In later analyses of biological samples containing corticosteroids using the same HPLC conditions, the original optimal MS detection conditions were readily reproduced by tuning according to the solvent cluster ion ratios only. These ratios also gave between chromatographic runs an accurate diagnostic of the quality and straightness of the jet of droplets. Therefore, it is advised that the LC–MS contributions should include, along with the mass spectra, a plot of solvent cluster ions[19] or, better, a simple table of the intensity of characteristic solvent tuning ions. This would greatly help other researchers to reproduce the original conditions, without

problems occurring with instrument-to-instrument variations in temperature, pressure or rough data[20].

Comparison of our data with those obtained in DLI LC–MS with different solvents and compounds, particularly in positive CI, shows that the most sensitive MS conditions are strongly dependent on the thermal stability of the solutes analyzed. While one group, on the basis of small volatile solutes (*e.g.* benzoic acid), concluded that "optimization... is best achieved by adjusting the DLI probe for the maximum solvent cluster ion intensity"[8], another group showed with penicillins that reducing "the extent of clustering ... yields higher sensitivity at higher ion source temperature"[21]. This illustrates the need for using a relatively high pressure and low temperature for thermally labile molecules, in order to maintain a thin solvent "blanket" around the solute molecules when they enter the source[22]. On the other hand, thermally stable molecules can withstand contact with hot source walls and be entirely vaporized while still giving abundant molecular weight information.

One also observes that the optimal pressures for negative and positive CI are not equal; in negative CI, an efficient electron capture ionization can be reached at 1 Torr pressure of reagent gas[23,24], while 0.05–0.3 Torr seems to be optimal in positive CI[8,20].

CONCLUSIONS

In DLI LC–MS an optimum tuning of the mass spectrometer is critical for reaching high sensitivity, especially for heat sensitive molecules. With a standard DSC, this can be achieved either at the cost of analysis time by using a low μLC flow-rate (10 μl/min) or at the cost of sensitivity and reproducibility by using a splitting gap that is difficult to adjust. The data presented in this paper demonstrate that positioning of the DLI probe relative to the source for maintaining an optimum CI pressure was achieved easier with the extension feature of the new DSCs. This allowed operating μLC–MS from 10 to 100 μl/min without having to split the LC eluent before it enters the mass spectrometer.

Furthermore, the data demonstrate that after removing a part of the vaporized solvent, the new solvent-stripping DSC promotes a more efficient transfer and milder CI ionization conditions for the solutes introduced into the ion source as high momentum droplets. The detection limits were in the picogram range in the full scan mode after a chromatographic separation of corticosteroids, which showed the level of sensitivity that the combined qualities of this new DSC allows.

The data reported here also demonstrate that a complete, accurate and easy control of the LC–MS conditions over broad tuning ranges can be achieved with the new DSC owing to its physical properties. Changes in pressure have a lesser effect on the CI reagent gas composition (*i.e.* the CI source is better "buffered") and another advantage is that one can use DLI diaphragms with pinholes larger than those used at 10 μl/min for pre-mass spectrometer splitting DLI interfaces. Moreover, the simple tuning procedure outlined in this paper demonstrates that although the same solvent has to be used for both LC separation and CI reagent gas, the flexibility of the new DSC permits the adjustment of LC–MS parameters somewhat independently towards maximum sensitivity. After a first round of tuning with repeated injections of a tuning solution, the ability of re-setting the pressure and com-

position of the CI reagent gas according to the intensity *ratios* of low- and high-mass solvent cluster ions (instead of maximizing the intensity of one ion only) was one major factor which made it possible to easily reproduce daily the original analysis conditions.

Stability and ease of tuning the system, combined with a low maintenance (cleaning of diaphragm after 2 days and cleaning of the ion source after 2 months of daily use) demonstrate clearly that DLI μLC–MS can reach the reliability needed for routine use. The analysis data for complex mixtures of biological materials after a crude sample preparation presented in this paper are an indication of what the full analytical power of the DLI μLC–MS technique will provide for high sensitivity detection and structural information on mixtures of unknown compounds.

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