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APPLICATION OF MICROBORE COLUMNS TO LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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1. INTRODUCTION

The major problem encountered in coupled liquid chromatography–mass spectrometry (LC–MS) is caused by the difference that exists between the flow-rate of the mobile phase through the LC column and the rate at which liquid can be continuously introduced into the ion source of a mass spectrometer. With conventional columns (4 mm I.D.) the flow-rate is *ca.* 1 ml/min, whereas only *ca.* 10 μl/min can be accepted into the mass spectrometer. This means that, when using the direct liquid inlet (DLI) technique developed by McLafferty and co-workers[1,2], only about 1 % of the solute injected onto the column can be introduced into the mass spectrometer. The consequence is that the system has relatively poor sensitivity, the detection threshold being in the nanogram range.

This is not a real problem if the total amount of sample available is not limited. A 4-mm I.D. column can easily support 10–100 μg of sample and thus there will be a reasonably useful dynamic range. When the sample is limited (*e.g.* blood sample, insect extract) it is absolutely necessary to achieve a better efficiency. The use of microbore-packed columns (1 mm I.D.), which have been recently developed[3–6] and are soon to become commercially available, can be considered as an attractive and efficient solution to the problem. At the limit, with such columns, all the mobile phase can be continuously injected into the ion source. Thus the sensitivity can be increased by a factor as high as 100. This is a consequence of the low flow-rate in microbore columns (between 5 and 50 μl/min) and explains the many methods recently reported for coupled high-performance micro liquid chromatography–mass spectrometry (HPMLC–MS)[7–11].

In order to use microbore columns in LC–MS coupling, we have developed a new interface based upon the DLI concept using a small pinhole as nebulizer and with

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a splitter. The splitter enables the sensitivity of the system to be increased and improves either the efficiency of the system or the speed of analysis. The uses and limits of the technique are discussed.

2. EXPERIMENTAL

2.1. *Chromatographic system*

For our experiments, we have modified a Waters 6000A pump to provide flow rates in the range 1–100 μl/min. Two modifications were tested. The first consisted in using a splitter at the outlet end of the pump, as shown in Fig. 1. The main portion of the liquid flow returns to the mobile-phase reservoir. The flow-rate in the columns is adjusted using a micro-needle valve and measured with a syringe, without a plunger, connected to the column through 1/16-in. PTFE tubing. The inconvenience of such a system is due to variations in the splitting ratio with time: the reproducibility of the experiments is not good enough for either quantitative or qualitative purposes. This phenomenon can be explained by the diameter of the small pinhole at the outlet end of the probe becoming modified as the experiment proceeds by fixation of small particles or by deposition of silica[12].

To obtain better reproducibility, we have tested another system similar to the solution described by Scott and Kucera[5], *i.e.* the pump is modified electronically. The flow-rate of the 6000A pump is controlled by the frequency of an oscillator in the speed-control circuit of the motor, the frequency of the sawtooth wave being determined by a resistance–capacitance system. To obtain a flow-rate in the range 1–99 μl/min, instead of 0.1–9.9 ml/min, we have multiplied the value of the capacitance by a factor of 100 using an external capacitance of *ca.* 1.5 μF connected to the internal capacitance as shown in Fig. 2. Such a modification is simple and inexpensive. The flow-rate delivered by the pump is fairly stable even in the microlitre-per-minute range as will be shown later.

The mobile phase from the pump passes directly to the injection valve (a Valco valve with an 0.5-μl internal sample loop or a Rheodyne 7525 valve with a 10-μl external sample loop). The microbore column projects directly into the valve housing

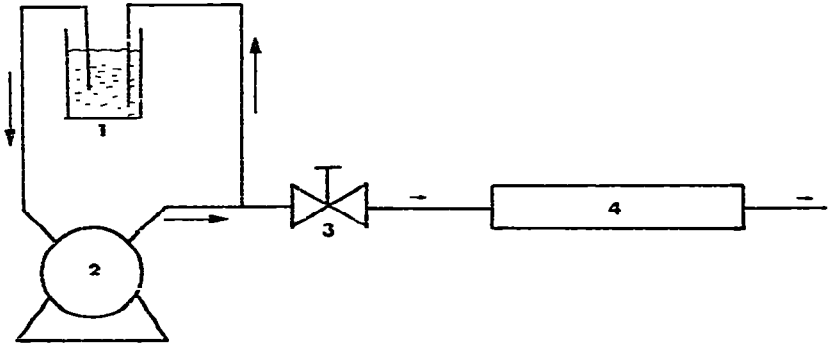

Fig. 1. Modification of the solvent-delivery system to obtain a flow-rate between 10 and 100 μl min in the microbore column. 1 = Solvent reservoir; 2 = HPLC pump; 3 = micro-needle valve; 4 = microbore column (1 mm I.D.).

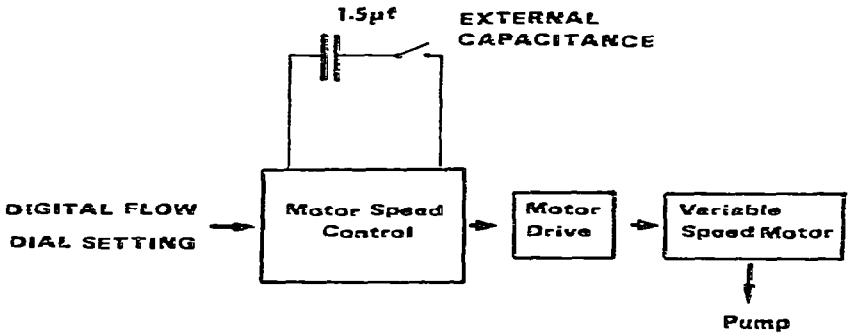

Fig. 2. Electronic modification of the HPLC pump in order to provide flow-rates in the range 1–100 μl min.

to eliminate any dead volume between the sample valve and the column in order to optimize the efficiency of the system[13], an arrangement similar to that devised by Scott and Kucera[5].

The columns are commercially available microbore-packed columns (Chrompack, Middelburg, The Netherlands), 30–50 cm × 1 mm I.D. The size of the packing material particles was 10 μm even for silica or reversed-phase materials. To increase the lifetime of the diaphragm a stainless steel frit of 0.5-μm porosity (instead of the classical 2-μm porosity filter) was inserted into the outlet end of the column.

2.2. *LC–MS interface*

For preliminary experiments we used an interface with no splitter so that all the mobile phase flowing through the column entered the ion source. The interface consisted in this case of a small diaphragm fitted against the frit at the outlet end of the column as shown in Fig. 3. The role of this diaphragm was to inject the liquid jet into the ion source as a fine spray. With such a geometry, any dead volume between the column and the detector was avoided, to optimize peak shape and the efficiency of the chromatographic system. However, with such a simple interface the analysis time is long, as will be shown later. This is the reason why we designed another DLI

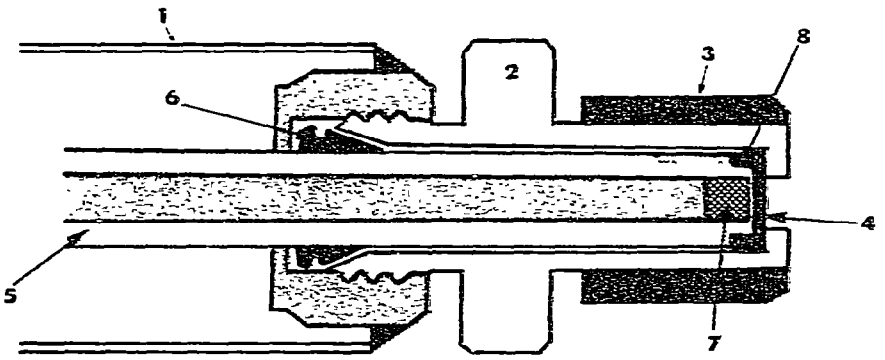

Fig. 3. Schematic drawing of the LC–MS probe used for direct coupling. 1 = LC–MS probe; 2 = probe head; 3 = insulator; 4 = diaphragm; 5 = microbore-packed column; 6 = Vespel ferrule; 7 = 0.5-μm porosity filter; 8 = PTFE seal.

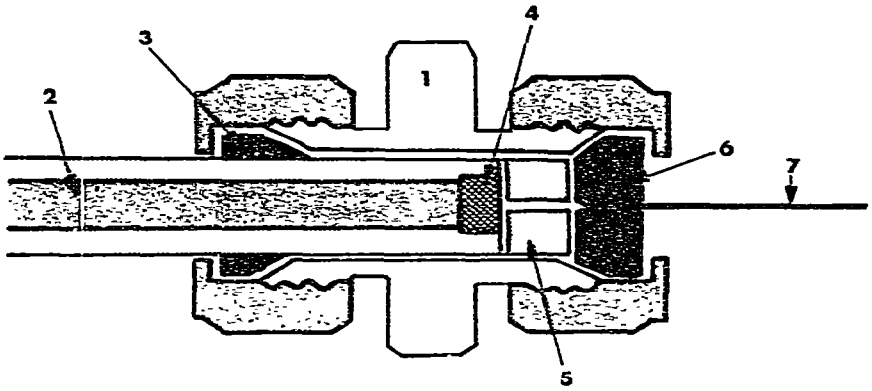

Fig. 4. Schematic drawing of the connection between the microbore column and the capillary tubing. 1 = Modified Swagelock union (1/16 in.); 2 = microbore-packed column (1/16 in. O.D., 1 mm I.D.); 3 = Vespel ferrule; 4 = 0.5-μm porosity filter; 5 = PTFE tubing (1/16 in. O.D., 0.2 mm I.D., length 5 mm); 6 = 1/16 in. Vespel ferrule; 7 = capillary tubing (0.3 mm O.D., 50 μm I.D.).

interface similar to that previously described[14]. The input tubing was modified: instead of 0.1 mm I.D. stainless-steel tubing, we used vitreous silica tubing (50 cm × 50 μm I.D.), in order to maintain a turbulent flow profile and to minimize peak tailing. The connection between the column and the silica capillary was optimized to eliminate any dead volume and is shown in Fig. 4.

At low flow-rates, *i.e.* below 80 μl/min, the presence of the capillary tubing does not significantly increase the pressure at the pump. However, with conventional columns and a liquid flow-rate of 1 ml/min, such tubing is unusable: the pressure drop along the tubing can be higher than the working limit of the pump pressure.

Analyses were performed on a Ribermag R 10 10 mass spectrometer coupled with a Sidar data system.

3 RESULTS

For the preliminary experiments we used the direct DLI interface. Typical results are shown in Fig. 5. An analysis of anthracene was performed on a 50-cm microbore column packed with Partisil 10. *n*-Hexane was used as mobile phase with a flow-rate of 10 μl/min; 0.5 μl of solution was injected through the Valco valve.

The chromatographic efficiency of the system was calculated using the band width at half height and the equation

$$N = 5.54\,(t_R/W_{1/2})^2$$

where t_R is the retention time of the peak at the apex and $W_{1/2}$ is the peak width at half height. In the case of anthracene, $k' = 0.8$, N was 16500 theoretical plates, *i.e.* a plate height, H, of 30 μm. The peak asymmetry factor, calculated using the classical approach[13], was 1.25. The elution time was very long (55 min) as can be seen from the chromatogram. Thus a compound with a k' value of 5 would be eluted after *ca.* 4 h.

Such a system is efficient but is not useful for general purposes. Therefore we have used, for the ensuing studies, a DLI interface with a splitter to decrease the elution time.

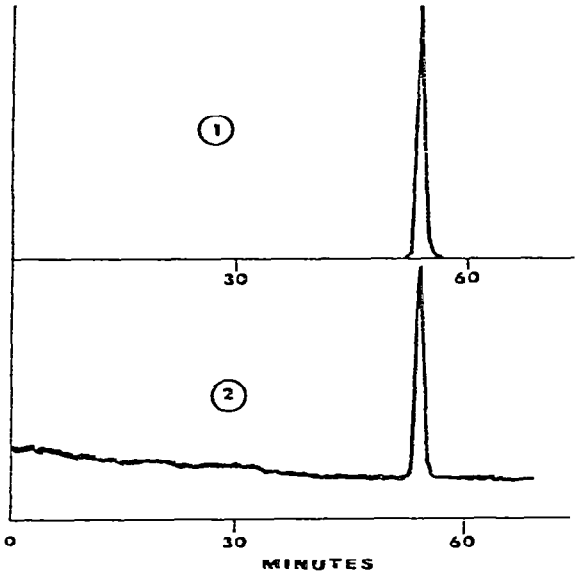

Fig. 5. Chromatogram of anthracene obtained with a microbore column (50 cm × 1 mm I.D.), packed with Partisil 10. Injected volume: 0.5 μl, mobile phase: hexane. Flow-rate: 10 μl/min. 1 = Reconstructed chromatogram on pseudo-molecular ion, m/e = 179; 2 = total ion current. Mass range: 150–300 a.m.u.

We first tested the second interface with a 30-cm reversed-phase column, using benzophenone (m/e = 182) as a test compound. Such a compound is readily detectable even by gas chromatography (GC)–MS, but our purpose was not the detection of non-volatile and/or thermolabile molecules. The mass of sample was no more than 500 ng (*i.e.* 0.5 μl injected) to avoid overloading on the column[5]. The mobile-phase velocity varied from 0.4 to 2 mm/sec (flow-rate in the range 10–50 μl/min). The mobile phase was acetonitrile.

The complete system was considered as optimized when the N values increase with a decreasing flow-rate (see Fig. 6). We observed that peak shape depends mainly on the position of the inlet end of the capillary tubing at the connection level (see Fig. 4). Optimal peak shape was obtained when the inlet end of the tubing was inserted in the Vespel ferrule. It is probable that, in such a configuration, the flow velocity profile in the Teflon tubing region is optimized.

With a flow-rate of 50 μl/min, N was equal to 7600; *i.e.* a plate height of *ca.* 40 μm for the reversed-phase column, and 35 μm for the silica-packed column. Higher values for N are obtainable with lower flow-rates. Unfortunately, it is not possible to have speed and resolution at the same time. This is the reason why to perform analysis under acceptable conditions, we have defined a standard flow-rate equal to 50 μl/min with which an unretained compound is eluted in less than 3 min on a 30-cm column. Under such conditions, the analysis time is about the same as with a conventional 25-cm column with a flow-rate of 1 ml/min.

We have used this second system for the study of juvenile hormones (JHs). Although JHs can be detected using a GC–MS system[15,16], the interest in using LC–

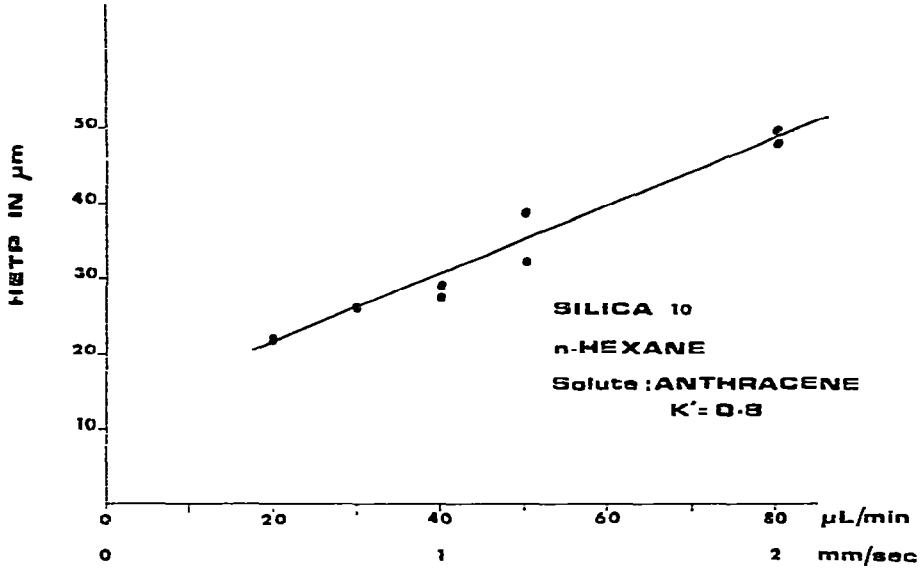

Fig. 6. Plot of HETP against velocity of mobile-phase and flow-rate.

MS is due to a simplification of the prepurification procedures: in the present case, purification by HPLC, which was time consuming, was replaced by purification on a C18 Sep Pack cartridge (Waters Assoc.)[17]. Thus using LC–MS instead of GC–MS permits a reduction in the time required for the analysis.

The first step was determination of the chromatographic conditions. JHs can be easily separated even by normal or reversed-phase chromatography. The former method requires non-polar solvents such as hexane–diethyl ether mixtures. With the latter method, JHs are eluted with methanol–water or acetonitrile–water mixtures.

From the DLI principle, solvent vapours are used for the chemical ionization of the solute. This means that chemical-ionization mass spectra can be significantly modified by changing the nature of the solvent[14]. A typical example is shown in Fig. 7 when using JH_1 as a test compound. In the second case, ammonia was added to the hexane vapours in the ion source through the GC–MS interface. In the last case, acetonitrile was used as mobile phase and reactant gas. The results clearly indicate that polar solvents are better reactant gases for affording higher intensities of the pseudo molecular ion, MH^+. Such an observation is quite normal when one considers that polar solvents have a higher proton affinity than non-polar solvents[18].

From an energetic point of view, proton transfer reaction between solvent and solute molecules can only occur when the proton affinity of the solute, PA_s, is higher than that of the solvent, PA_S. At the limit, when $PA_s \gg PA_S$, the internal energy of the protonated solute molecule, after the proton transfer, can be high enough to produce fragmentation. Thus, we may presume that the best chemical-ionization mass spectra will be obtained when the difference between PA_s and PA_S is as small as possible. This is the reason why detection of mixture of JHs was performed on the microbore reversed-phase column using acetonitrile–water (75:25) as mobile phase. The total ion chromatogram of the four JHs is shown in Fig. 8. This corresponds to an injection

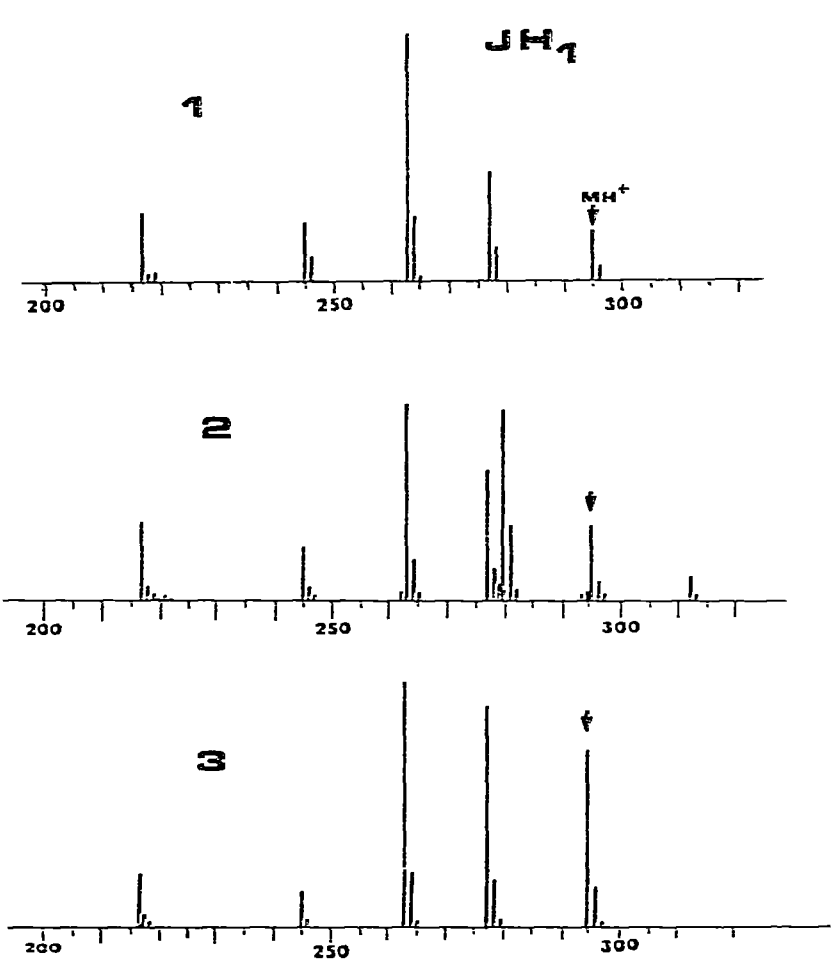

Fig. 7. Influence of the mobile phase on the chemical-ionization mass spectra of JH_1 (m/e = 294). Peaks: 1 = hexane; 2 = hexane plus ammonia which was injected directly into the ion source through the GC–MS interface; 3 = acetonitrile.

of 50 ng for JH_3 and JH_2 and of 25 ng for JH_1 and JH_0 (*i.e.* 10 ng and 5 ng, respectively, injected into the ion source).

To increase the sensitivity of the system for the detection of JHs in biological samples, we have used the multiple selected-ion-monitoring detection mode. Since chemical ionization mass spectra were reduced to three or four ions[17], selection of two ions gave good specificity. Thus, with only four ions selected, detection and identification of JHs was possible even at low concentration levels as indicated in Fig. 9. The results show that with standard mixtures and selected-ion monitoring 10 pg of each hormone can be detected. The limit of sensitivity appears to be due to background noise corresponding to the solvent vapour ions.

The stability of the flow-rate was controlled during these analyses. The good reproducibility for different retention times (see Table I) indicates that the modified 6000 A pump delivers solvent with a fairly stable flow-rate.

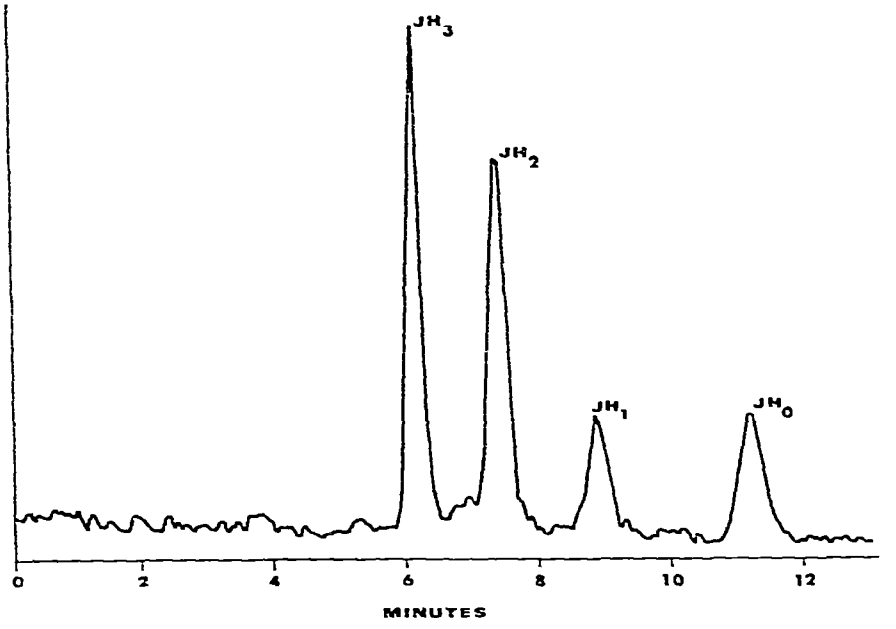

Fig. 8. Analysis of the four JHs separated on the reversed-phase column. Mobile phase: acetonitrile–water mixture (75:25). Flow-rate: 50 μl min.

Fig. 10 shows the analysis of a biological sample (extract of *Pieris brassicae* L. haemolymph from larvae) under the conditions described previously. In this sample, only JH_1 was detected.

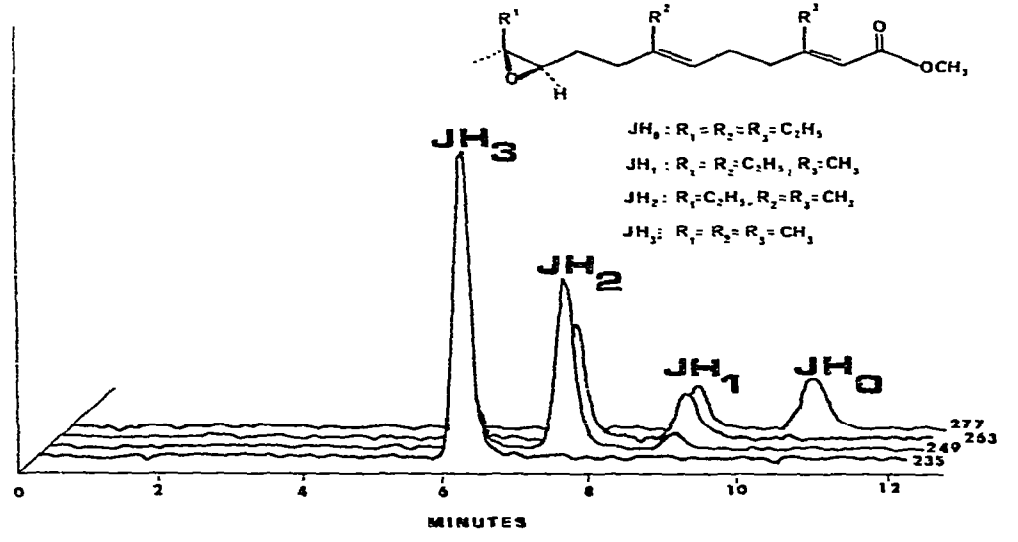

Fig. 9. Multiple selected-ion-monitoring detection of the four JHs. Same conditions as for Fig. 8. JH_0, JH_1: 200 pg injected. JH_2, JH_3: 400 pg injected.

TABLE I

STABILITY OF THE SOLVENT DELIVERY SYSTEM

Test compound: mixture of juvenile hormones.

Compound	*Retention times (min:sec)*				*Mean value*	*Standard deviation (min)*
	Run No. 1	*Run. No. 2*	*Run No. 3*	*Run No. 4*		
JH_3	6.15	6:14	6:14	6:19	6:16	0 02
JH_2	7:30	7·37	7:32	7 37	7·34	0.04
JH_1	9:10	9.14	9 04	9:09	9.09	0.04
JH_0	11·20	11:35	11:25	11:35	11·29	0 07

4. DISCUSSION

As we have demonstrated in this paper, the adaptation of an LC–MS interface to microbore-packed columns is simple and problems due to the elimination of dead volumes can be easily overcome. The use of a splitter at the interface permits monitoring of the analysis time. As an example, with a column length of 30 cm and a flow-rate of *ca.* 50 μl/min, the elution time of an unretained compound is *ca.* 3 min: an acceptable value. If very high efficiency or sensitivity is required, it is possible to choose a lower flow-rate. In this case, the splitting ratio can be close to 1. Considering that with 1-mm I.D. columns a high linear mobile-phase velocity can be realized, then at relatively low solvent flow-rates, high speed separations can be obtained[6]. In these cases, the maximum flow-rate is directly related to the maximum pressure of the HPLC pump and to the evaporation of non-volatile molecules in the ion source[11].

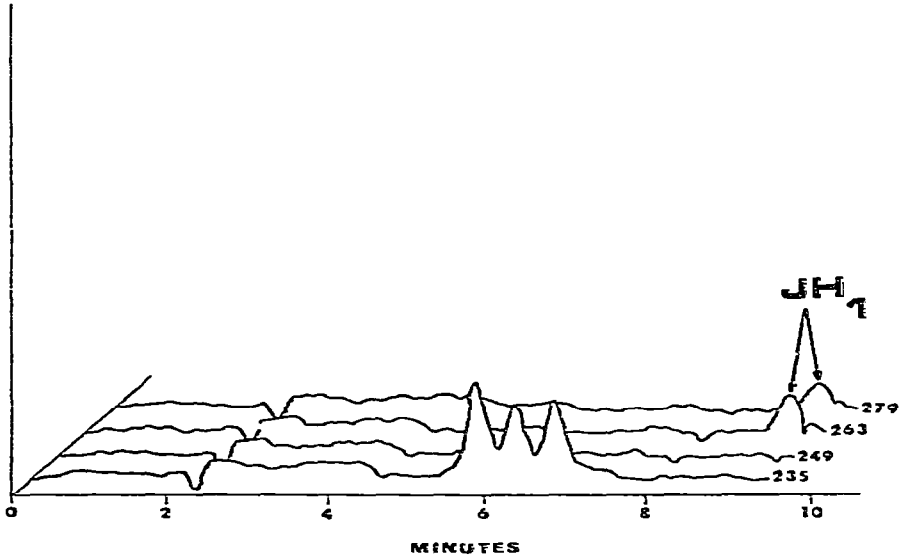

Fig. 10. Multiple selected-ion-monitoring of an extract of *Pieris brassicae* L. Haemolymph from larvae. Only JH_1 was detected in this sample.

The problems we encountered were essentially due to the microbore column itself. First, we observed that these columns have a short lifetime: the number of theoretical plates can decrease dramatically in one month. The cause of such a short lifetime is not well understood. However, we can presume that the phenomenon can be due partially to the fragility of the column. The thickness of the column walls is fairly low, *ca.* 0.3 mm. Consequently, it is easy to bend a column. Thus a column bed disturbance can occur. To avoid this, the simplest solution would be to increase the thickness of the walls to give a greater rigidity: a 4-mm O.D. tubing could be used instead of the classical 1/16-in. O.D. tubing[19]. The second problem is due to the time required to obtain convenient special columns from the manufacturers. This especially applies to the need for 0.5-μm, or better 0.2-μm, porosity frits.

However, it must be remembered that microbore columns are still under development. Thus we may presume that the problems observed will be solved in the near future.

5. CONCLUSION

The use of microbore columns for LC–MS coupling is an attractive and efficient way of increasing the sensitivity of the system in terms of quantities injected. The simplest interface we have studied, which consists in a direct coupling between the column and the mass spectrometer, permits optimization of the sensitivity of the apparatus but, with such a system, analyses are time-consuming or would need 0.5-mm I.D. columns which are not available. To avoid such an inconvenience, we have developed a new interface, still based upon the DLI concept but with a diaphragm and splitter. The advantage of the splitter is that flow-rates may be varied over a large range. Thus, it is possible also to increase either the sensitivity of the system or the speed of elution. Finally this technique permits the detection of trace-level components with analysis times about the same as with conventional columns.

6. ACKNOWLEDGEMENTS

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7. SUMMARY

The advantages of microbore columns are seen when the amount of sample is limited (biological sample) and/or when a short analysis time is required. The advantages and drawbacks of microbore-packed columns for high-performance liquid chromatography–mass spectrometry are presented and discussed. To decrease the analysis time a new interface based upon the direct liquid inlet principle with a splitter and a diaphragm has been developed. The advantage of such an interface is the possibility of modifying the analytical conditions: depending on the problem to be solved, either the sensitivity or speed of elution can be increased. Thus the problem of long elution times is solved while sensitivity remains high enough for the detection of compounds at low concentrations. Selected ion monitoring provides a 10 pg detection limit for juvenile hormones injected into the mass spectrometer.

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