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LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES ON RIFAMYCIN ANTIBIOTICS

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SUMMARY

The use of a direct liquid introduction type liquid chromatographic–mass spectrometric interface to study highly thermally labile rifamycin antibiotics is described. Using negative ionization, abundant molecular ions were observed, and the spectra also contained structurally significant fragments. Variation of the high-performance liquid chromatographic parameters did not change the spectra, thus making it easy to change chromatographic conditions. In quantitative studies, a surprising correlation was found, indicating that the mass spectrometric signal was proportional to the square of the sample concentration.

INTRODUCTION

Rifamycins are a well known family of antibiotics obtained by fermentation and successive chemical modification. The most widely used member of this group is rifampicin; there are also some important new derivatives, such as rifapentine (Fig. 1). Their activity against both Gram-positive and Gram-negative bacteria, and in particular *Mycobacterium tuberculosis*, makes some of these antibiotics widely employed in therapy. Recently, interesting activity has been observed against *Mycobacterium avium* complex, the causative agent of an infection common in patients with autoimmune deficiency syndrome (AIDS).

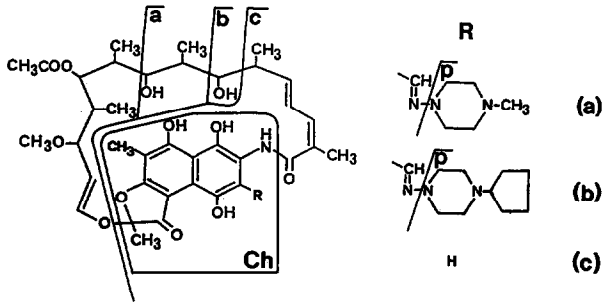

Fig. 1. Structure and main fragmentations of (a) rifampicin, (b) rifapentine and (c) rifamycin SV. a, b, c, p and Ch indicate main fragmentation processes (see also Figs. 2, 3 and 5).

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Mass spectrometric studies of these antibiotics are hindered by their thermal lability. Electron impact (EI) using direct inlet[1–5] gave useful results but also indicated serious limitations. Whereas certain rifamycins give interpretable EI spectra on some mass spectrometers, there are many cases where extensive thermal decomposition occurs, resulting in spectra which may not only lack the molecular ion, but which also depend strongly on the instrument used. Structurally significant ions may be absent, and in some cases the spectra show peaks at every mass unit, with rapidly decreasing abundance towards higher masses. Our preliminary studies indicate that the same is true for chemical ionization using direct inlet. Rifamycins were also studied by other ionization methods. Field desorption[5,6] gives the molecular weight but little structural information. Laser desorption[7] and fast atom bombardment[8] have recently been used, giving promising results.

In our laboratory, a direct liquid introduction (DLI) type liquid chromatography–mass spectrometric (LC–MS) connection is used, and has proved very useful for studying thermally labile compounds, even if chromatographic separation is not required[9,10]. Contrary to some comments in the literature[11] this method is suitable for the study of non-volatile compounds, as also illustrated by this report. In DLI (for reviews see refs. 9, 12 and 13), liquid flows at 10–50 μl/min through a *ca.* 5 μm diameter orifice diaphragm, forming a liquid jet. The jet very soon breaks up into small droplets, from which the solvent evaporates. This process requires heating: not to warm up the liquid, but to stop it from freezing due to fast evaporation. The only part of the instrument where thermal decomposition is likely is the ion source. Even here the sample molecules normally should not make contact with the walls of the source, only with the reagent gas (in low vacuum). This provides a less efficient heat transfer, and only for a few milliseconds. However, some of the sample molecules may (temporarily) be adsorbed on the walls, especially if the jet is bent, and this may lead to thermal decomposition[14]. It is also worth mentioning that solutes of low volatility are probably not evaporated in this process, but evaporation of volatile solvents from the droplets may leave them in the gas phase.

In the present work, MS characteristics and optimization of experimental conditions to study rifamycins are discussed. The main purpose is partly to use DLI as a sample introduction method for these thermally labile compounds and partly to establish LC–MS conditions to analyse various derivatives, metabolites and impurities. The study focuses principally on the behaviour of rifapentine. Our experience with rifampicin, rifamycin SV (Fig. 1) and various other derivatives indicates that these results can be generalized for most rifamycins.

EXPERIMENTAL

Samples

Rifapentine, rifampicin and rifamycin SV Lepetit analytical reference substances were used. Unless otherwise indicated, 50 μg of sample dissolved in 10 μl of methanol was injected on to the HPLC column. As rifamycins slowly decompose in solution, fresh stock solutions were prepared each day.

Liquid chromatographic–mass spectrometric system

An HP-1090 HPLC system was connected to an HP-5985B quadrupole mass

spectrometer system of 1000 dalton mass range using a direct liquid introduction interface (5 μm orifice water-cooled stainless-steel diaphragm). Unless otherwise stated, methanol was used as the mobile phase at 1 ml min^{-1} flow-rate with a Hewlett-Packard Hypersil C_{18} column (100 × 4.6 mm) with the addition of a precolumn. The eluate from the column passed through a Perkin Elmer LC-15 UV detector (254 nm) and a 5-μm filter frit before entering the mass spectrometer. The mass spectrometer was used in chemical ionization mode; the ion source pressure was *ca.* 1 Torr (measured in the otherwise unused gas chromatographic transfer line of the HP-5985B system) and the ion source temperature was 250°C. The LC eluent was used as reagent gas.

RESULTS AND DISCUSSION

Mass spectral characteristics

Positive chemical ionization of rifamycins using DLI gives protonated molecular ions and some structurally characteristic fragments (Fig. 2). The main fragment ions are analogous to those described previously[1,2] but, as the molecular ion is clearly identifiable, this method is a significant improvement over direct inlet methods. However, sensitivity is poor, and due to a high background, fragment ions are difficult to identify below *m/z* 300.

Negative ionization with DLI (Fig. 3) also gives abundant molecular ions together with some fragments. The sensitivity is 100–1000 times better than in positive ionization, background ions are smaller, so lower mass fragments can also be studied. For these reasons, negative ionization proved more suitable than positive ionization for the analysis of rifamycins, and only the former has been studied in detail.

In negative ionization, $M^{-\cdot}$ is formed with high abundance, indicating an electron attachment process and a correspondingly high electron affinity for rifamycins. This also suggests that the eluent acts as a moderator gas. It is interesting to note that $[M-H]^-$ type ions, which are more usually encountered in negative chemical

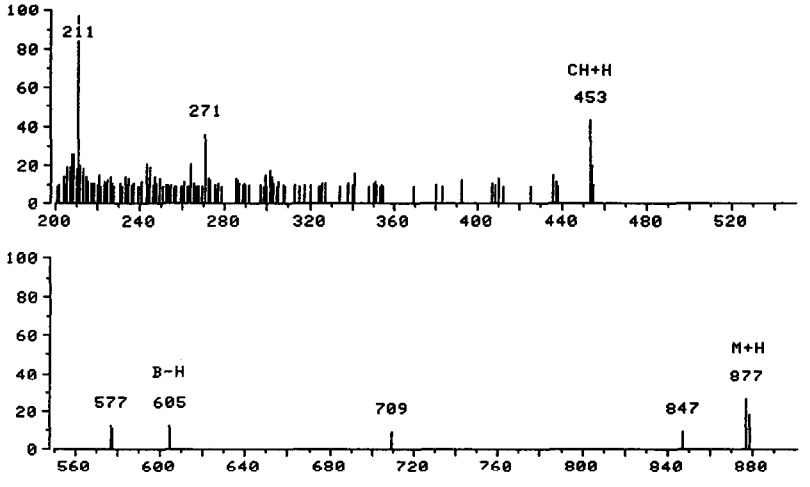

Fig. 2. Positive ion DLI spectrum of rifapentine.

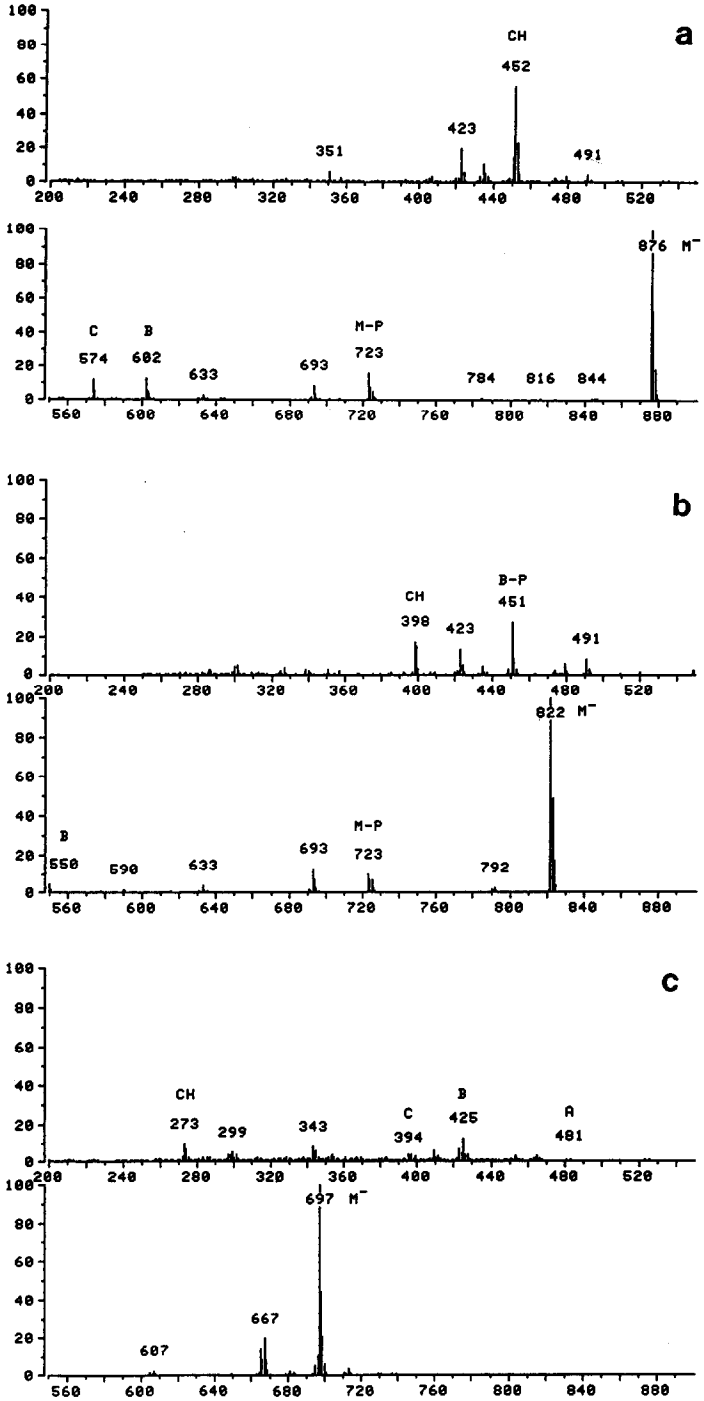

Fig. 3. Typical negative ion DLI spectra of (a) rifapentine, (b) rifampicin and (c) rifamycin SV obtained at the top of the molecular ion chromatograms.

ionization, are absent in the spectra of rifamycins. Recently, use of chlorine-containing additives in the mobile phase was suggested to increase sensitivity in negative ionization DLI[15]. In the case of rifamycins it has an opposite effect: even traces of chlorine-containing solvents reduce the abundance of rifamycin-related ions by several orders of magnitude, probably by quenching the supply of low velocity electrons necessary for electron attachment. This finding suggests that halogen-containing additives should generally be avoided if negative ionization occurs by electron attachment.

In the spectra of rifamycins (Fig. 3) there are a number of significant fragment ions. One of the most abundant is the so-called "chromophoric" ion[1,2] (denoted Ch in Fig. 1, *m/z* 452 for rifapentine, *m/z* 398 for rifampicin, *m/z* 273 for rifamycin SV). There are small but significant fragments characteristic of a, b and c type cleavages first noticed in electron impact studies[1,2] (Fig. 1); these give information about the "ansa" part of the molecule (the saturated part of the macrocycle). There is fragmentation at the piperazino ring in rifampicin and rifapentine (p in Fig. 1), which has not been reported previously, and there are losses of small molecules such as CH_2O, CH_3OH and CH_3COOH from various ions listed above. Fragmentations such as these can be helpful in determination of the structure of synthetic modifications of known rifamycins. There is also an abundant ion at *m/z* 150, not previously observed, present in the spectra of all derivatives studied. This might originate from the ansa chain, though we could not locate which part. As the abundance of *m/z* 150 is very variable, partly due to thermal decomposition, it was decided to show spectra above *m/z* 200 in Figs. 2, 3 and 5.

Thermal decomposition and reproducibility

There are several features in the behaviour of rifamycins suggesting that thermal decomposition occurs to some degree under DLI conditions. In the case of rifapentine, for example, the chromatographic response obtained at the UV detector comprised

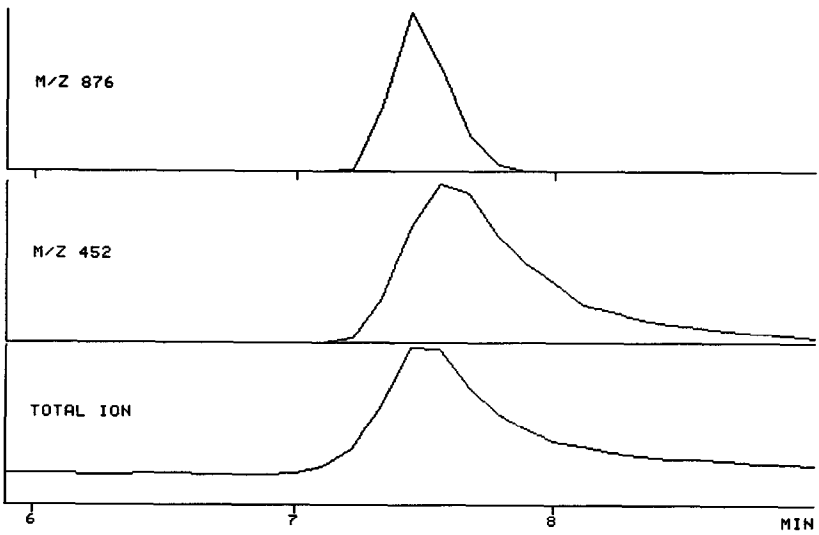

Fig. 4. Ion chromatograms of rifapentine in negative ion DLI LC–MS.

a symmetrical peak *ca.* 14 s wide at half height. The ion chromatogram of the negative molecular ion was likewise fairly symmetrical (Fig. 4) with a similar half-width. The total ion chromatogram and ion chromatograms of several fragment ions (*e.g.*, *m/z* 452), on the other hand, were about twice as wide (Fig. 4), showing significant tailing. As the residence time of gases in the source is in the order of 0.01–0.1 s, this can be explained easily only by an adsorption/desorption process. Such a process could occur either on the hot walls of the source or, perhaps, on the diaphragm, desorption occurring with thermal decomposition. Using the different time behaviour of the various peaks, it is possible to deconvolute them to obtain spectra for two main

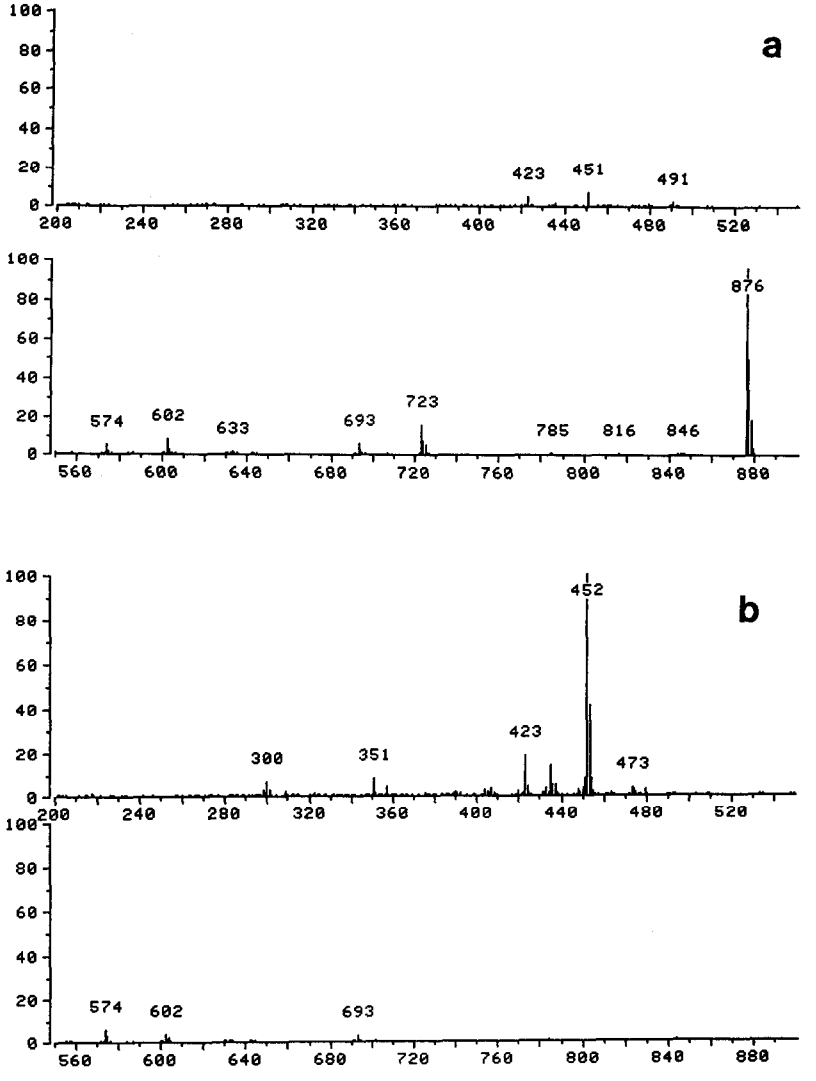

Fig. 5. Deconvoluted negative ion DLI spectra of rifapentine pure electron attachment spectrum of (a) rifapentine; (b) spectrum of the main thermal decomposition product.

components, as shown in Fig. 5. It is worth noting that the ion at *m/z* 452, probably the structurally most useful ion indicating the "chromophore" of rifamycins[1,2], seems to arise from thermal decomposition. This ion is absent in the deconvoluted "pure electron capture" spectrum of rifapentine (Fig. 5a), while there is a small but significant ion at *m/z* 451. This might be interpreted as thermal decomposition to produce a molecule which in turn gives an odd electron ion, while fragmentation following electron capture might produce an even electron ion of similar structure.

Temperature dependence of the spectra has been studied between 150 and 300°C. As might be expected, the abundance of most fragments relative to the molecular ion is higher at higher source temperatures. While this increase is moderate for most fragments, the abundance of *m/z* 452 increases about 100–1000 fold. This is in accordance with its formation by thermal decomposition. As the diaphragm is water cooled (and therefore its temperature is constant), this large variation suggests that thermal decomposition occurs on the walls of the ion source. This is further supported by the observation that, if the jet through the diaphragm is bent, so that the liquid may hit the walls of the source before complete evaporation, the intensity of the molecular ion decreases dramatically, while that of *m/z* 452 does not change significantly.

Optimum ion source temperature depends on various factors, such as contamination in the ion source and type of information sought. In general, lower source temperatures are preferred if detection of the molecular ion is desired, whereas at higher temperatures fragment ions have higher abundance. However, below about 200°C, buildup of contaminants in the ion source and incomplete evaporation of the droplets produced by the liquid jet result in low sensitivity and unstable operation of the instrument.

Because many parameters affect thermal decomposition processes, care should be taken not to change instrumental conditions during a set of experiments. When spectra taken at the top of a chromatographic peak are compared, the short term reproducibility (within a few hours or a day) is good, about 10–20% (relative). It is not significantly worse than long term or interlaboratory reproducibility of mass spectra in general, when no special care is taken. Long term reproducibility (within a few months, during which period the ion source is cleaned) is much worse. Ions which are supposed to be formed following ionization (*e.g.*, those at *m/z* 723 and 693), and which are seen in the "deconvoluted" spectrum of rifapentine in Fig. 5a, could be reproduced well (20%). The relative abundance of the ion at *m/z* 452, which is thought to be formed by a thermal decomposition process, varies widely (by a factor of 100) even if the source is kept at constant temperature. The abundance of other peaks, which could be formed partly by thermal decomposition and partly following ionization (most other peaks including b and c) vary usually within a factor of two or three. When the spectra were studied throughout the elution time, they could be deconvoluted as shown in Fig. 5. In contrast to spectra taken at the top of the chromatographic peak, the long term reproducibility of these "deconvoluted" spectra is good, *ca.* 20–30%.

Apart from the source temperature, the most important parameters affecting thermal decomposition seem to be the condition (probably the catalytic effect) of the ion source and the quality of the liquid jet. A clean ion source with a straight liquid jet gives practically no thermal decomposition, and the spectrum obtained at the top of the chromatographic peak is practically the same as the "deconvoluted" spectrum shown in Fig. 5a. On the other hand, spectra obtained with a very dirty ion source may

be dominated by decomposition products, even with a straight jet. The relative abundance of the molecular ion may drop below 1% if a dirty source is used with a bent jet.

Quantitative studies

To extend LC–MS analysis to quantitative or semi-quantitative determination of rifamycin derivatives, the relationship between the sample amount injected and the MS signal was studied. Surprisingly, instead of a linear response, a quadratic relationship was observed. This is shown in Fig. 6 using a double logarithmic scale. The range of sample amount extended from about 2 to 100 nmol of rifapentine injected, and the signals at the maximum of the ion chromatograms were recorded. The low end of this range corresponded to the appearance of rifapentine-related signals (using full scans), while at high levels the HPLC peak (as observed on the UV trace) started to broaden significantly. As we are not aware of previous observation of a quadratic response in LC–MS, the instrument performance was checked thoroughly, as follows.

(1) The response of the LC–MS system was checked using two standards: the $[M-H]^-$ ion of hydroquinone and the M^- ion of nitrobenzene (these ions represent over 70% of the total ion current in the respective spectra), in the same molar concentration range. The response of the mass spectrometer was found to be linear within a factor of 1.5; the data for nitrobenzene are shown in Fig. 6. The response for some other classes of antibiotics were also checked, and approximately linear relationships were found.

(2) For rifapentine, both the molecular ion and the chromophoric ion at m/z 452 showed a quadratic response (Fig. 6). This was reproduced on several occasions, even when the ratio between the molecular ion and m/z 452 differed by a factor of 10–100 (due to varying conditions of the ion source and the liquid jet, as described above).

(3) While the MS signal showed a quadratic relationship with the amount of rifapentine, the UV trace showed the expected linear response. (At high levels slight deviations from linearity and quadraticity were observed, owing to peak broadening.)

(4) Beside rifapentine, some other rifamycins were also checked, and similar quadratic relationships were found.

The observed quadratic relationship between amount of rifamycin and mass spectrometer response suggests that interaction between two rifamycin molecules is necessary for the ionization process. However, in the range studied there are *ca.* 10^5 to 10^6 solvent molecules for each rifamycin molecule present, even at the top of the chromatographic peak! This makes intermolecular interactions in the gas phase unlikely. In the DLI process, however, small droplets are formed, from which the solvent evaporates. In the process the liquid phase becomes more concentrated. This might explain the interaction of two sample molecules. Solute clustering in solution or enhanced surface concentrations might also be involved. It is not possible at present to offer a detailed explanation of this unexpected relationship.

Effect of HPLC conditions on mass spectra

As discussed above, the molecular ion is probably formed by electron attachment, where the solvent used in HPLC acts as a moderator gas by thermalizing electrons. In accordance with this mechanism, changes of solvent do not significantly alter either the relative ion abundances or the sensitivity for rifamycins. Various

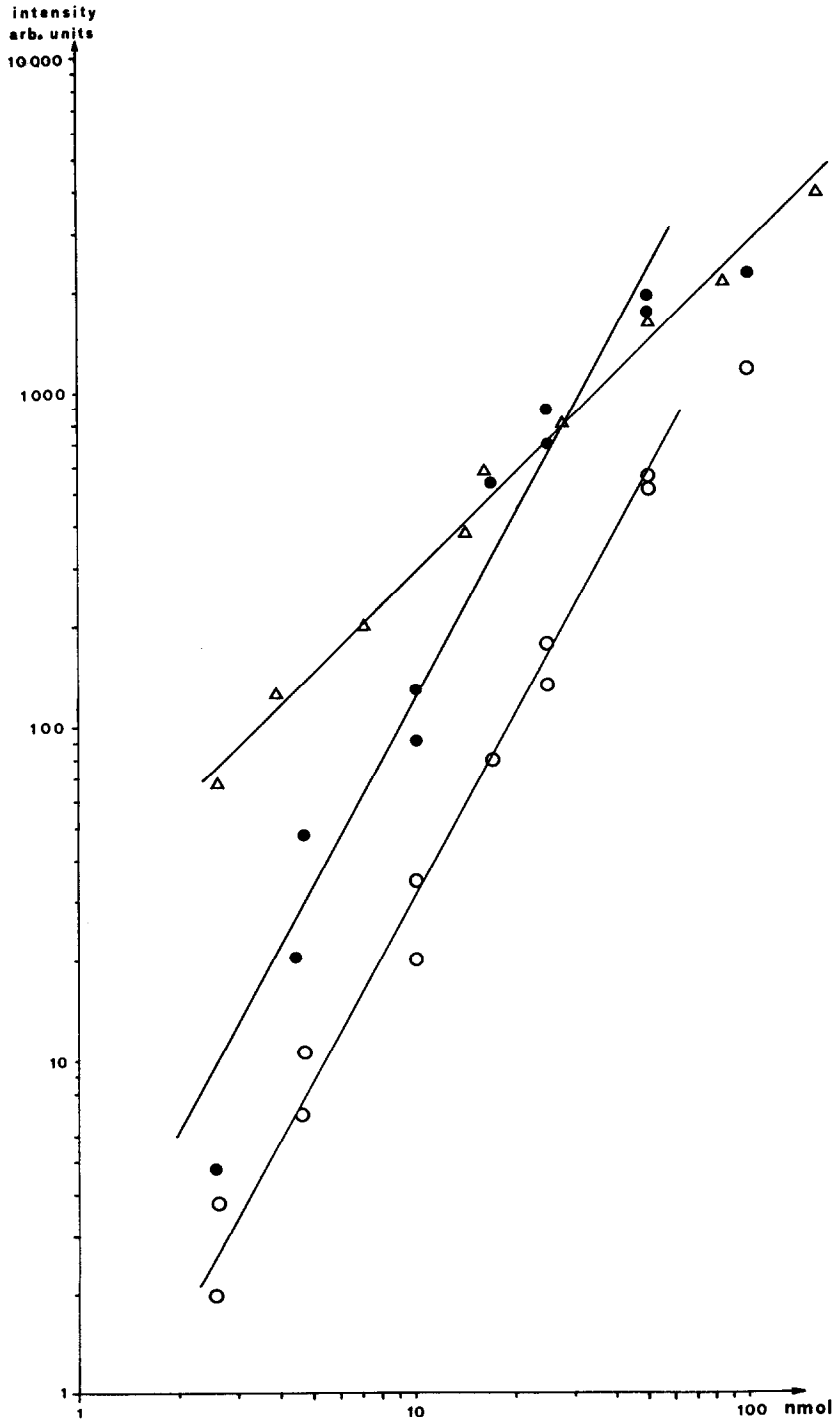

Fig. 6. Correlation between the mass spectrometric signal and the sample amount injected on to the HPLC column, shown on a double logarithmic scale. Negative ionization DLI LC–MS. Molecular ion (*m/z* 876) of (●) rifapentine; *m/z* 452 of (○) rifapentine; molecular ion (*m/z* 123) of (△) nitrobenzene.

combinations of water, methanol, acetonitrile and toluene were used as eluents, and only differences in the low mass solvent clusters were observed, usually below m/z 100. The addition of volatile acids (*e.g.*, CH_3COOH) or ammonium formate buffers with pH from 4.3 to 8.0 also did not change the spectra significantly. However, as a general restriction when using a DLI interface, non-volatile buffers cannot be used. For reasons discussed above, halogen-containing additives should be avoided.

The flow-rate in HPLC may be varied between *ca.* 0.5 and 2 ml/min without significant effect on the spectra. However, when the flow-rate is altered the pressure in the ion source may also change. If it becomes too high (*ca.* 2 Torr in our instrument) sensitivity may drop significantly, probably due to scattering in the analyser region. In such a case it is necessary to lower the amount of liquid getting into the ion source by shortening the liquid jet.

CONCLUSION

A DLI type LC–MS interface using negative ionization gave very good results for rifamycin antibiotics. This demonstrates that DLI is very useful for studying non-volatile and thermally labile compounds. Beside an abundant molecular ion the spectra show various fragments suitable for structural characterization of rifamycins.

Changes in the spectra during a chromatographic peak, temperature dependence and long term reproducibility of the spectra suggest that a structurally important fragment, the so-called chromophoric ion, is formed by thermal decomposition. This specific process, however, may not always occur, *e.g.*, when the ion source is properly cleaned. As the chromophoric ion gives valuable structural information, the ironic consequence is that sometimes a dirty source is a desirable feature!

Short term reproducibility is reasonable (*ca.* 20%), but over a longer period the relative abundance of the chromophoric ion may change over two orders of magnitude. To obtain comparable mass spectra of rifamycins, it is suggested that at the beginning of each day a standard (*e.g.*, rifampicin) should be used to determine the extent of thermal decompositions, and the unknown rifamycin spectra should be compared with the spectrum of this standard.

Chromatographic conditions do not seem to affect the mass spectra significantly, making it easy to change solvents or pH or to use gradient methods. The only real restriction is that non-volatile buffers cannot be used. However, the unexpected quadratic relationship found between the MS signal and the sample amount injected makes quantitative analysis and trace determination of rifamycins difficult.

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