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On-line coupled reversed-phase high-performance liquid chromatography–gas chromatography–mass spectrometry

A powerful tool for the identification of unknown impurities in pharmaceutical products

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ABSTRACT

An alternative approach to HPLC–MS involving direct coupling of reversed-phase HPLC to GC–MS is described for the identification of unknown impurities in pharmaceutical products. Conventional-sized reversed-phase HPLC (column of 4.6 mm I.D., flow-rate 2 ml/min) was coupled to GC–MS. Liquid sample volumes of 500 μl were transferred. In contrast to LC–MS coupling techniques, the proposed method technique experiences no problems with low-molecular-mass solutes or any buffer salts in the reversed-phase LC eluent. An example illustrating the use of this technique in analytical research and development in the pharmaceutical industry is presented. An unknown impurity observed in the HPLC of a stressed sample of a pharmaceutical product was identified by directly transferring the LC fraction of interest to a GC–MS system. The transfer problems arising from the content of water and buffer salts in the LC eluent were circumvented by on-line coupling of liquid–liquid extraction by means of a sandwich-type phase separator, which was coupled to a loop-type LC–GC interface. It is shown that this technique can be improved by lowering the extraction temperature below the freezing point.

INTRODUCTION

In the pharmaceutical industry, there is a great demand for on-line coupling of reversed-phase HPLC to MS, because by this means structural information about unknown impurities observed in HPLC during the development of pharmaceutical products can be obtained in a very economical way. Therefore, LC–MS has become well established in analytical research and development in most pharmaceutical companies. Nevertheless, LC–MS coupling techniques show clear limitations when solutes of interest have a certain volatility and when the LC eluent contains non-volatile buffer salts (such as the commonly used phosphate buffer). In contrast, LC–GC–MS, using the instrumental set-up presented here, experiences no problems with low-molecular-mass solutes (with the exception of very volatile compounds, eluting at a temperature less than *ca.* 80°C above the transfer temperature in GC [1]) or any buffer salts in the LC eluent. Hence, LC–GC–MS is a valuable complement to common LC–MS.

As reversed-phase HPLC clearly dominates over normal-phase HPLC in pharmaceutical analysis, there is a particular interest in transferring aqueous LC fractions to GC columns. In our approach, the

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transfer problems arising from the content of water and buffer salts in the LC eluent were circumvented by on-line coupling of liquid–liquid extraction by means of a sandwich-type phase separator, as described by Van Zoonen *et al.* [2]. The on-line connected laboratory-made LC–GC interface was set up according to Grob and Stoll [3]. The technique applied for sample introduction into the GC column was concurrent eluent evaporation [4].

Van Zoonen *et al.* [2] reported that the extraction efficiency of their on-line liquid–liquid extraction device was *ca.* 65%. We tried to improve the yield of the organic phase with the same device by cooling the phase separator. Initial results obtained with this approach are presented in this paper. In addition, we present an example illustrating the use of LC–GC–MS for analytical research and development in the pharmaceutical industry.

EXPERIMENTAL

The instrumental set-up for LC–GC–MS is shown in Fig. 1.

Instrumental set-up

For the HPLC system, a Kontron (Zürich, Switzerland) Model 420 pump, a manual injection valve (Rheodyne, Berkeley, CA, USA) and a Kontron Uvicon 725 detector were used. The extraction solvents were delivered by a separate Kontron Model 420 pump via a 100 mm × 4.6 mm I.D. column packed with 5-μm Spheri 5 silica (Brownlee Labs., Applied Biosystems, Santa Clara, CA, USA) serving as a restriction only. LC eluents and the extraction solvent were mixed together in a Valco (Houston, TX, USA) 0.75 mm I.D. T-piece and pushed through a 3 m × 0.8 mm I.D. PTFE capillary, which was connected to a sandwich-type phase separator (purchased from the Free University of Amsterdam, Amsterdam, Netherlands).

The phase separator was cooled by a Haake (Karlsruhe, Germany) F3C cryostat filled with polyethylene glycol. As indicated in Fig. 1, the UV detector was installed either before or after the liquid–liquid extraction unit, depending on whether it was intended to detect the solutes in the LC eluent or in the extract. A needle valve restrictor was used to regulate the flow of the organic extract.

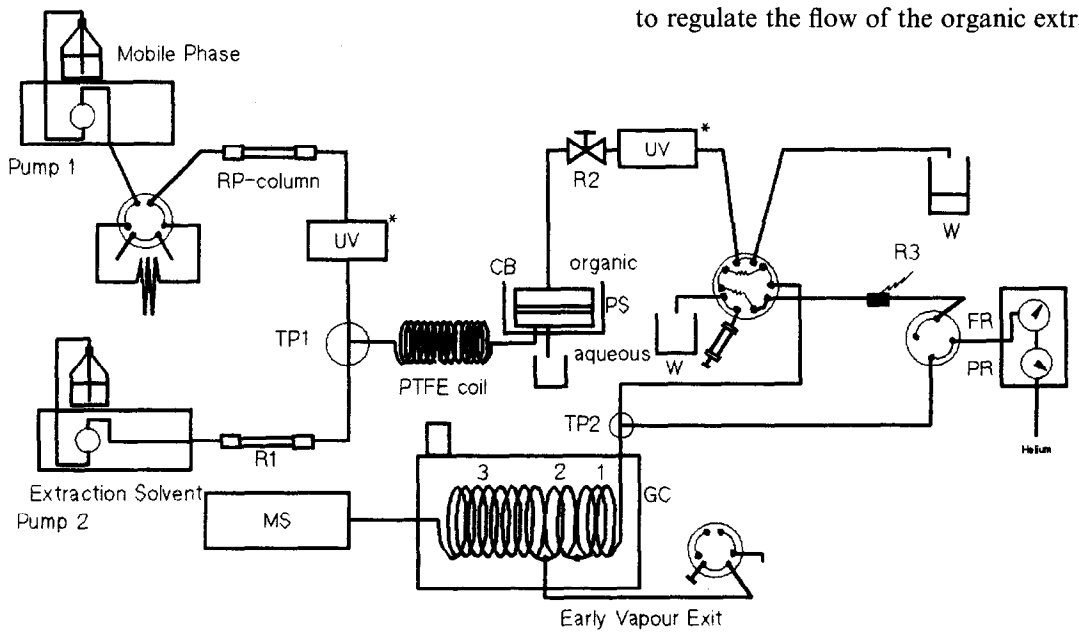


Fig. 1. Instrumental set-up for on-line coupled LC–GC–MS. 1 = Retention gap; 2 = retaining precolumn; 3 = analytical capillary; R1 = LC column (restriction); FR = flow regulator; R2 = needle valve restrictor; R3 = capillary (0.075 mm I.D.); PS = phase separator (sandwich type); CB = cooling bath; PR = pressure regulator; W = waste; TP1 = T-piece 1; TP2 = T-piece 2; UV = UV detector. * = The position of the UV detector is either before or after the liquid–liquid extraction unit. During the transfer of the LC extract to the GC column the UV detector is installed behind the extraction unit.

The LC–GC interface consisted of a Valco ten-port valve, equipped with a 500-μl sample loop. The other two valves were Rheodyne (Berkeley, CA, USA) six-port valves. As described by Grob and Stoll [3], serially connected pressure and flow regulators [Porter (Hatfield, PA, USA) 8286 and Porter DFC 1400, respectively] regulated the carrier gas. T-piece 2 in Fig. 1 was a glass press-fit connector.

A Hewlett-Packard (Palo Alto, CA, USA) Model 5890 gas chromatograph equipped with a Hewlett-Packard Model 5971A mass-selective detector was used. The GC–MS interface was an open-split connection (ratio *ca.* 1:11) with a 50 cm × 0.11 mm I.D. fused-silica capillary.

Chromatographic conditions

LC separations were performed on a 125 mm × 4.0 mm I.D. LiChroCART RP-18 (5 μm) column (Merck, Darmstadt, Germany) using methanol–tetrahydrofuran–water (50:5:45, v/v/v) as eluent at a flow-rate of 2.0 ml/min. UV detection was performed at 230 nm.

After on-line liquid–liquid extraction with *n*-pentane (for conditions, see below), 500 μl of the extract, cut by the loop, were transferred to the GC–MS system. Hence always the total LC peak was transferred.

The oven temperature of the gas chromatograph during transfer was 75°C, leading to an observed maximum pressure of 1.8 bar. The early vapour exit (see Fig. 1) was kept open for 7.95 min after start of the transfer and was closed subsequently.

Gas chromatographic separation was carried out on a 15 m × 0.32 mm I.D. DB-17 fused-silica capillary, 0.5 μm film thickness (J&W Scientific, Folsom, CA, USA), the head of which was connected to a retaining precolumn (3 m × 0.32 mm I.D. DB-17 fused-silica capillary 0.5 μm film thickness) via a Y-shaped press-fit connector, the third leg of which was connected to the temporarily opened early vapour exit (see above). A 3 m × 0.32 mm I.D. fused-silica retention gap, deactivated by phenyldimethylsilylation (J&W Scientific), was connected to the retaining precolumn and left the GC oven through a small hole. It was connected to the LC–GC interface via T-piece 2. The oven temperature was initially kept at 75°C for 10 min, then increased to 260°C at 15°C/min. After termination of the transfer, the pressure of the carrier gas was 1.1 bar.

MS conditions

The ionization mode was electron impact (EI) (70 eV). The filament was switched off for 10 min after start of the transfer.

On-line coupled liquid–liquid extraction

The experimental parameters for on-line coupled liquid–liquid extraction which have an influence on the yield of organic phase were studied. The LC flow-rate was set to 2.0 ml/min. The maximum flow-rate of the organic extract that was achievable without causing turbidity due to non-separated water was measured as functions of the composition of the LC eluent, of the kind of extraction solvent used and of the temperature of the phase separator. The extraction solvents tested were *n*-pentane, *n*-hexane and dichloromethane.

Solvents

Methanol, acetonitrile (both of LiChrosolv grade), dichloromethane, *n*-pentane, *n*-hexane and tetrahydrofuran (all of analytical-reagent grade) were obtained from Merck. The water used was obtained with a Milli-Q water purification system (Millipore).

RESULTS AND DISCUSSION

Liquid–liquid extraction studies

Two common reversed-phase mobile phase systems (methanol–water and acetonitrile–water) were investigated with respect to their suitability for on-line liquid–liquid extraction. The extraction solvents tested were *n*-pentane, *n*-hexane and dichloromethane. Attempts were made to establish what the limits of the concentration of organic solvent in the LC eluent were. In addition, the effect of temperature on the phase separation was studied, aiming at high extraction efficiences even for mobile phases with a high percentage of organic component. It had been expected that lowering the temperature of the phase separator could have a beneficial effect on phase separation, mainly for two reasons: (1) the stability of emulsions is known to be bad at low temperatures and (2) the miscibility of aqueous solutions with alkanes or dichloromethane decreases

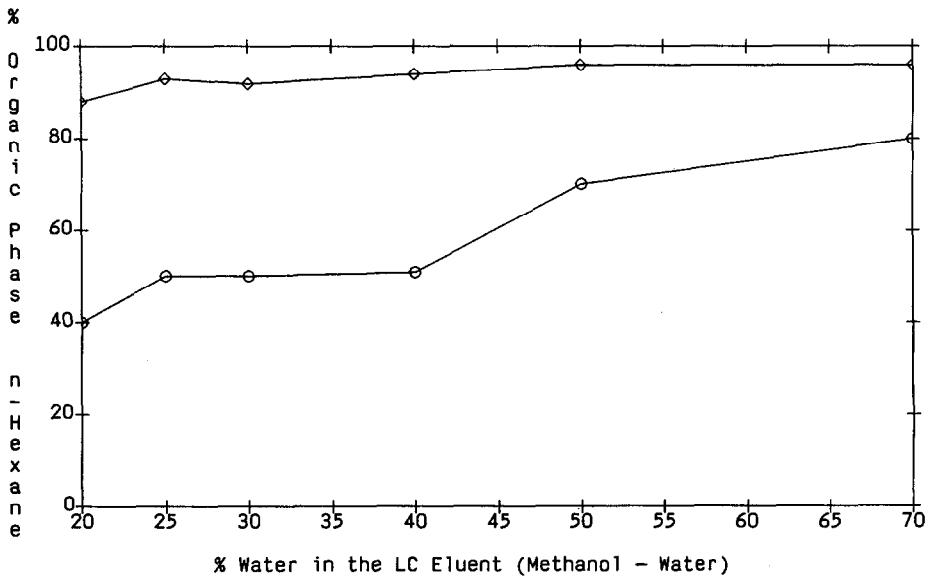


Fig. 2. Extraction solvent *n*-hexane: yield of organic phase *vs.* ratio of water + methanol in the LC eluent. ◇ = −15°C; ○ = room temperature.

on lowering the temperature. It was felt that the extraction process itself should not be affected by our manipulations as the analyte transfer from the aqueous to the organic phase is assumed to take place in T-piece 1 and the subsequent section of the PTFE capillary (see Fig. 1), both being exposed to room temperature. Only the sandwich-type phase separator is immersed in the cooling bath.

This work was focused on the influence of temperature on the yield of organic phase as a function of the water content in the LC eluent. The extraction yield itself (yield of analyte) will be investigated in a separate study.

As expected, the results of this preliminary study show that the yields of organic phase are generally higher with a higher percentage of water in the LC eluent. The positive influence of low temperature during phase separation on the yield of organic phase was demonstrable in all instances, with the exception of aqueous acetonitrile eluents containing high percentages of acetonitrile (*ca.* 70%), which were extracted with *n*-pentane.

In detail, the following results were obtained:

LC eluent methanol–water

Extraction solvent n-hexane (Figs. 2 and 3). Using *n*-hexane as an extraction solvent, methanol–water eluents can be extracted well, even with proportions of methanol in the region of 80%, on cooling the phase separator to −15°C (Fig. 2). The yields of organic phase are in the range 88–96%.

Extraction solvent dichloromethane (Fig. 5). Using dichloromethane as an extraction solvent is very problematic with methanol–water eluents. Even a concentration of 50% methanol results in a yield of organic phase of only 15%.

The recommended temperature of the phase separator is −20°C.

LC eluent acetonitrile–water

Extraction solvent n-pentane (Fig. 4). Using *n*-pentane as an extraction solvent, the yield of organic phase depends strongly on the percentage of acetonitrile in the LC eluent: the higher the percentage of acetonitrile, the lower is the yield. The influence of the temperature on the yield of organic phase is less pronounced for acetonitrile–water than for methanol–water eluents. Cooling the phase separator below 0°C does not have any beneficial effect on the extraction yield. Whereas the extraction of LC eluents containing *ca.* 30% of acetonitrile always gives a constant yield below 0°C, just the opposite

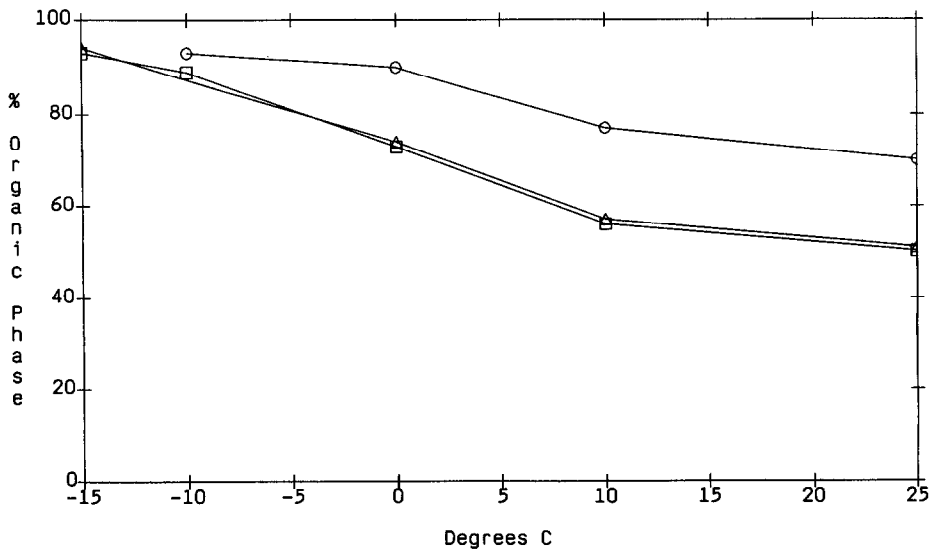


Fig. 3. Extraction solvent *n*-hexane: dependence of the yield of organic phase on the temperature of the phase separator. Methanol–water: ○ = 50:50; △ = 60:40; □ = 75:25.

occurs with LC eluents containing a high proportion of acetonitrile (70%): the yield of organic phase decreases drastically when the temperature is lowered to −20°C.

Extraction solvent dichloromethane (Fig. 5). With respect to the suitability of dichloromethane as an extraction solvent, the situation looks much better for acetonitrile–water than for methanol–water eluents. The extraction of an eluent containing 70% of acetonitrile still results in a yield of organic phase of 60%.

A prerequisite for satisfactory phase separation is

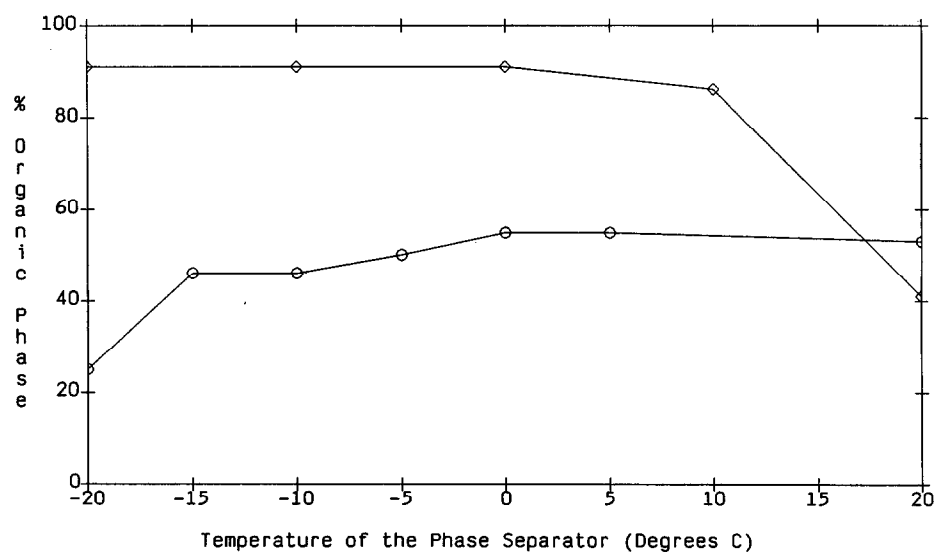


Fig. 4. Extraction solvent *n*-pentane: dependence of the yield of organic phase on the temperature of the phase separator. Acetonitrile concentration: ◇ = 30%; ○ = 70%.

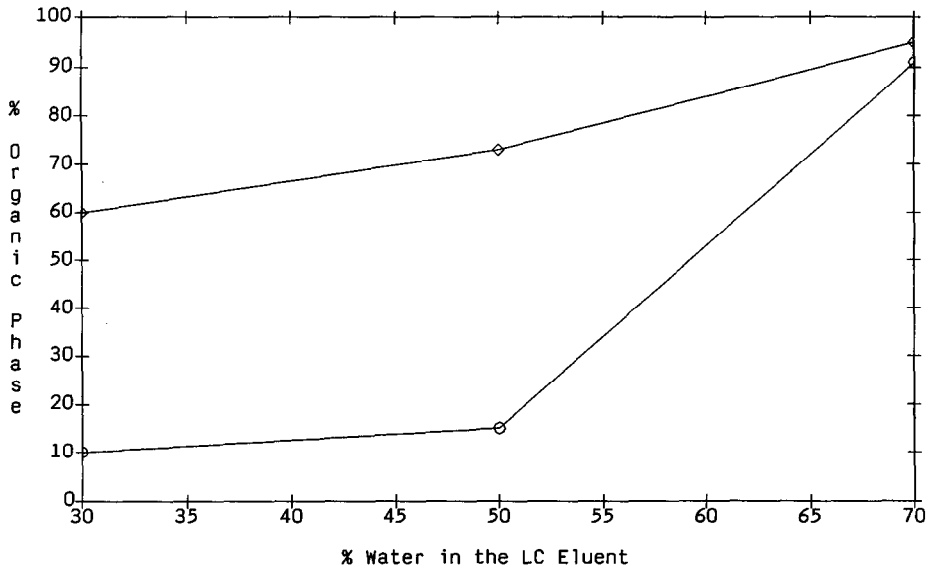


Fig. 5. Extraction solvent dichloromethane: yield of organic phase *vs.* percentage of water in the LC-eluent. ◇ = Acetonitrile–water; ○ = methanol–water.

cooling of the phase separator to −20°C. This applies with all ratios of water and acetonitrile, in contrast to the anomalous behaviour of *n*-pentane extractions (see above).

Transfer of aqueous LC fractions of a particular drug product

The value of LC–GC–MS in the pharmaceutical industry is illustrated by the following example. Particular stress samples of a liquid formulation of a particular drug substance (concentrate for infusion) showed an unusual degradation product giving peak 2 in reversed-phase HPLC (Figs. 6–8). For reasons which are not yet clear, the application of HPLC–MS did not give a mass spectrum that was of any use for the identification of the component of interest. In contrast, the application of LC–GC-MS resulted in a high-quality EI mass spectrum of this unknown degradation product of the active ingredient (Fig. 8), which led to the proposal of a structure for this substance. In the same way, peak 1 (a known degradation product) and peak 3 (active ingredient) were cut and transferred on-line to the GC–MS system (Figs. 6 and 7). The corresponding mass spectra were of similar quality and were in accordance with the known structures of the substances.

The eluent of the reversed-phase system, which had been elaborated for the analysis of the drug product, was methanol–tetrahydrofuran–water (50:5:45, v/v/v). This eluent was also amenable to liquid–liquid extraction. The extration solvent used was *n*-pentane and the phase separator was cooled to *ca.* −20°C. At higher temperatures, the yield of organic phase decreased dramatically.

The volumes of the LC fractions transferred were 500 μl. In accordance with the work of Grob and co-workers, current eluent evaporation [3,4] again proved to be a powerful technique that focuses solutes to narrow bands, even when extremely large sample volumes are transferred to a capillary GC column. As a result, sharp GC peaks were obtained (Figs. 6–8). When such large liquid sample volumes are applied to a GC column, one has to cope with the vast amount of vapour created during the sample transfer. The following measures were taken in order not to affect the functioning of the mass spectrometer: (a) the major part of the solvent vapour was split off by the temporarily opened "early vapour exit", and (b) the filament of the MS ion source was temporarily switched off (for 10 min); it

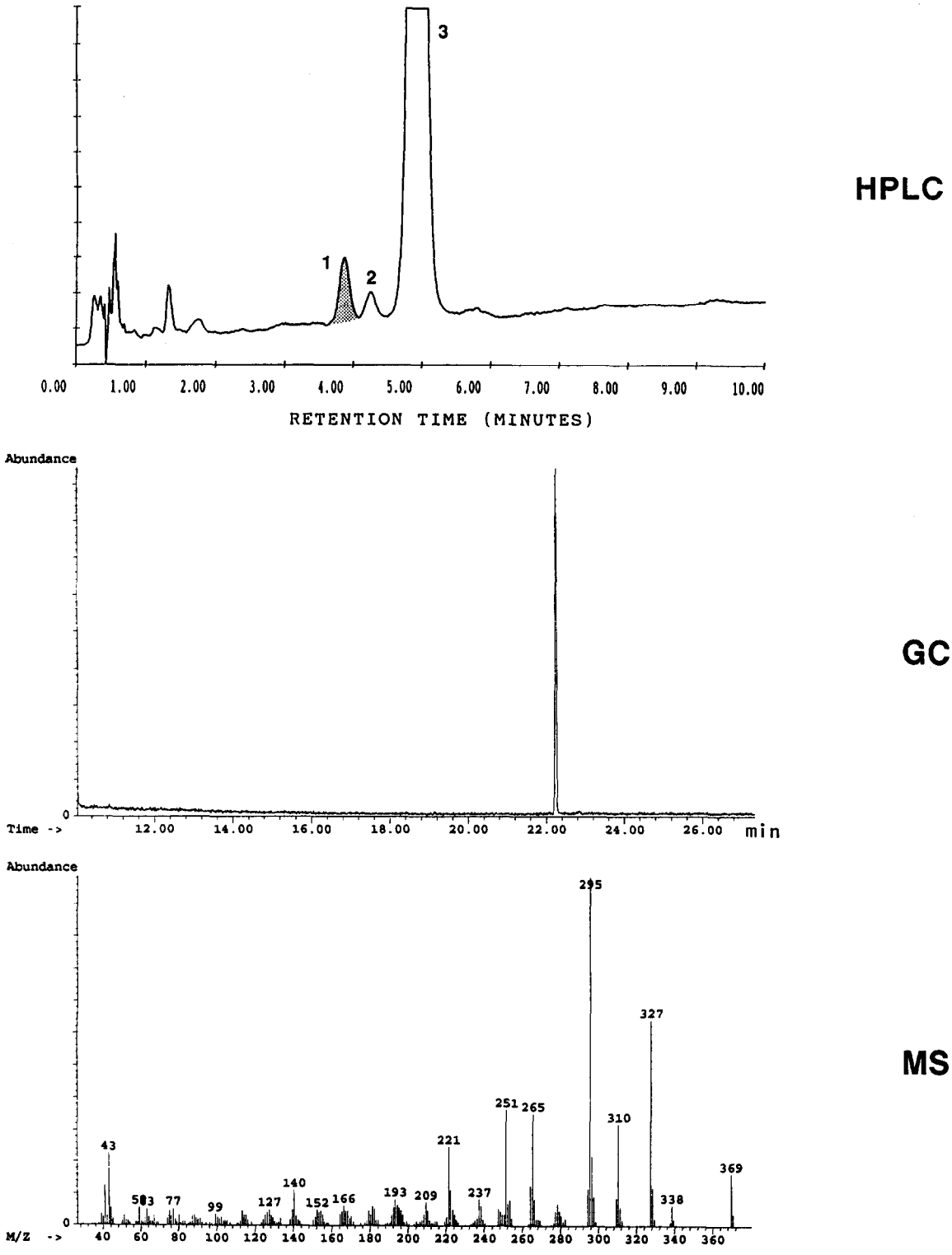
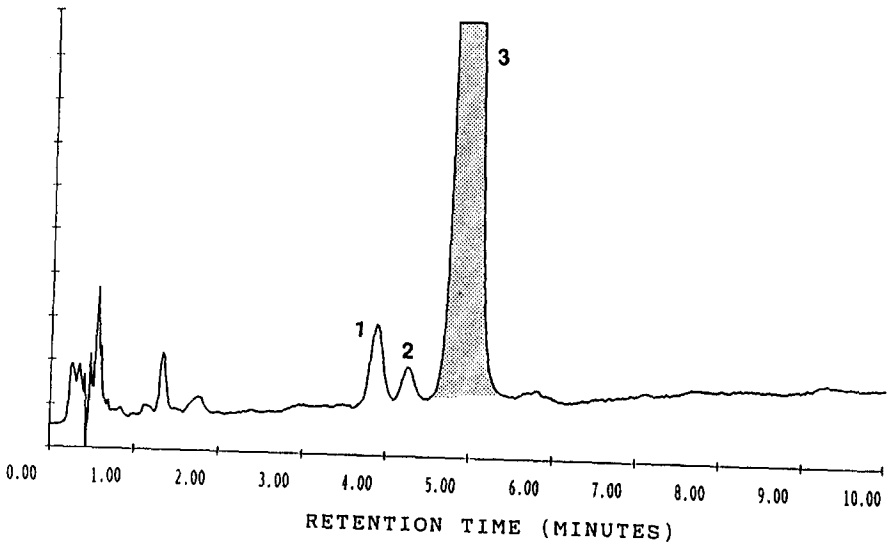
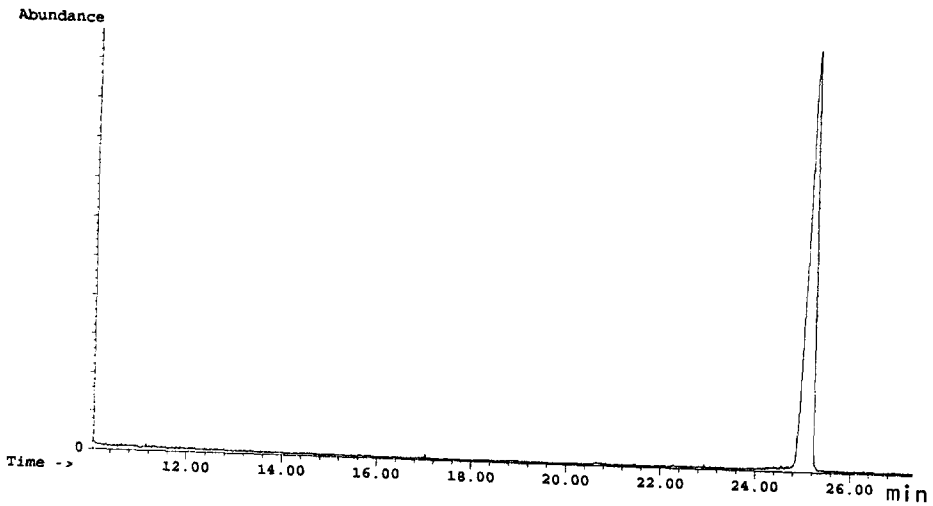


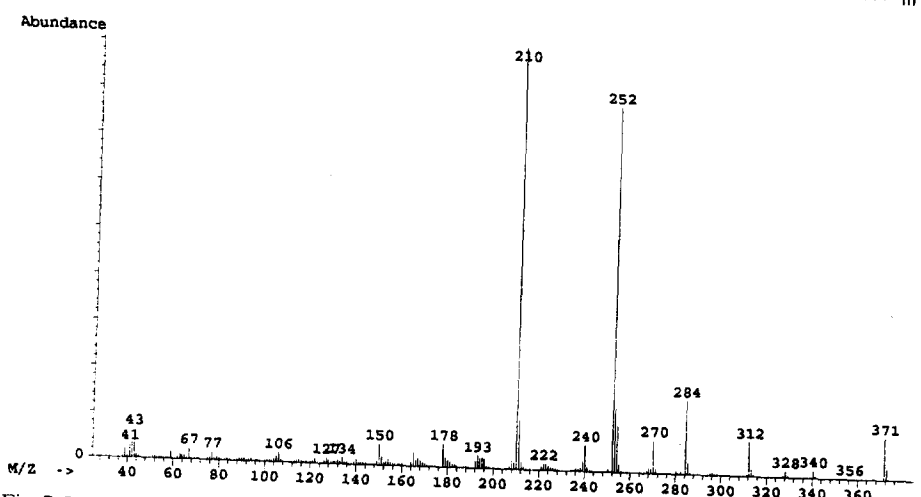
Fig. 6. LC–GC–MS: transfer of an LC fraction (1) of a stressed sample of a drug product. Peaks: 1 and 2 = degradation products; 3 = active ingredient.



HPLC

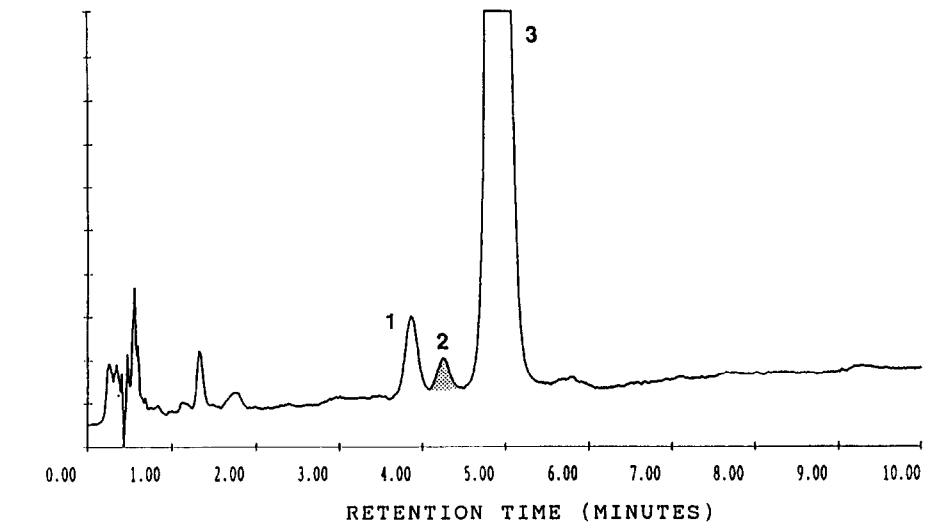


GC

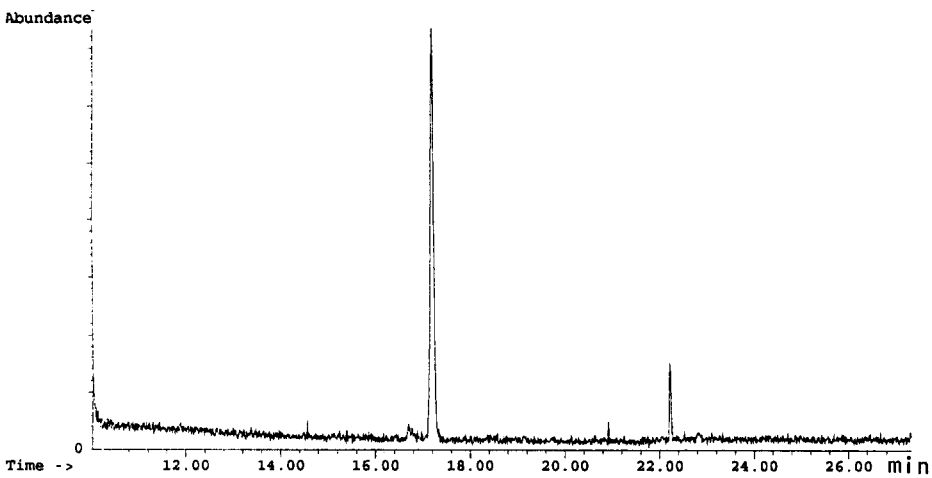


MS

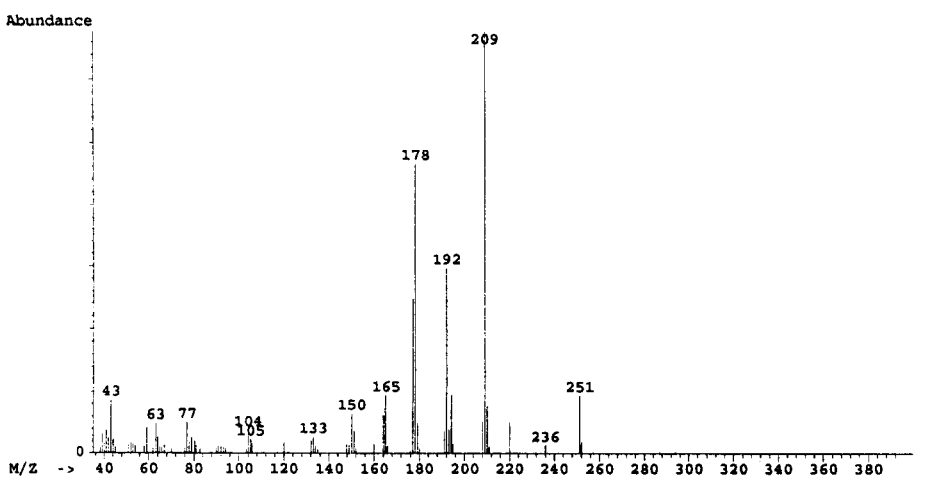
Fig. 7. LC–GC–MS: transfer of an LC fraction (3) of a stressed sample of a drug product. Peaks: 1 and 2 = degradation products; 3 = active ingredient.



HPLC



GC



MS

Fig. 8. LC–GC–MS: transfer of an LC fraction (2) of a stressed sample of a drug product. Peaks: 1 and 2 = degradation products; 3 = active ingredient.

took *ca.* 8 min after the start of the transfer of the LC fraction until the temporarily collapsed vacuum in the ion source was re-established.

CONCLUSIONS

From the results obtained by the application of the technique described, the following can be concluded.

Degradation products that occurred in stressed samples of a particular drug product (concentrate for infusion) during development can easily be identified by LC–GC–MS. No problematic isolation steps are necessary, as LC, cutting of the LC fraction of interest, liquid–liquid extraction and transfer to a GC–MS system take place on-line.

Even poorly resolved LC peaks can be selectively transferred to the GC–MS system. The result is one GC peak and one mass spectrum per component. This fact can also be utilized for proving peak purity (see Fig. 6).

As the LC peaks of interest are completely transferred the sensitivity of the method is good.

In contrast to all known LC–MS techniques, LC–GC–MS yields true EI mass spectra of components in reversed-phase LC. The appearance of a spectrum obtained from a large LC fraction does not differ from that obtained from a small sample volume as usually used in GC–MS (0.5 μl). This allows library searching, which could be an aid in the identification of unknown compounds.

Transfer volumes of 500 μl of aqueous LC eluents do not create any problem with the technique described. Hence conventional-sized HPLC (flow-rate of *ca.* 2 ml/min) can be coupled to capillary GC–MS.

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