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Scanning thin-layer chromatography–liquid secondary ion mass spectrometry and its application for investigation of drug metabolites

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ABSTRACT

Thin-layer chromatography–mass spectrometry including both direct and scanning thin-layer chromatography–liquid secondary ion mass spectrometry, has been used to obtain good separation and good mass spectra of non-volatile and thermally unstable mixtures. The methods are very simple, useful and easy to perform, and they can overcome the problems associated with indirect and direct coupling of liquid chromatography and mass spectrometry. Scanning thin-layer chromatography–liquid secondary ion mass spectrometry can be carried out like gas chromatography–mass spectrometry, and gives good quality chromatograms and mass spectra. These methods were used to analyse drug metabolites and mixtures of other organic compounds.

INTRODUCTION

Studies of drug metabolism are often hindered by non-volatile and thermally unstable mixtures. Our aim was to find a way of obtaining good mass spectra of such samples. As thin-layer chromatography (TLC) is a very simple process that offers excellent resolution of mixtures, we combined it with mass spectrometry (MS) to develop a powerful system for analysing organic compound mixtures [1–3]. Unger *et al.* [4] reported a TLC–MS method for obtaining secondary ion mass spectra of mushroom alkaloids. They neutralized the surface of the chromatographic support by flooding it with low-energy electrons and/or adding an electrolyte to the sample matrix. Two similar suggestions have been proposed for combination of TLC with MS. Chang *et al.* [5] suggested transferring the TLC spot to the fast-atom bombardment (FAB) probe tip covered by double-faced tape. Iwabuchi *et al.* [6] proposed attaching a TLC plate to the silver tip of the liquid secondary ion (LSI) MS ion source by sandwiching a double-faced tape. However, with both methods, the sample would not have sufficient electrical contact with the chamber wall, because the sample material in the matrix was isolated from the metallic target plate by a sheet of double-faced tape, and the sample solution in the matrix only occasionally overflowed to the metallic target

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plate to make electrical contact. The secondary ion current generated would thus not be stable.

We independently investigated the basic concept of TLC–LSIMS. Successive ionization along the chromatogram on the TLC plate is very important, and we have devised a system of scanning TLC–LSIMS. Kushi and Handa [7] independently reported a prototype of the scanning TLC–LSIMS almost at the same time as we did. Their system uses the same method of attachment as the two described above. Our system has been successfully applied to the investigation of drug metabolites. This paper describes our scanning TLC–LSIMS system, with practical examples.

EXPERIMENTAL

Materials

Cephalosporins (7-benzylamido-deacetylcephalosporic acid, cephalexin methyl ester = methyl 7-(D-α-amino-α-phenylacetamido)-3-methyl-3-cephem-carboxylate, and cephaclor = 3-chloro-7-D-(2-phenylglycinamido)-3-cephem-4-carboxylic acid) were synthesized in our laboratories. Rilmazafone, its metabolites and their authentic samples were synthesized as previously described [8]. Aminoglycosides were obtained from Sigma (St. Louis, MO, U.S.A.). Croconazole metabolites were obtained as previously described [9].

To prepare metabolite samples of (±)-5-(*Z*)-7-(3-*endo*-phenylsulphonylaminobicyclo[2.2.1]hept-2-*exo*-yl)heptenoic acid (S-145), the metabolite mixture was chromatographed on a Nucleosil $5C_{18}$ column (15 × 0.46 cm I.D.) with CH_3CN–MeOH–50 m*M* $KH_2PO_4 \cdot H_3PO_4$ (pH 3.0) (20:5:45–60, v/v/v) as a mobile phase at a flow-rate of 1.5 ml/min at 25°C. The metabolites were detected at a wavelength of 220 nm using a Shimadzu LC-6A liquid chromatography system.

TLC plates and mass spectrometry

A sintered glass TLC plate was prepared using a mixture of silica gel (Kieselgel 60 HF or LiChrosorb® Si 60), soda lime glass powder and Zn_2SiO_4/Mn (1:1.5–2.5:0.5) in acetone suspension [1,2]. The suspension was applied to the glass plate with an applicator, the acetone was evaporated, and the plate was baked at 700°C for 10 min to obtain a thin layer of thickness 200 μm. Our TLC plate has a glass support with many linear grooves engraved into the rear surface. Each groove serves as a cutter guide [10].

The 'TLC aluminium sheet silica gel' (E. Merck, Darmstadt, F.R.G., No. 5583) and HPTLC silica gel 60 F_{254} (E. Merck, No. 5628, glass plate) were also used.

For reversed-phase chromatography, RP-8 $F_{254}S$ (E. Merck, No. 15 684, glass plate) was used. Because reversed-phase aluminium plates are not commercially available, they were prepared from normal phase aluminium plates by the proce-

dure of Gilpin and Sisco [11]. The carbon contents for the latter were 11% (RP-8) and 17% (RP-18). The cleaved strip of a glass TLC plate or an aluminium sheet TLC plate can be placed on a conventional holder for LSIMS.

The TLC solvent systems are described in the figure legends for each example.

A Hitachi M-68 mass spectrometer (ion-optically equivalent to an M-80 mass spectrometer) was used for LSIMS measurements and for scanning TLC–LSIMS. A strip of the TLC plate was held by the primary-ion target holder of the LSIMS ion source, as shown in Fig. 1. For the measurements, glycerol was used as a matrix.

Driving system of TLC plate for scanning TLC–LSIMS

The scanning TLC–LSIMS probe (Fig. 2) was designed and constructed by ourselves. It can accommodate a plate of up to 85 mm in length and scan up to 65 mm of it. A glass, aluminium or plastic plate can be stably mounted. A special holder for a thicker plate, such as a glass plate, can be used to retain the plate surface in a suitable position. A metallic mounting beam was designed to embrace the plate, to allow the sample-loaded adsorbent phase to maintain good electrical contact with the metallic beam and the ionization chamber wall. The sample introduction probe, part of the ion-source housing, and part of the ionization chamber were reconstructed. An electric motor was used to drive the probe at either a constant or a variable-programmed speed [3]. The drive can be stopped at any point of the chromatogram for a mass spectrum to be taken. This device can be regarded as equivalent to a good GC–MS system, including the data processing (Fig. 2).

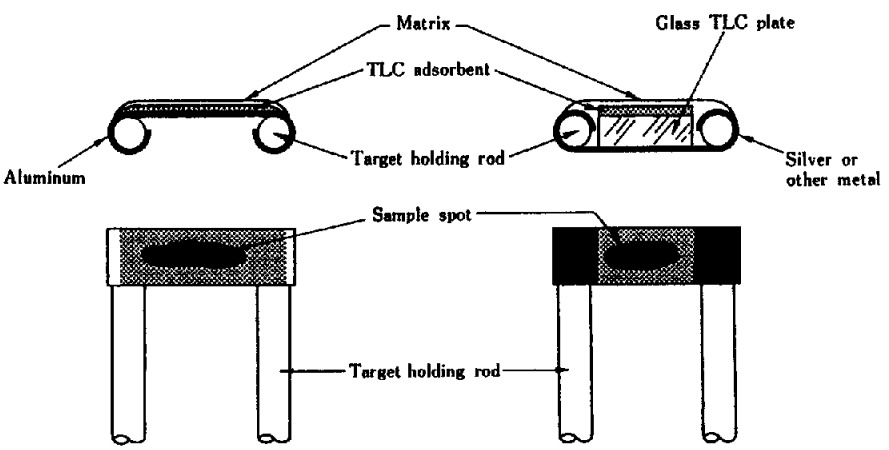

Fig. 1. A piece of TLC aluminium sheet plate and a piece of TLC glass plate mounted for direct TLC–LSIMS.

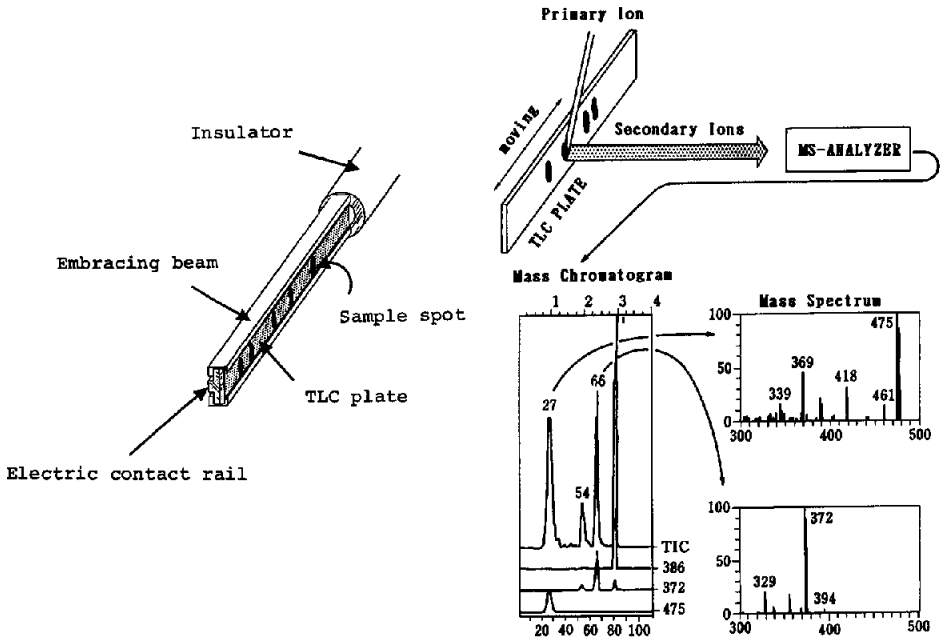

Fig. 2. Diagram of scanning TLC–LSIMS system.

RESULTS AND DISCUSSION

TLC–LSIMS

The technique combining TLC with LSIMS enables very simple yet precise measurement of samples of thermally unstable mixtures. The technique uses a small strip of TLC aluminium sheet coated with silica gel, taken from conventionally developed TLC plates or our plate described in Experimental, as the primary ion target instead of the usual silver plate. Since it was essential that the sample and chamber wall were at the same potential, particular attention was paid to maintaining this electrical contact, especially when a piece of the TLC sintered glass plate was used as the sample holder.

A small piece of aluminium sheet, which is the support of the 'TLC aluminium sheet silica gel', was compared with the usual silver plate with respect to the sensitivity of the secondary ion. The sensitivity of the aluminium sheet is more than 80% of that of the silver plate. The reproducibility and stability of the secondary ion current from the aluminium TLC plate gave almost the same results as those from the conventional silver plate [2], showing that TLC–LSIMS can be applied to quantitative analysis using a stable-isotope-labelled internal standard.

No background spectra that might interfere with the spectra were found in the mass spectra of TLC–LSIMS using silica gel, alumina or cellulose as adsorbent. Reversed-phase adsorbent also gave no background spectra.

A mixture of cephalosporins was examined with good results (Fig. 3). Each spectrum obtained by TLC–LSIMS was superimposed on the equivalent one from the conventional silver plate (data not shown). Good results were also obtained with other drug metabolites, such as rilmazafone, croconazole, *etc.* (data not shown). Thus LSI mass spectra generated from the sample on the TLC plate are comparable with those from the conventional silver substrate.

Scanning TLC–LSIMS

In TLC–LSIMS, successive ionization along the TLC chromatogram is very important, and was achieved by reconstructing the ion-source housing *etc.* Movement of the TLC plate, mounted on the TLC–LSIMS probe, led to progressive impact of the primary ion with the spots to generate secondary ions, as described in Experimental.

A mixture of a sleep inducer (rilmazafone) and its metabolites was subjected to analysis on a 'TLC aluminium sheet silica gel', which was set on the TLC plate holder of the TLC–LSIMS probe and moved at 10 mm/min. The repeated magnetic scanning (mass chromatographic) measurement (at 0.5 s/decade) gave a good TLC chromatogram in which the distance between the minimum and maxi-

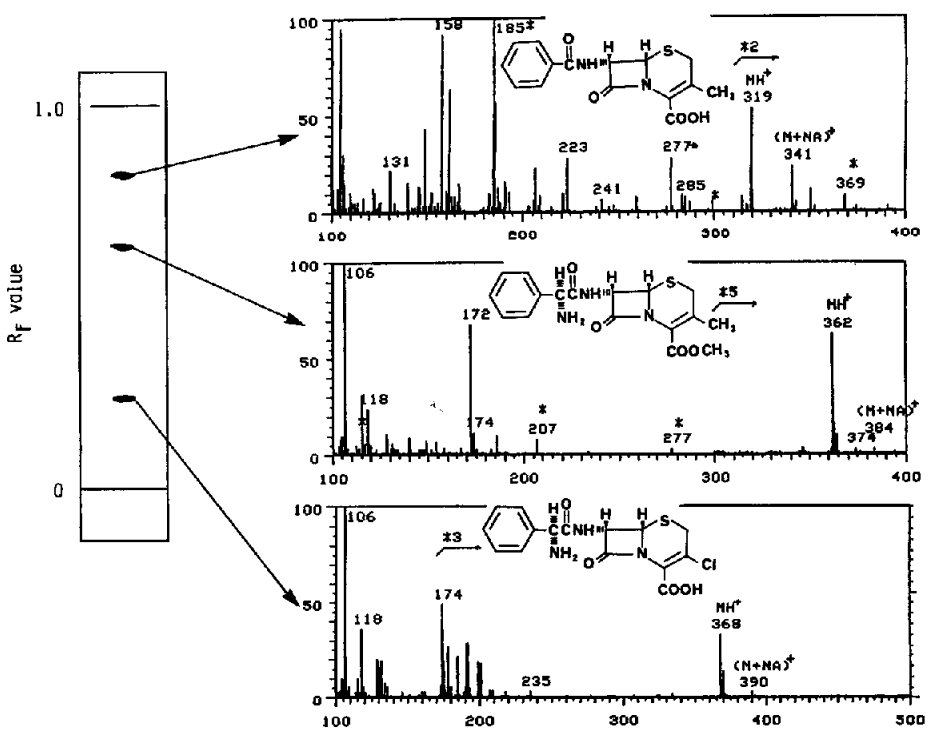

Fig. 3. Direct TLC–LSIMS of cephalosporins. TLC plate, silica gel 60 F_{254} (aluminium sheet, No. 5583, E. Merck); solvent, ethyl acetate–acetic acid–water (30:10:5, v/v/v).

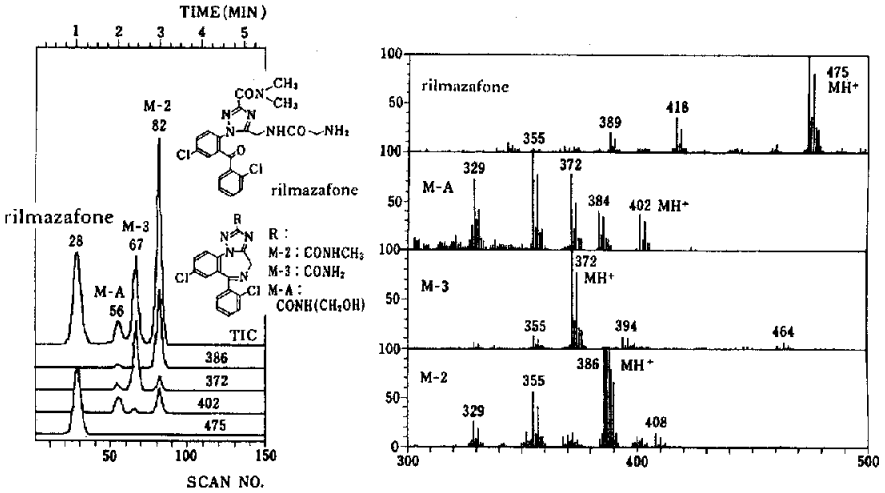

Fig. 4. Scanning TLC–LSIMS of rilmazafone (450191-S) and its metabolites. TLC plate, silica gel 60 F_{254} (aluminium sheet, No. 5583, E. Merck); solvent, benzene–*n*-propanol–28% ammonia (90:30:1, v/v/v).

mum R_F value spots was 45 mm in this example (Fig. 4). Each spectrum was identical with that of the authentic compound observed by conventional LSI–MS.

A mixture of aminoglycosides was also analysed on a TLC aluminium sheet coated with silica gel and on a TLC glass plate, which provided a good chroma-

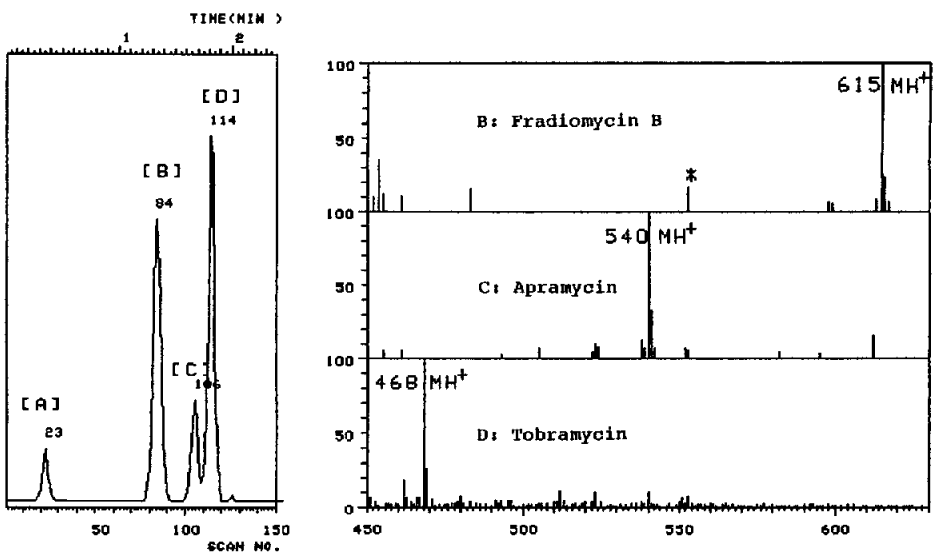

Fig. 5. Scanning TLC–LSIMS of aminoglucosides. TLC plate, silica gel 60 F_{254} (glass plate, No. 5628, E. Merck); solvent, 2-propanol–28% ammonia–water, 2:1:1, v/v/v).

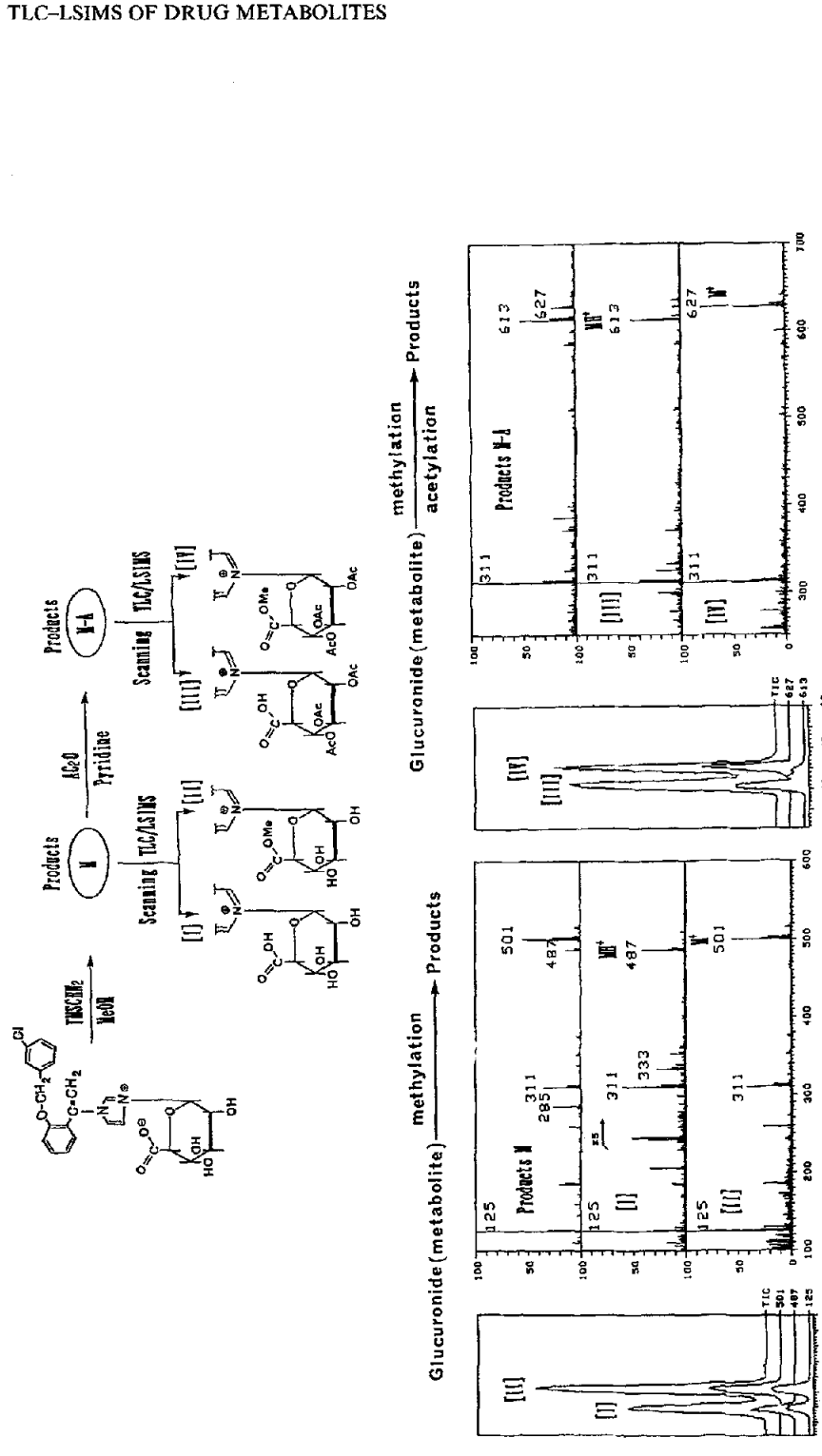

Fig. 6. Scanning TLC–LSIMS of reaction products of croconazole metabolites for structure determination. TLC plate, silica gel 60 F_{254} (aluminium sheet, No. 5583, E. Merck); solvent, *n*-butanol–acetic acid–water (4:1:1, v/v/v).

togram and mass spectra (Fig. 5). A sintered glass TLC plate also gave good results (data not shown).

Structure determination of croconazole glucuronide was carried out by scanning TLC–LSIMS. The reaction product mixtures of methylation products (M) and methylation and acetylation products (MA) were analysed by this system (Fig. 6).

A good example of an analysis of drug metabolites was a preliminary examination of a small amolunt of a drug, (±)-5-(*Z*)-7-(3-*endo*-phenylsulphonylaminobicyclo[2.2.2]hept-2-*exo*-yl)heptenoic acid (S-145). The metabolite mixtures (*ca.* 1 μg, in all) were analysed by scanning TLC–LSIMS (Figs. 7 and 8). Although the spots detected by UV fluorescence appeared to be overlapping, and the positive-ion and the total-ion mass chromatograms of the scanning TLC–LSIMS were not well resolved, the mass spectra could indicate the structures. The negative-ion chromatograms and spectra also contributed to the structure elucidation. The negative-ion mass chromatograms were examined by using a small amount of sample (0.3 μg), and the structure of the taurate metabolite of the drug could be determined (Fig. 9). This information was directly useful in the metabolism studies. Alternatively, the metabolite mixture was first separated by reversed-

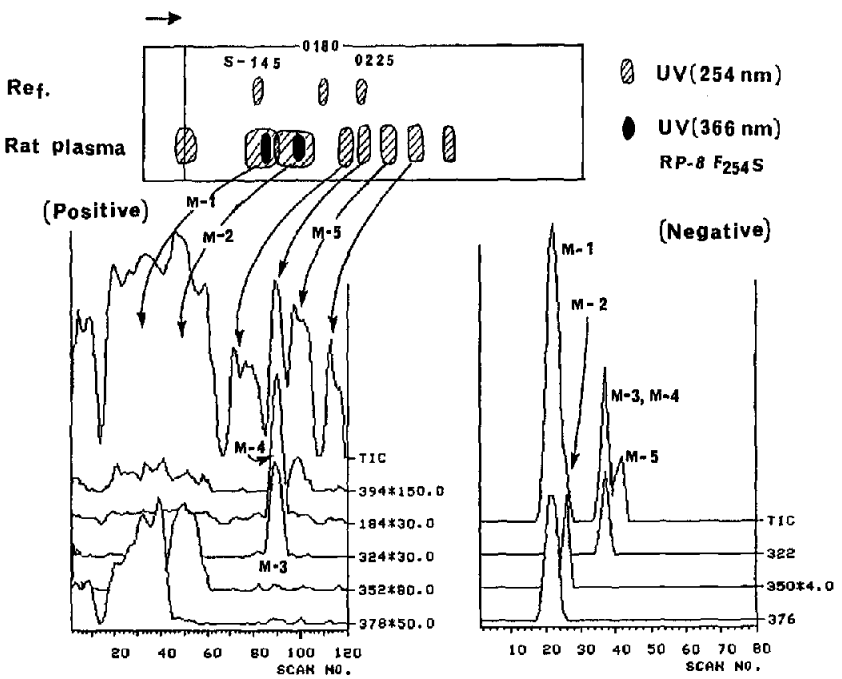

Fig. 7. Scaning TLC–LSIMS of S-145 metabolites: thin-layer chromatograms and mass chromatograms. TLC plate, RP-8 F_{254} (glass plate, No. 15 584, E. Merck); solvent, methanol–water–acetonitrile–acetic acid (1:1:0.5:0.005, v/v).

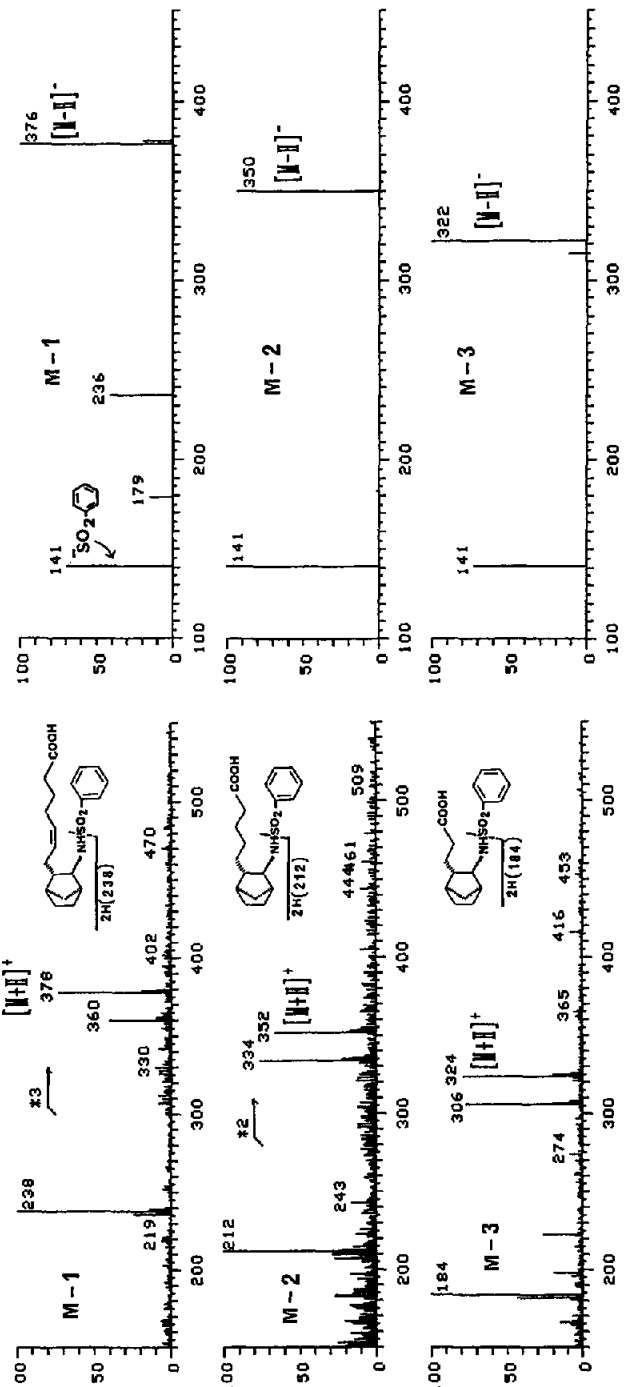

Fig. 8. Scanning *TLC–LSIMS* of *S-145 metabolites*: mass spectra.

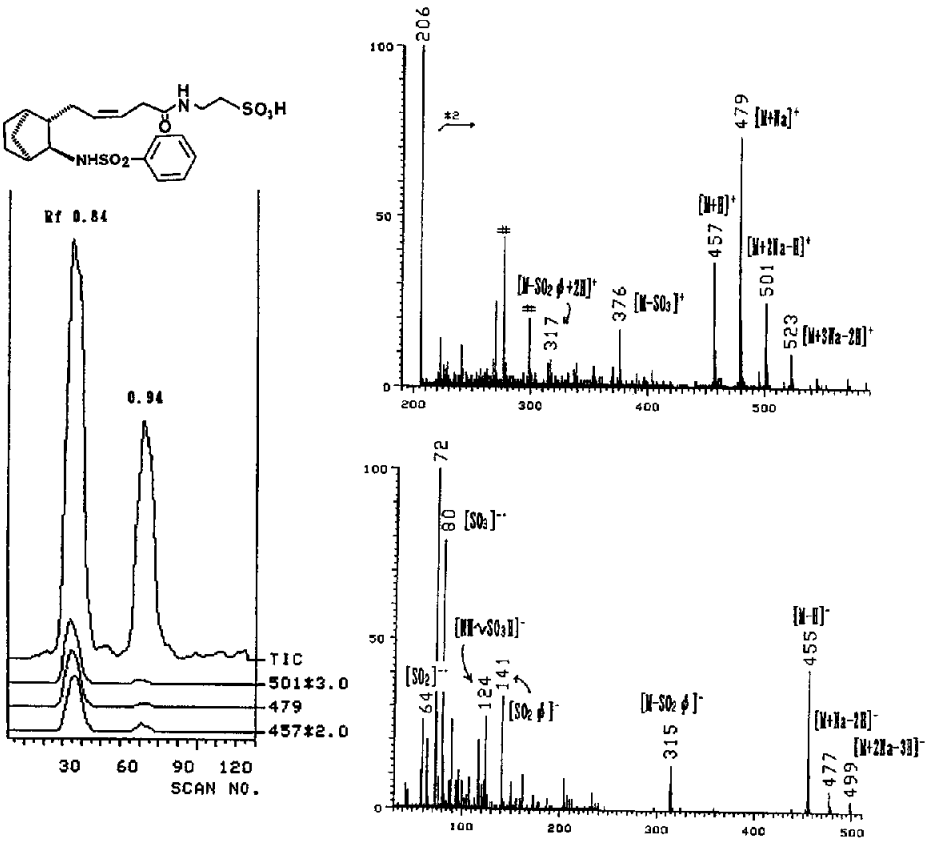

Fig. 9. Scanning TLC–LSIMS of the taurate metabolite of S-145: mass chromatograms, and positive-ion and negative-ion LSIMS. TLC plate, silica gel 60 $WF_{254}S$ (aluminium sheet, No. 16 484, E. Merck); solvent, chloroform–methanol– acetic acid water (13:10:1:3, v/v).

phase liquid chromatography and then each fraction was analysed by scanning TLC–LSIMS. In this manner some contaminants could be removed, and good mass spectra of pure compounds obtained.

LC often uses buffer solutions that cannot be analysed by LC–MS. Our scanning TLC–LSIMS method can be used to analyse samples that have been developed in buffer solutions, *e.g.* some peptides and conjugates.

Another example of drug conjugates is a mixture of glucuronide and sulphate conjugates of a protoberberine (9,10-dimethoxy-3-hydroxy-7-methyl-5,6,7,8,13, 14-hexahydrodibenz[*c,g*] azecine). Figs. 10 and 11 show the mass chromatogram of this mixture before and after computer-processing. The good resolution of the components is apparent.

The only limitations of TLC–LSIMS, including batch-processing direct TLC–LSIMS, and scanning TLC–LSIMS, arise when TLC cannot separate the mixture

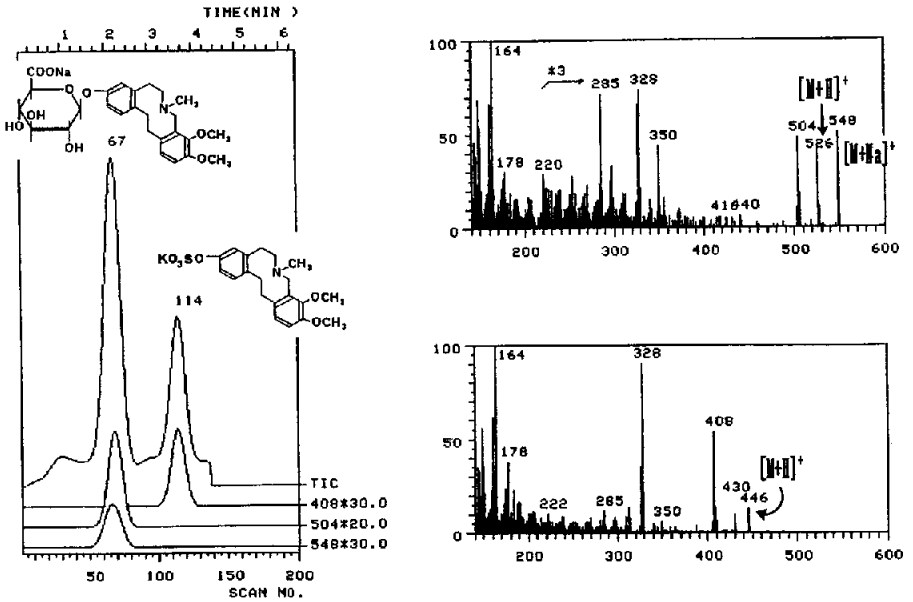

Fig. 10. Scanning TLC–LSIMS of protoberberine conjugates before computer processing. TLC plate, silica gel 60 $WF_{254}S$ (aluminium sheet, No. 16 484, E. Merck); solvent, *n*-butanol–acetic acid–water (4:1:1, v/v/v). Upper: protoberberine glucuronide sodium salt. Lower: protoberberine sulphate potassium salt.

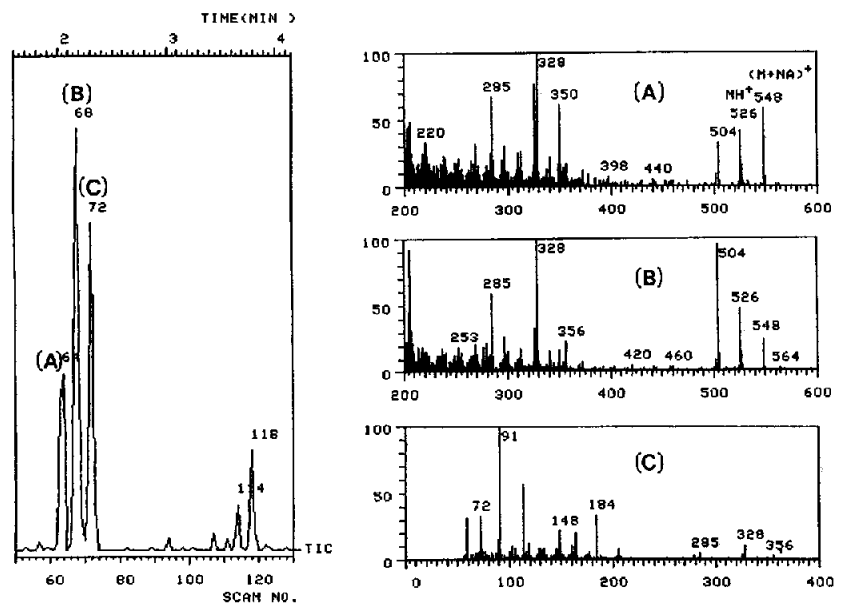

Fig. 11. Scanning TLC–LSIMS of protoberberine conjugates after computer processing. For experimental conditions see Fig. 10.

or when conventional LSIMS measurement of the pure component sample cannot provide a spectrum.

CONCLUSION

The excellent features of TLC and MS were combined for our systems of direct TLC–LSIMS, and scanning TLC–LSIMS, which can be used to analyse non-volatile and thermally unstable mixtures in biomedical studies. The chromatograms and mass spectra obtained by scanning TLC–LSIMS are of excellent quality, very similar to those of authentic compounds observed individually by conventional methods. The scanning TLC–LSIMS system is especially good for analysing mixtures of drug metabolites. Although TLC has a lower resolution than other chromatographic systems, the scanning TLC–LSIMS system can overcome the defects in LC–MS, and the handling simplicity and versatility should make it a very powerful tool in the health sciences.

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