Journal of Chromatography, 394 (1987) 171–181
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 205

STRUCTURE ELUCIDATION OF DRUG METABOLITES USING THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

TIMOTHY J. A. BLAKE
Department of Drug Metabolism, Smith Kline and French Research Ltd., The Frythe, Welwyn, Herts. AL6 9AR (U.K.)

SUMMARY

Thermospray liquid chromatography–mass spectrometry (LC–MS) has been used to provide structural information both from *in vitro* and *in vivo* experiments. This paper will describe the more salient aspects of the technique that have emerged. The ability of the interface to handle gradients was essential for its successful application to metabolism studies, owing to the wide range of compound polarity involved. The examples discussed in this paper include the use of LC–MS in the analysis of *in vitro* incubations of drugs with hepatocyte cell cultures and the direct analysis of plasma samples from *in vivo* studies in the dog.

INTRODUCTION

Xenobiotics are generally eliminated from living species by the formation of more polar molecules. This typically involves conjugate formation such as glucuronidation or sulphation or the generation of hydroxylated substrates, which can then be eliminated from circulation. The polar nature of metabolites has resulted in the extensive use of reversed-phase high-performance liquid chromatography (HPLC) methods to separate the various species concerned. Although it has been possible to combine directly HPLC with mass spectrometry for about a decade[1–3], severe problems have been encountered with mobile phases having a high aqueous content.

The development of the thermospray interface[4,5] has resulted in the first reliable method of coupling these two techniques, thus enabling aqueous mobile phases to be handled at conventional LC flow-rates. The interface acts both as the physical link between the two methodologies and as the primary source of ionisation. This means that external modes of ionisation are not necessary for most applications. However, the majority of commercially available thermospray interfaces now have a filament incorporated that allows the generation of a chemical ionisation type plasma to enhance the ionisation of solutes that do not run well under natural thermospray conditions. The thermospray interface has greatly extended the ease with which liquid chromatography–mass spectrometry (LC–MS) data can be acquired and has allowed mixtures encompassing compounds of widely differing polarity to be handled as the interface easily accommodates gradient elutions. The usefulness of this tech-

0021-9673/87/$03.50

nique will be demonstrated by its application to the study of the metabolism of two compounds.

EXPERIMENTAL

HPLC

All materials used were AnalaR grade or better. HPLC was performed on a Hewlett-Packard 1090A fitted with a filter photometric detector. The column used was a Waters C_{18} Novapak 15 cm × 3.9 mm I.D. packed with 5-μm sized particles. It was operated at a flow-rate of 1.4 ml min^{-1} at ambient temperature. The mobile phases were 0.1 *M* ammonium acetate (natural pH; filtered through a 0.45-μm filter) and acetonitrile. The gradient system used for the SK&F 95018 hepatocyte sample was 10% acetonitrile for 2 min then linear ramps to 30% at 15 min and 60% at 20 min followed by a 3-min hold at 60%.

The gradient system used for the SK&F 93944 plasma sample was 10% acetonitrile for 2 min followed by a linear ramp to 40% at 20 min and then a hold of 2 min. UV detection was at 280 nm and was performed in-line with the mass spectrometer in all cases.

Mass spectrometry

The mass spectrometer used was a Finnigan 4610 with an Incos 2300 data system. A Finnigan thermospray LC–MS interface coupled the liquid chromatograph to the mass spectrometer. A typical vapouriser temperature was 120°C with a jet temperature of 220°C. The vapouriser temperature was lowered to around 108°C by the end of the gradient run. In each case the instrument was tuned on the parent compound of interest, by adding it to the HPLC buffer and continuously introducing it into the mass spectrometer via a short loop, prior to running the main samples.

SK&F 95018 hepatocyte incubations

Male dog hepatocytes were prepared by the method of Strom *et al.*[6]. SK&F 95018 was made up to 50 μM in Williams Medium E (total 1 ml) and added to the hepatocytes. Incubation was for 24 h at 37°C. The supernatant was removed, filtered through a 0.45-μm filter and concentrated (approximately 10 times) by blowing down with nitrogen. The sample was injected in a total volume of 100 μl.

SK&F 93944 plasma samples

SK&F 93944 was dosed orally (100 mg kg^{-1}) by gastric intubation. An 8 h blood sample was taken (3 ml) and spun down to yield plasma. The plasma was protein precipitated by the addition of an equal volume of acetonitrile, and spun down. The supernatant was filtered through a 0.45-μm filter and 100 μl was injected onto the column.

RESULTS AND DISCUSSION

In the first example, this technique was used to study the behaviour of potential drugs in hepatocyte cell cultures. The hepatocytes were prepared by the technique of Strom *et al.*[6]. Once the hepatocyte cells had been obtained it was a simple procedure

to incubate them with the compound of interest. The cells were bound to collagen which allowed the supernatant to be easily removed, filtered and concentrated ready for injection.

The structure of SK&F 95018 (ref. 7) is shown in Fig. 1. The molecule is an antihypertensive agent comprised of a vasodilator portion (the left-hand side of the molecule) and a β-blocker moiety (the right-hand side). The drug was incubated with male dog hepatocytes in culture medium for 24 h and the supernatant was analysed by thermospray LC–MS. The UV trace, monitored at 280 nm, is shown in Fig. 2. The corresponding reconstructed total ion current (TIC) trace is shown in the bottom of Fig. 3 with summed selected ion currents in the top trace. The mass spectrometric acquisition was started 3.8 min after injection to allow the polar endogenous material in the incubation medium to go to waste. The chromatographic integrity of the interface is clearly demonstrated. The large peak centred on scan 302 is an endogenous compound that is UV transparent at 280 nm.

The metabolites have been labelled A–F, with the parent compound labelled G, and their structures, deduced from their thermospray mass spectra, are summarised in Fig. 4. From the UV trace (Fig. 2) it is clear that the acids A and C (Fig. 4) are two of the major metabolites produced. Both of the precursor alcohols were detected although the ions from the latter were too weak to show up in the upper trace of Fig. 3. This latter alcohol eluted between metabolites E and F.

The positive ion thermospray [TSP(+)] mass spectrum of SK&F 95018 (Fig. 1) showed the protonated molecular ion [$(MH)^+$] at m/z 523. Thermal cleavage of the molecule as it passed through the interface produced two major neutral species which on subsequent protonation yielded the observed ions at m/z 258 and 266. The

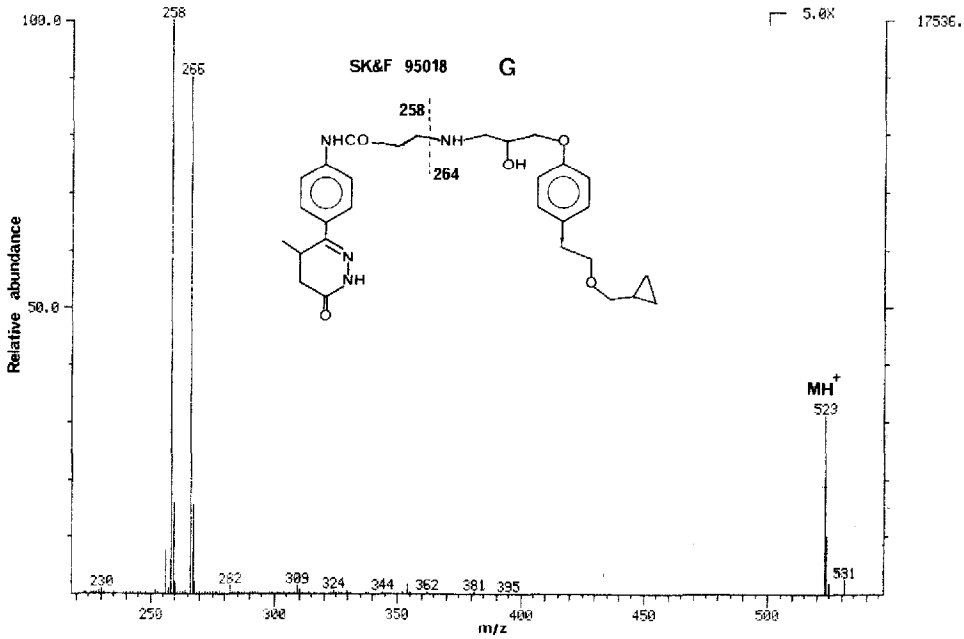


Fig. 1. The structure and TSP(+) mass spectrum of SK&F 95018 (peak G in Fig. 2).

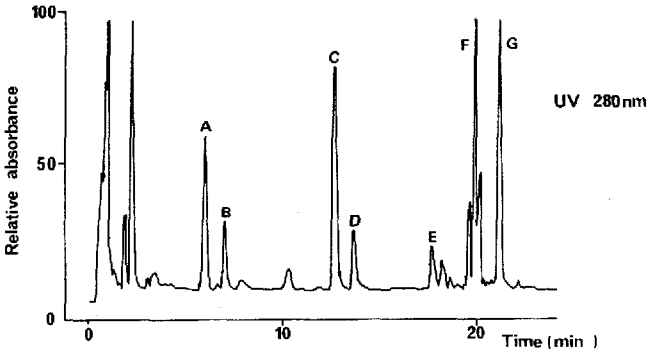


Fig. 2. UV trace, at 280 nm, of SK&F 95018 incubated with male dog hepatocytes. The letters A–F indicate the position of metabolites and G denotes SK&F 95018.

ion at *m/z* 266 was assigned to the primary amine generated from the β-blocker portion of the molecule, whilst *m/z* 258 originated from the vasodilator portion. Monitoring the mass shifts of these ions in the spectra of the metabolites yielded the assigned structures shown in Fig. 4.

The TSP(+) mass spectrum of metabolite C is shown in Fig. 5. The $(MH)^+$ ion was observed at *m/z* 483. The molecule underwent the same thermal cleavage as SK&F 95018 resulting in ions at *m/z* 258 and 226. The former ion indicated that the vasodilator portion of the molecule was intact and the latter ion showed that the β-blocker moiety had been modified (*cf. m/z* 266 in Fig. 1). In the case of metabolite

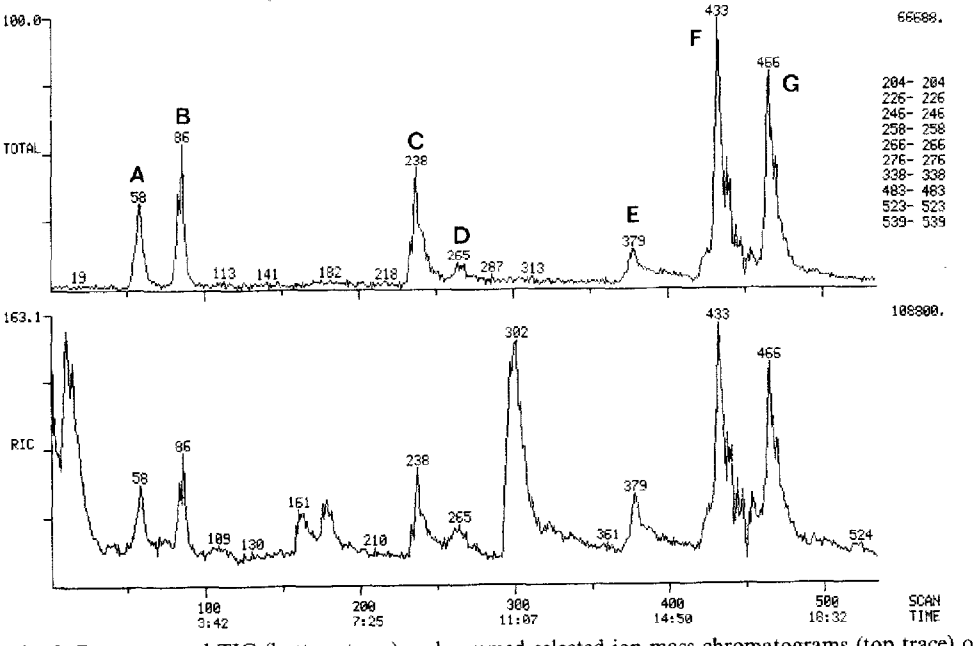


Fig. 3. Reconstructed TIC (bottom trace) and summed selected ion mass chromatograms (top trace) of SK&F 95018 incubated with male dog hepatocytes. Start time was 3.8 min with respect to the UV trace.

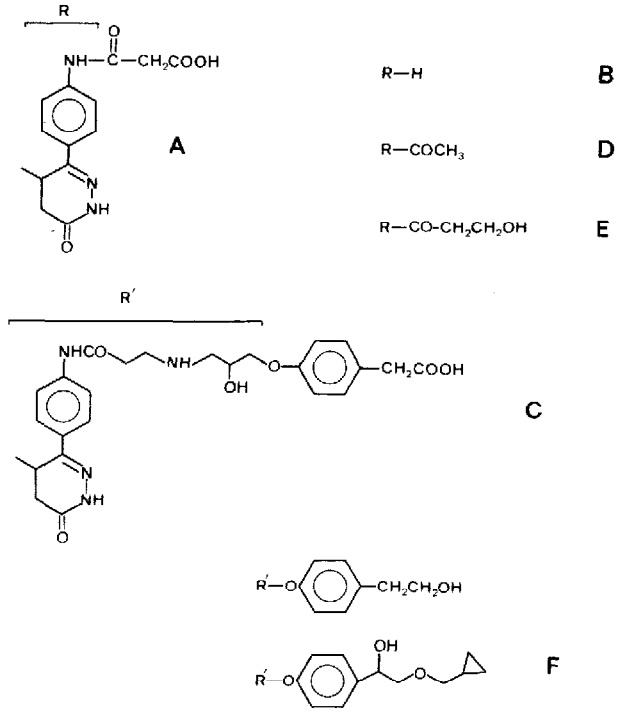


Fig. 4. Structures of the dog hepatocyte metabolites of SK&F 95018 determined from their TSP(+) mass spectra.

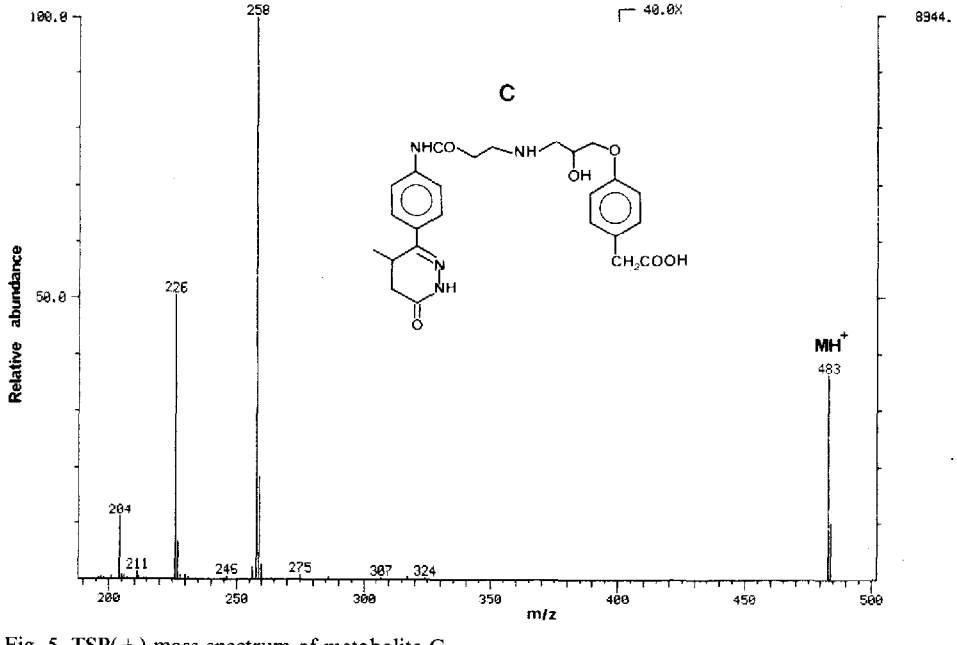


Fig. 5. TSP(+) mass spectrum of metabolite C.

A (Fig. 6) the $(MH)^+$ ion was observed at m/z 290 and decarboxylation yielded the base peak at m/z 246.

The method was fast and gave extensive structural information without the necessity of individual compound isolation. However, due to the nature of the thermospray process, detailed stereochemical information was often not available from the mass spectrum alone. For example, the site of hydroxylation in metabolite F was assigned by analogy with the known metabolism of similar β-blockers and could not be deduced from the mass spectrum directly.

The second example came from the analysis of blood plasma obtained from male dogs which had been dosed with SK&F 93944, an H_1-antagonist that has been shown to have negligible ability to penetrate into the central nervous system[8].

The structure of SK&F 93944 is shown in Fig. 7. The presence of a bromine atom in the molecule was useful as it acted as an internal isotopic label which aided the mass spectrometric assignment of compound related peaks. The UV trace, at 280 nm, of the dog plasma extract (100 mg kg^{-1}; oral dose (P.O.); 8-h sample) is shown in Fig. 8. The major metabolites present are marked on the trace and their structures are shown in Fig. 7 (the numbering of the metabolites is a result of the chronological order in which they were isolated and fully characterised).

This paper will not discuss the overall metabolism of SK&F 93944 in the dog, but will highlight the specific contribution that thermospray LC–MS has made to the study of the metabolism of this compound. The reconstructed TIC trace is shown in Fig. 9 and again shows that the interface does not significantly degrade the chromatographic resolution of the system. The mass spectrometric acquisition was started 2.5 min after injection to allow the initial endogenous material to go to waste. Com-

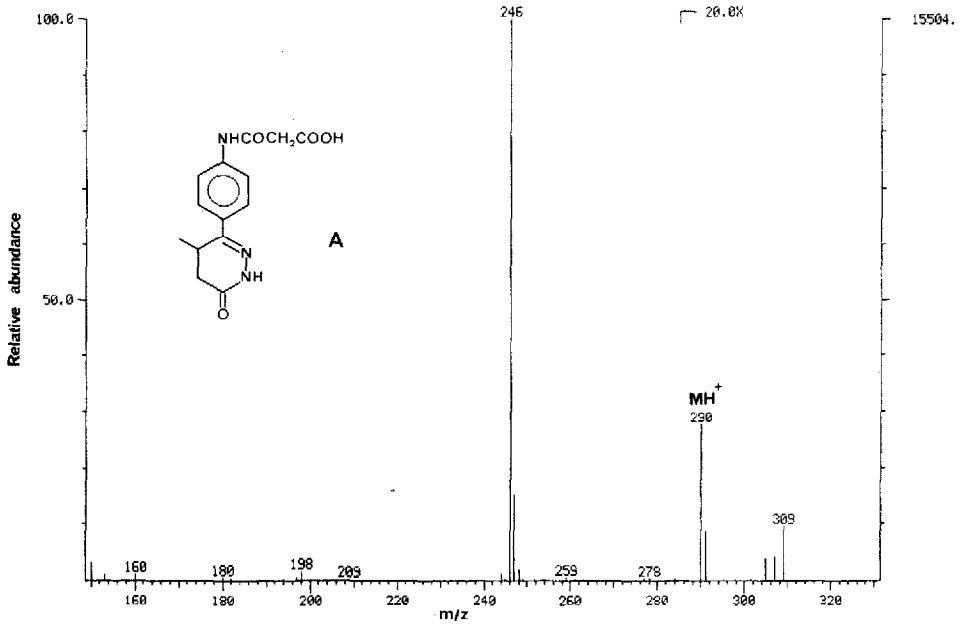
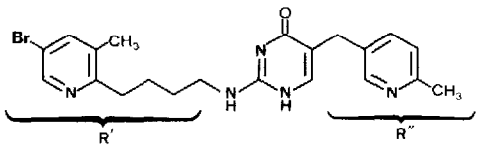
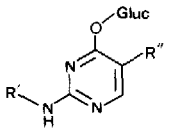


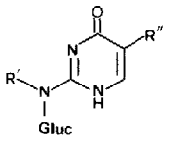
Fig. 6. TSP(+) mass spectrum of metabolite A.



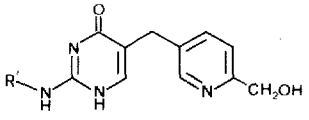
SK&F 93944



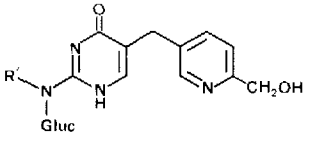
MET-II



MET-XI



MET-VII



MET-X

Fig. 7. Structure of SK&F 93944 and selected metabolites. MET and Gluc are abbreviations for metabolite and glucuronide, respectively.

parison of the UV and TIC traces (Figs. 8 and 9, respectively) shows the variation in the absolute responses between the two methods of detection. As the absolute sensitivities derived from thermospray ionisation seem to vary widely and unpredictably, the relative areas as determined by UV should be used as an indication of relative abundance, given the usual caveats. The peak running after SK&F 93944, centred on scan number 503 (Fig. 9) was an endogenous compound that was UV transparent at 280 nm and served as a useful illustration of the complimentary nature of the two modes of detection.

Fig. 10 shows selected ion mass chromatograms displayed from scan 160 to scan 490 of the total run. The species which gave rise to the ions at m/z 243, 442 and 458 all contained a bromine atom and therefore appeared as doublets (1:1 relative intensity) reflecting the naturally occurring isotopic abundance of bromine. Only the ^{79}Br isotope containing ions from each doublet are shown for the sake of clarity. The species which gave rise to the ions at m/z 218 and 234 did not contain bromine.

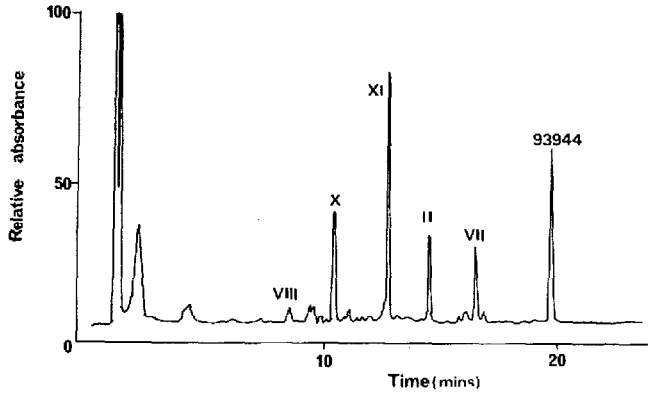


Fig. 8. UV trace, at 280 nm, of plasma extracted from dogs dosed with SK&F 93944 (100 mg kg^{-1}; P.O. dose; 8 h sample). The Roman numerals represent metabolite numbers.

Under TSP(+) conditions no molecular ion data was obtained for any of the glucuronide metabolites studied. In fact, the highest mass species observed corresponded to the protonated aglycone in each case, indicative of thermal degradation of the glucuronides as they passed through the thermospray interface. No evidence for the glucuronic acid portion of the molecules was observed in the mass spectra obtained. Full characterisation of these metabolites required isolation and analysis by ^{1}H NMR and discharge ionisation secondary ion mass spectrometry.

The ion mass chromatogram of m/z 442, which corresponded to the ^{79}Br isotope containing $(MH)^{+}$ ion from SK&F 93944, showed responses (apart from the

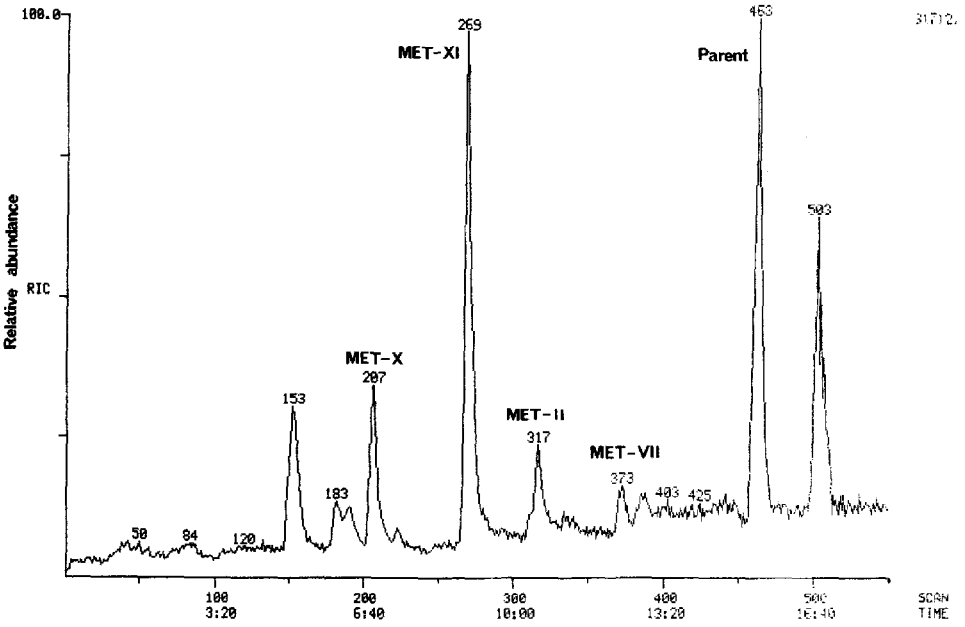


Fig. 9. Reconstructed TIC corresponding to Fig. 8. Start time was 2.5 min with respect to the UV trace.

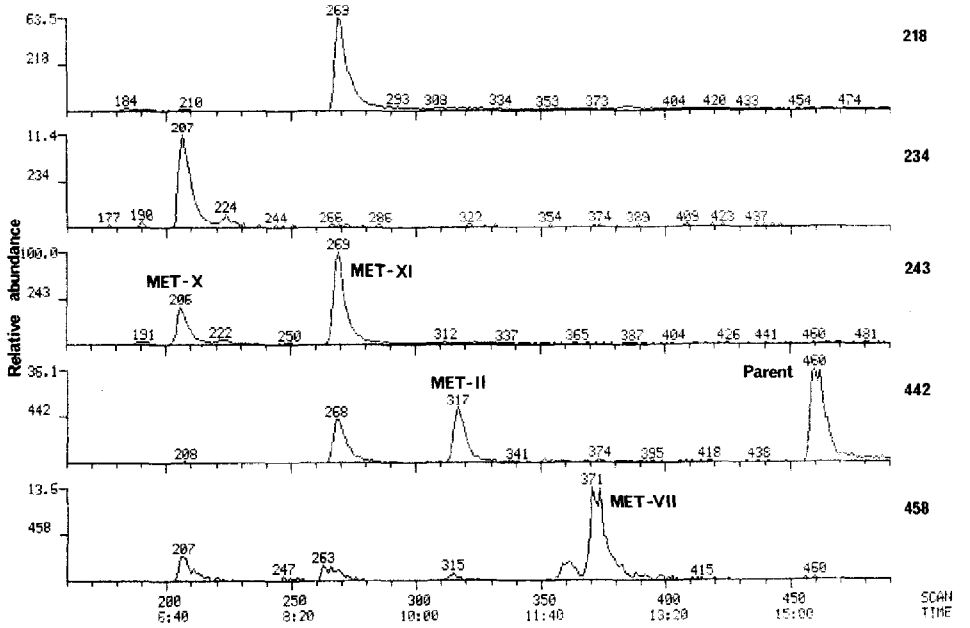


Fig. 10. Selected ion mass chromatograms of plasma obtained from dogs dosed with SK&F 93944.

parent compound itself) for the O-glucuronide (metabolite II) and the N-glucuronide (metabolite XI) which originated from the protonated aglycone species derived from these metabolites. The protonated aglycone ion which originated from metabolite X, the N-glucuronide of metabolite VII, was observed at m/z 458 centred on scan number 207 (Fig. 10). The peak centred on scan number 263 probably indicated the presence of the O-glucuronide of metabolite VII which eluted just in front of the N-glucuronide of SK&F 93944 (metabolite XI).

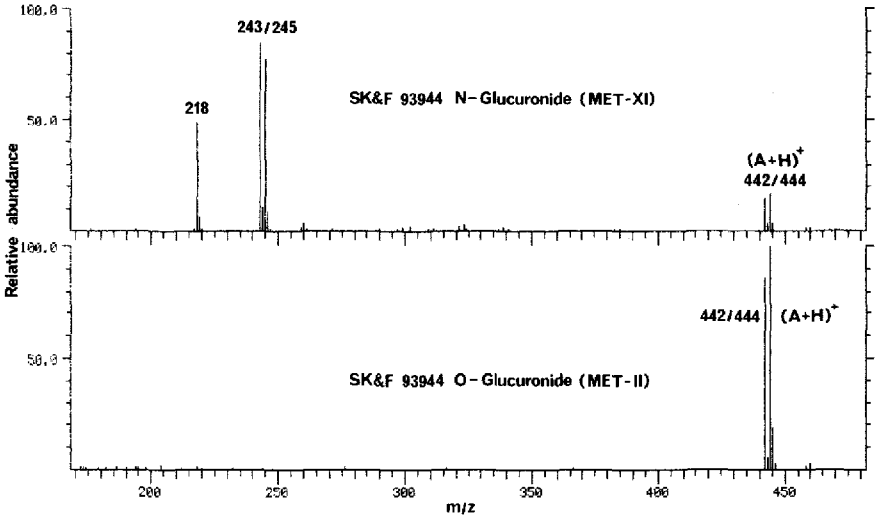


Fig. 11. TSP(+) mass spectra of metabolites XI and II of SK&F 93944.

The ion mass chromatogram of *m/z* 243 (Fig. 10) provided the most interesting trace as this ion originated from a thermally induced cleavage reaction only undergone by N-glucuronides of this compound. Note that a response was observed for both of the N-glucuronide metabolites, X and XI, but that nothing was seen for the O-glucuronide metabolite II centred on scan 317. This has provided a very quick and unexpected way of distinguishing between N- and O-glucuronidation for this compound. The other fragment resulting from this reaction was also observed. For metabolite XI the ion occurred at *m/z* 218 and for metabolite X it shifted to *m/z* 234 (Fig. 10), showing the general location of the site of hydroxylation in the molecule.

The TSP(+) mass spectra of the N- and O-glucuronides, metabolites XI and II, are shown in Fig. 11. The striking difference between the behaviour of the two types of glucuronide under thermospray conditions is clearly shown. In both spectra the $(A + H)^+$ ions represented the protonated aglycone doublets at *m/z* 442 and 444. For the N-glucuronide, the protonated products of thermal degradation dominated the mass spectrum with the bromine containing doublet at *m/z* 243 and 245 and the ion at *m/z* 218.

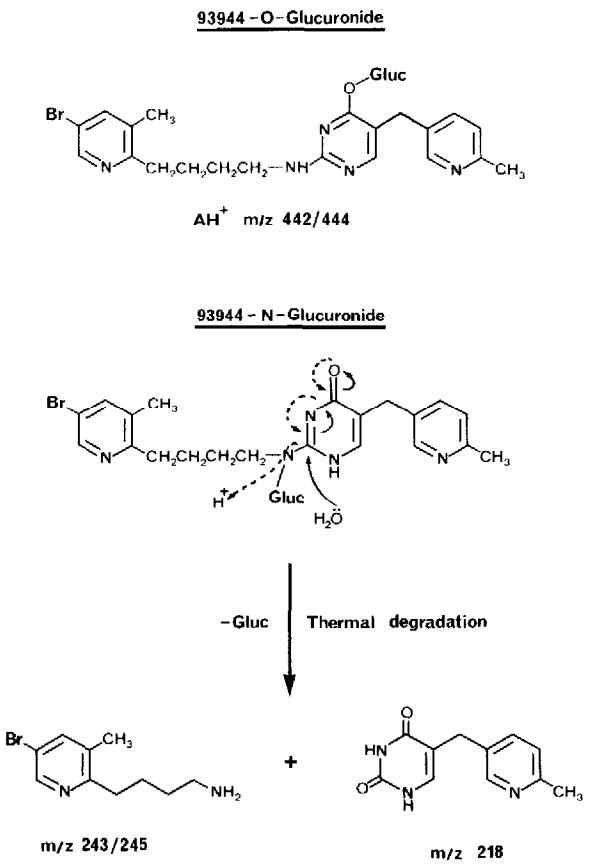


Fig. 12. Rationalisation of the behaviour of the N- and O-glucuronides of SK&F 93944 as they pass through the TSP interface.

Fig. 12 shows a rationalisation of this behaviour. It would appear that the ketonic form of the isocytosine ring was required to enable the thermal cleavage to occur. Nucleophilic attack by water resulted in the cleavage of the molecule as shown, using the carbonyl functionality as an electron sink. The glucuronic acid group was also lost in the process which yielded a primary amine and a substituted uracil as the final products. The former protonated to yield the doublet at *m/z* 243 and 245 whilst the latter gave rise to the ion at *m/z* 218. This ion occurred at *m/z* 234 in the spectrum of metabolite X. Unfortunately, neither fragment was observed with the sugar portion attached. The site of glucuronidation in these metabolites has been ascertained, from their ^{1}H NMR spectra, to be the exocyclic nitrogen atom or the oxygen atom of the isocytosine ring.

The presence of the glucuronic acid must play a role in activating this mechanism since the reaction did not occur with the parent compound, although it exists almost exclusively in the ketonic form. In the case of the O-glucuronide this degradation pathway was not available to the molecule.

CONCLUSION

The thermospray LC–MS interface has provided a routine and reliable link between the liquid chromatograph and the mass spectrometer. It has enabled substantial quantities of data on the structure of metabolites to be acquired without the requirement of individual compound isolation. An unexpected benefit has been demonstrated in the study of the metabolism of SK&F 93944 in being able to differentiate clearly between N- and O-glucuronidation in the isocytosine ring of the molecule. This is a result of the substantial quantity of thermal energy that is imparted to compounds as they pass through the thermospray interface.

Use of hepatocyte cell incubations of potential drug candidates, as highlighted by SK&F 95018, has allowed rapid appraisal of their metabolism in a variety of species. When human hepatocytes are available the results have added significance to the drug development process.

ACKNOWLEDGEMENTS

The SK&F 95018 hepatocyte cell incubations were performed by the group of Dr. R. Chenery and the SK&F 93944 plasma sample was prepared by the group of Dr. P. Cox, both of the Department of Drug Metabolism.

REFERENCES

1 P. J. Arpino, M. A. Baldwin and F. W. McLafferty, *Biomed. Mass Spectrom.*, 1 (1974) 80.
2 R. P. W. Scott, C. G. Scott, M. Munroe and J. Hess, Jr., *J. Chromatogr.*, 99 (1974) 395.
3 W. H. McFadden, H. L. Schwartz and S. Evans, *J. Chromatogr.*, 122 (1976) 389.
4 C. R. Blakley, J. J. Carmody and M. L. Vestal, *J. Am. Chem. Soc.*, 102 (1983) 5931–5933.
5 C. R. Blakley and M. L. Vestal, *Anal. Chem.*, 55 (1983) 750.
6 S. C. Strom, R. L. Jirtle, R. S. Jones, D. L. Novicki, M. R. Rosenberg, A. Novotny, G. Irons, J. R. McLain and G. Michalopoulous, *J. Nat. Cancer Inst.*, 68 (1982) 771.
7 R. A. Slater, *Eur. Pat.*, 0168921 A_2 (1986).
8 G. J. Durant, C. R. Ganellin, R. Griffiths, C. A. Harvey, R. J. Ife and D. A. A. Owen, *Br. J. Pharmacol.*, 82 (1984) Suppl. 232P.