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APPLICATION OF THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY AND LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY FOR THE IDENTIFICATION OF CYNOMOLGUS MONKEY AND HUMAN METABOLITES OF SK&F 101468, A DOPAMINE D_2 RECEPTOR AGONIST

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SUMMARY

A combination of thermospray liquid chromatography–mass spectrometry (LC–MS) and LC MS–MS has allowed the structural elucidation of a number of metabolites of 4-[2-(dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one (SK&F 101468) in monkey urine. By using LC–MS–MS with the third quadrupole (Q3) set up in multiple ion detection (MID) mode, a number of metabolites were subsequently detected in the human urine and plasma samples despite very low dosing regimes. This was achieved with minimal sample preparation, *e.g.* for the urine sample centrifugation was the only preparative step, in order to remove particulate matter, prior to analysis.

The good signal-to-noise ratio obtained for the human samples, using LC–MS–MS with Q3 set up for MID, raised the possibility of a LC–MS–MS quantitative assay. As a result, the detection limit of this method for SK&F 101468 when dissolved in methanol was determined to be in the region of 20 pg on column.

INTRODUCTION

Potential drug candidates when administered to animals or humans are generally metabolised quite rapidly, producing a variety of more polar compounds that can then be eliminated from the body. This is normally achieved in the animal via a number of oxidative reactions such as hydroxylation, oxidation of alcohols, N-oxidation and N-dealkylation. These metabolites can then be excreted from the body or they can undergo further metabolism such as conjugation reactions, normally with glucuronic acid, sulphate, glutathione and in rare cases amino acids.

The oxidative reactions are normally classed as Phase I metabolism, which also includes any reductive reactions, while Phase II metabolism covers the conjugation reactions and also includes methylation and acetylation[1].

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As a result of the wide range of polarities that the drug and its metabolites cover, the most widely used method for their analysis is by reversed-phase high-performance liquid chromatography (HPLC) utilising gradient elution in order to ensure good separation of all the components.

The most commonly used methods of detection for HPLC are fixed or scanning UV detectors or, if the drug is ^{14}C-labelled, a radiochemical detector. However, the information that these detectors can provide is limited with the radiochemical detector mainly being used for quantitative work. Identification of potential metabolites would therefore require isolation and analysis by NMR and mass spectrometry.

One method that can provide both structural and molecular weight information on metabolites is coupling HPLC with mass spectrometry (LC–MS). In the early days this was achieved by using a moving belt interface[2–4]. This interface enjoyed some success when used for applications that required normal-phase HPLC and where the compounds of interest were relatively volatile and thermally stable.

However, for reversed-phase HPLC systems the moving belt interface was not as successful. The major problems were associated with its inability to handle mobile phases with a high aqueous content and the vapourisation of non-volatile, thermally labile compounds. The introduction of a spray device for deposition of the HPLC eluant onto the belt largely eliminated the problem associated with high aqueous mobile phases[5–6].

Both of these problems were effectively eliminated when the thermospray LC–MS interface was introduced[7,8]. This interface was capable of handling a flow-rate of 1–2 ml min^{-1} of aqueous solvent but required a volatile buffer, normally ammonium acetate, in order to effect ionisation of the solute. This removed the requirement for an external source of ionisation, although this is now available on commercial interfaces for compounds that do not ionise under thermospray conditions.

One feature of thermospray LC–MS, is that in many cases it produces a protonated molecular ion, MH^+, as the base peak with little or no fragmentation resulting in a lack of structural information. At present there are two ways that fragment ions can be induced. One is to use an external source of ionisation which imparts more energy into the system, usually resulting in increased fragmentation. The other is to use MS–MS and collisionally activate the MH^+ ion to produce daughter ions that are structurally significant.

The objective of this paper will be to demonstrate the potential of thermospray LC–MS and LC–MS–MS for the structural analysis of drug metabolites. In addition, it will also illustrate the usefulness of LC–MS–MS with multiple ion detection (MID) for the identification of metabolites at low levels. This will be achieved by describing its application to the study of the *in vivo* metabolism of 4-[2-(dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one (SK&F 101468), a dopamine D_2 receptor agonist which has potential for the treatment of Parkinson's disease.

EXPERIMENTAL

SK&F 101468 urine and plasma samples

A male cynomolgus monkey was dosed 15 mg kg^{-1} of SK&F 101468 *per os* (p.o.) and the 0–6-h urine was collected for analysis. The human urine sample was obtained by dosing a male volunteer 640 μg of SK&F 101468 p.o. and the 0–6-h urine was collected.

Both urine samples were centrifuged at 9450 *g* for 10 min to remove particulate matter. A 50-μl volume of the monkey and 100 μl of the human supernatants were then used for the analysis.

The human plasma sample was obtained after dosing a male volunteer 1.25 mg of SK&F 101468 p.o. and taking a blood sample after 2 h. The plasma obtained from the blood was worked up prior to analysis by taking 500 μl of plasma and removing the protein by precipitation with 500 μl of acetonitrile. The protein was then removed by centrifugation at 9450 *g* for 20 min after which the supernatant was taken and the acetonitrile evaporated under a stream of nitrogen leaving a final volume of approximately 100 μl. This was then stored at −20°C prior to analysis when the whole sample was used.

High-performance liquid chromatography

HPLC was carried out on a Hewlett-Packard 1090L fitted with a filter photometric detector. The chromatographic separation was carried out on a C_{18} Hewlett-Packard 100 × 4.6 mm I.D. column packed with 5-μm sized particles which was kept at room temperature. The mobile phases used were 0.1 *M* ammonium acetate (pH unadjusted) filtered through a 0.45-μm filter and acetonitrile. The system was run at a flow-rate of 1.4 ml min^{-1}. The gradient system used to effect the separation consisted of a 3-min hold at 0% acetonitrile followed by a linear ramp up to 35% acetonitrile over 17 min with a further 3-min hold at 35%. The wavelength of the UV detector was set at 254 nm and was connected in line with the mass spectrometer in all cases.

Mass spectrometry

A Finnigan MAT TSQ 46 mass spectrometer equipped with a Nova 4X SuperIncos data system was used. The chromatograph was coupled to the mass spectrometer with a Finnigan MAT thermospray interface. The typical vapouriser temperature was 120°C with a jet temperature of 300°C.

The MS–MS was carried out using argon as the collision gas at a pressure of approximately 1.4 mTorr.

Prior to any analysis by LC–MS or LC–MS–MS the system was tuned by the continuous introduction of a solution of SK&F 101468. This was made up in 0.1 *M* ammonium acetate–acetonitrile (4:1, v/v) at an approximate concentration of 2 μg ml^{-1}. All mass spectra shown were background subtracted.

RESULTS AND DISCUSSION

Due to the relatively "soft" nature of the thermospray ionisation process, thermospray LC–MS is an analytical method that is capable of providing molecular weight information on drug metabolites in biological matrices with the minimum of sample preparation. As a result of this, the amount of structural information available in thermospray mass spectra tends to be limited due to the lack of any fragment ions. This means that the sites of any structural modifications to the parent drug cannot be readily identified. However, if the mass spectrometer has a MS–MS capability, this problem can be overcome by carrying out collisionally activated decomposition (CAD) experiments to fragment the MH^+ ion and obtain structurally meaningful

daughter ions. For the discussion a conventional mass spectrum will be termed a Q1 mass spectrum while a CAD daughter ion mass spectrum will be termed a Q3 mass spectrum.

One example where this approach was used was SK&F 101468 (Fig. 1). This compound was found to be metabolised extensively by the monkey and to a lesser extent by man. The metabolites identified by LC–MS and LC–MS–MS in the monkey and human samples are listed in Fig. 1. The results of the monkey urine analysis will be discussed initially.

The UV trace and totalled selected ion chromatogram trace obtained for the monkey urine are shown in Fig. 2. The two large peaks in Fig. 2, at scan nos. 273 and 277, are spurious peaks which were produced as a result of temporary pressure fluctuations in the ion source which artificially enhanced the sensitivity. These peaks were absent from subsequent LC–MS runs of this sample. The identities of metabolites 4, 7 and 11, SK&F 96990, 104557 and 101468, respectively, were confirmed by comparison of their mass spectra and retention times with authentic standards.

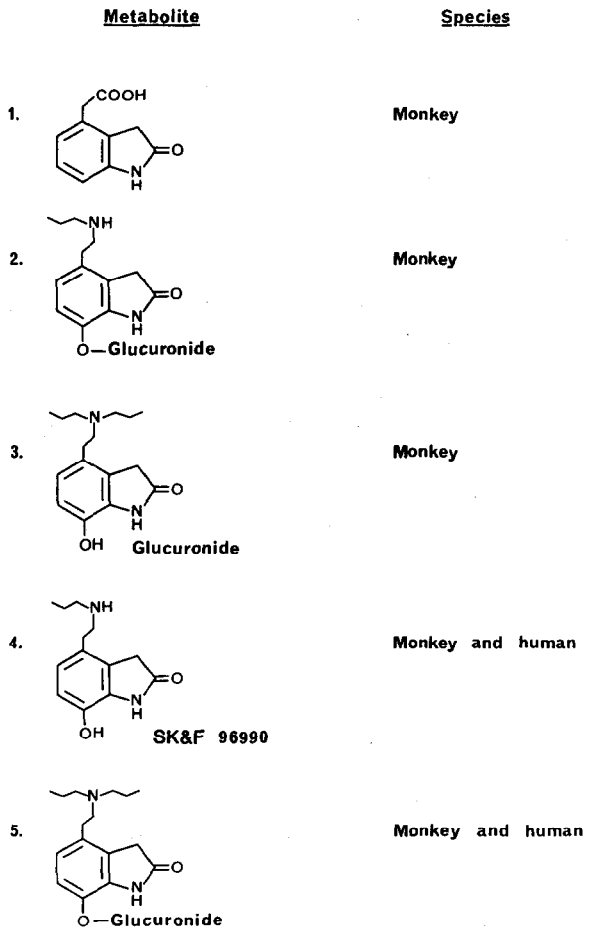

Fig. 1.

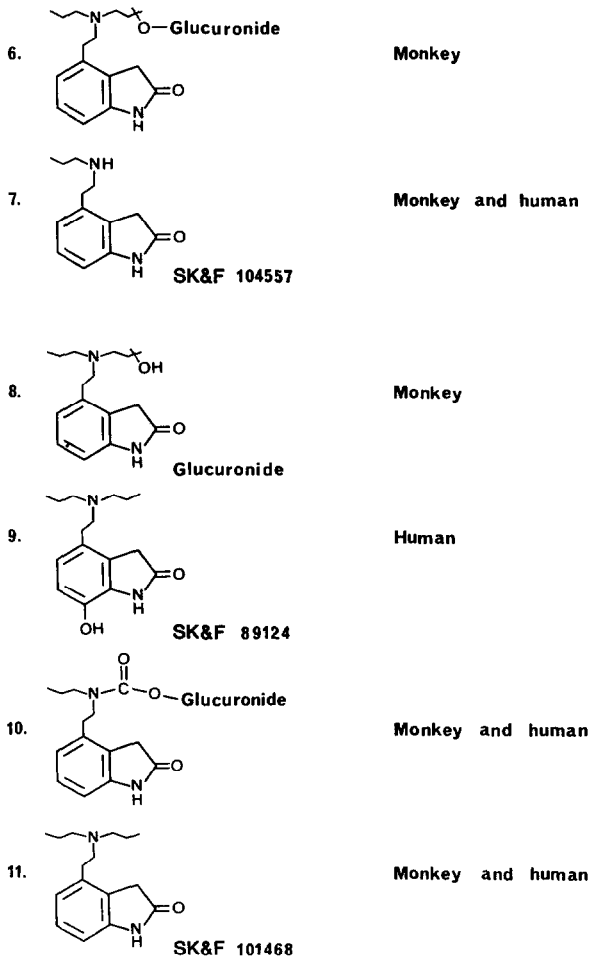

Fig. 1. Structures of SK&F 101468 and its metabolites that were found in the urine of the cynomolgus monkey and man.

The Q1 and Q3 mass spectra for metabolite 1 are shown in Fig. 3. The Q1 spectrum showed a single MH^+ ion at *m/z* 192 with no evidence of any fragmentation. However, collisional activation of *m/z* 192 resulted in the fragment ion *m/z* 146, a loss of 46 a.m.u. This corresponded to a loss of formic acid thus confirming the presence of the carboxylic acid group. This asignment was confirmed after the metabolite was isolated and analysed by NMR.

Metabolite 2 was assigned as the O-glucuronide of SK&F 96990. The presence of the glucuronide was confirmed by the ion at *m/z* 411 in the Q1 spectrum (Fig. 4a) with the aglycone ion *m/z* 235. The site of hydroxylation was narrowed down to the indolone ring system by performing CAD of *m/z* 235, (Fig. 4b), for comparison to the CAD spectrum obtained for authentic SK&F 96990.

The major fragment ions produced were *m/z* 72, 120, 148 and 176. The ion at *m/z* 72 corresponded to cleavage of the carbon–carbon bond of the ethyl link with charge

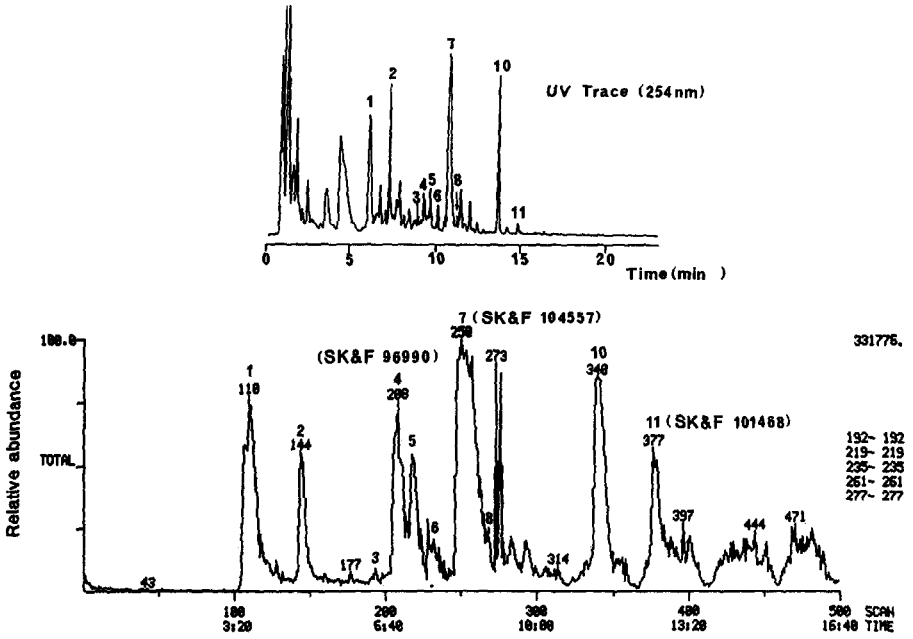

Fig. 2. UV chromatogram (254 nm) and totalled selected ion chromatogram obtained for the 0–6-h monkey urine.

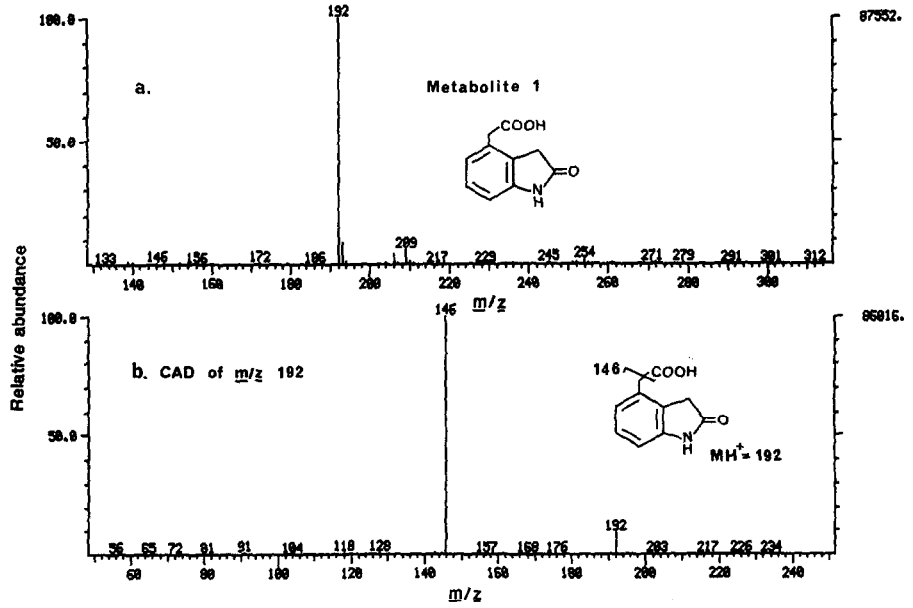

Fig. 3. (a) Thermospray Q1 mass spectrum of metabolite 1, (b) daughter ion mass spectrum of metabolite 1.

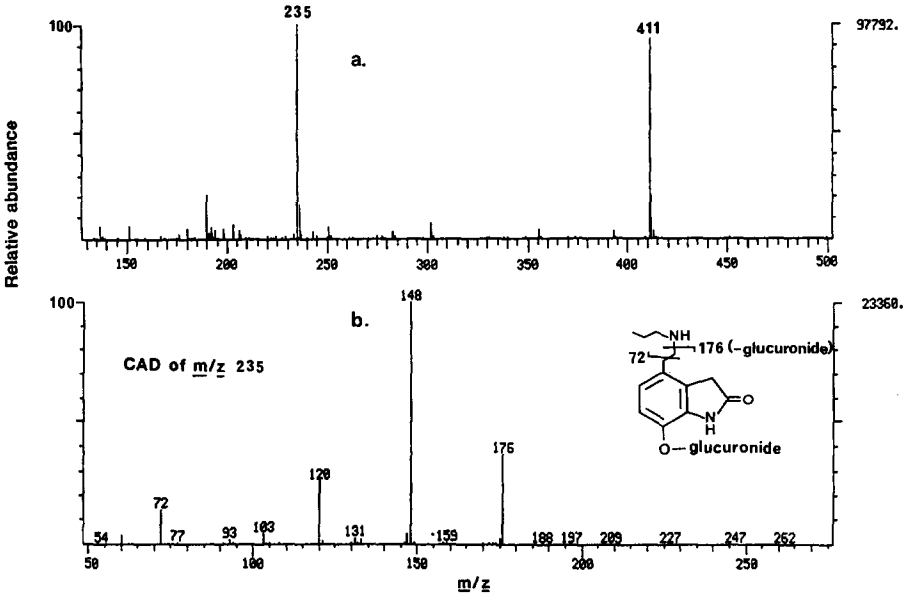

Fig. 4. (a) Thermospray Q1 mass spectrum of metabolite 2, (b) Q3 mass spectrum obtained for m/z 235, the aglycone MH^+ of metabolite 2.

retention by the propylamine fragment. Cleavage of the carbon–nitrogen bond gave rise to the ion at m/z 176 confirming that the hydroxyl group was on the indolone ring system. The two consecutive losses of 28 a.m.u. forming m/z 148 and 120 are thought to be due to successive losses of CO from the lactam and the phenolic hydroxyl group. The site of hydroxylation was assigned to position 7, as shown, for two reasons. (1) The results obtained on incubation of the sample with β-glucuronidase showed that the glucuronide peak (metabolite 2) decreased with a corresponding increase in SK&F 96990 (metabolite 4). (2) The CAD spectrum was identical to that obtained for authentic SK&F 96990.

The Q1 spectra for metabolites 3 and 5 (Fig. 5a and b) are virtually identical, with a protonated MH^+ for the glucuronide conjugate at m/z 453 with the more intense aglycone ion at m/z 277. Daughters of m/z 453 (Fig 5c and d) produced the same fragment ions although the ion ratios varied. Both metabolites produced m/z 114 and 176 which confirmed that the site of hydroxylation was on the indolone ring. This was assumed to be on position 7, as shown, for both metabolites as metabolite 5 had previously been found in rat bile, isolated and then characterised by NMR as the O-glucuronide of SK&F 89124. The site of glucuronidation for metabolite 3 is unknown.

The Q1 spectra for metabolites 6 and 8 (Fig. 6a and b) also indicated that they were both glucuronides of hydroxylated SK&F 101468. However, both compounds were able to lose water from the protonated MH^+ ion of the aglycone, m/z 277. This implied that the site of hydroxylation was on one of the aliphatic chains. This was confirmed by collisionally activating m/z 453 (spectra shown in Fig. 6c and d).

The aglycone ion, m/z 277, was the base peak which underwent loss of water to

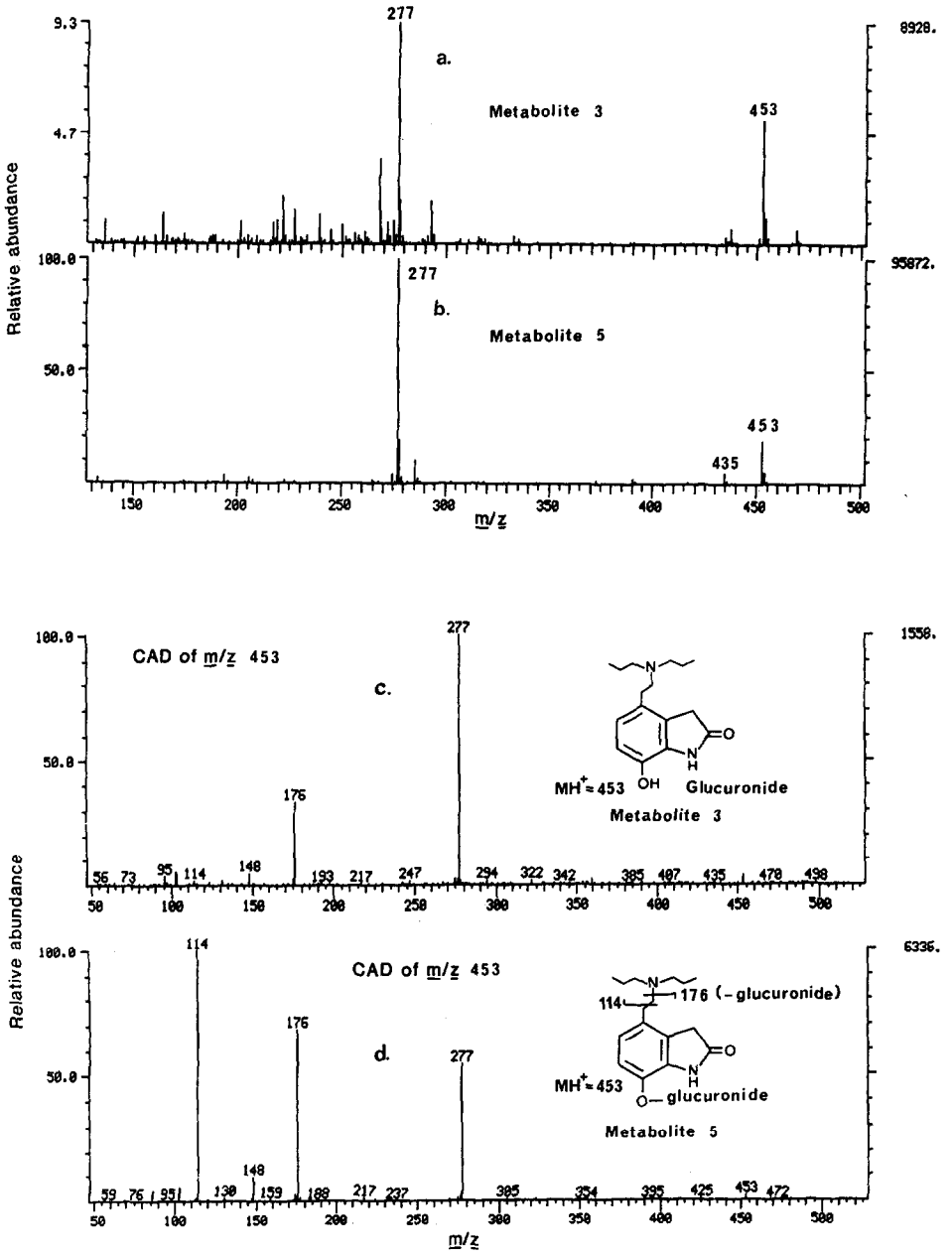

Fig. 5. (a,b) Thermospray Q1 mass spectra obtained for metabolites 3 and 5, respectively, (c,d) daughter ion mass spectra of *m/z* 453, the MH^+ ion of both metabolites 3 and 5, respectively.

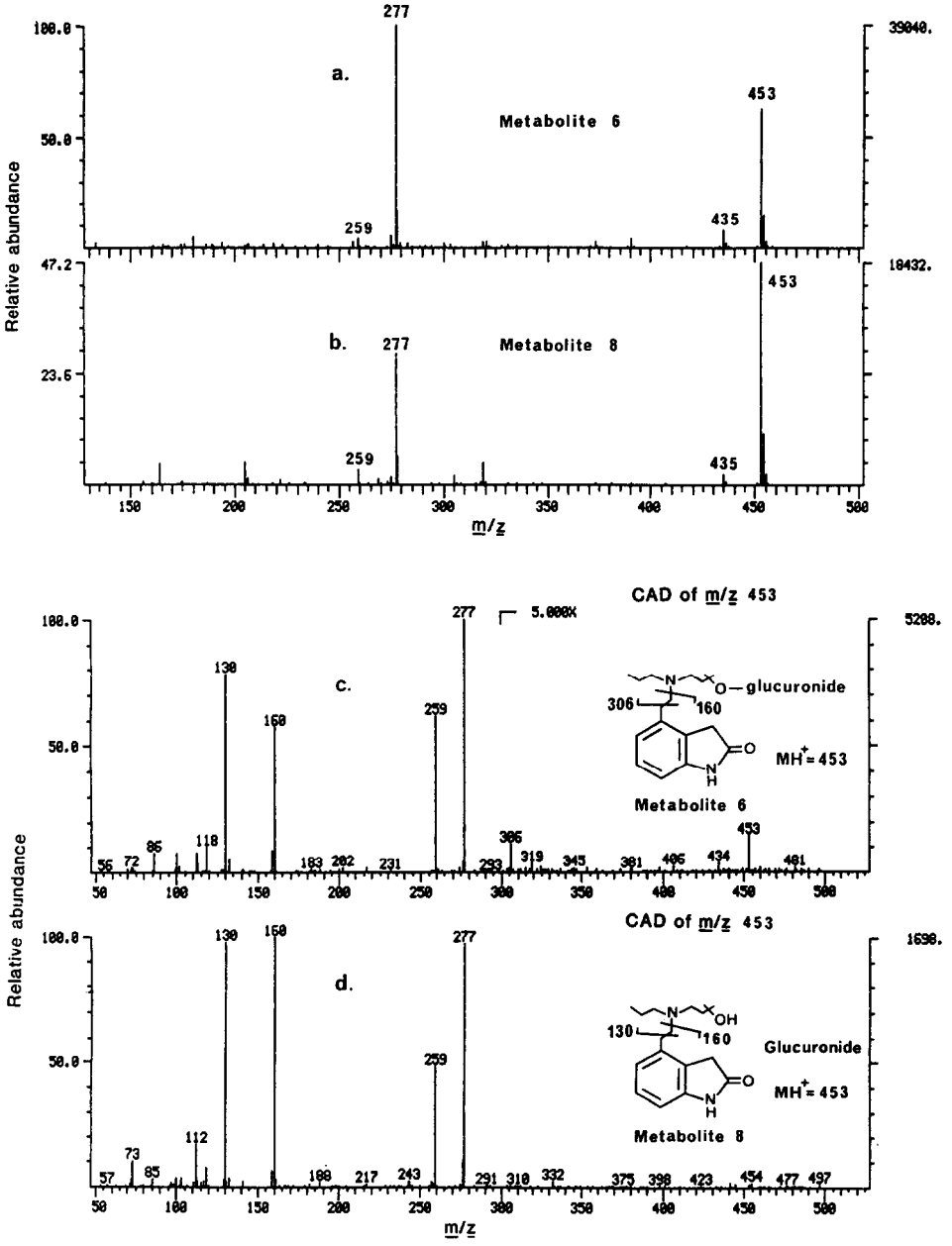

Fig. 6. (a,b) Thermospray Q1 mass spectra of metabolites 6 and 8, respectively, (c,d) Daughter ion mass spectra of *m/z* 453, the MH^+ ion of both metabolites 6 and 8, respectively.

m/z 259. The origin of the two other major fragment ions, *m/z* 130 and 160, were analogous to *m/z* 114 and 176 found for metabolites 3 and 5 with the exception that the site of hydroxylation is now on one of the propyl chains.

Metabolite 6 also produced an additional fragment ion at *m/z* 306 which represented the hydroxylated dipropylamine fragment with the glucuronide moiety still attached implying that the site of glucuronidation was on the hydroxyl group. The site of glucuronidation for metabolite 8 is unknown.

The final metabolite found in the monkey urine was metabolite 10. The Q1 spectrum (Fig. 7a) produced *m/z* 219 as the major ion which suggested a structure similar to SK&F 104557. However, two weak higher mass ions were observed at *m/z* 263 and 439. The ion at *m/z* 263 corresponded to an increase of 44 a.m.u. over *m/z* 219 and may be due to the addition of CO_2. Addition of glucuronic acid to *m/z* 263 accounted for *m/z* 439, thus confirming that the metabolite was a glucuronide conjugate.

Collisional activation of *m/z* 439 (Fig. 7b) produced the fragment ions *m/z* 263, 221, 219 and 160. Loss of the glucuronide along with proton transfer to the aglycone moiety resulted in the ion at *m/z* 263. This then demonstrated a loss of 44 a.m.u., presumably due to loss of CO_2, with concomitant proton transfer leaving the ion at *m/z* 219. The presence of the ion at *m/z* 160 confirmed that the indolone ring was present intact. The other weak fragment ion, *m/z* 221, corresponded to the glucuronide still attached to the carboxylic acid group which would suggest that the metabolite was conjugated through the acid functionality.

The metabolite was then isolated and analysed by NMR spectroscopy. It was confirmed that the substructure was SK&F 104557 and was conjugated with

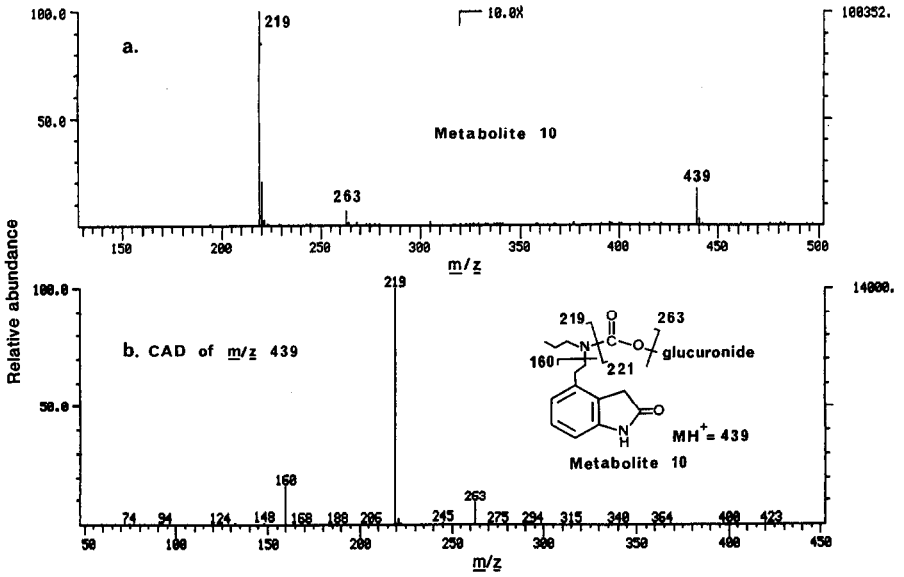

Fig. 7. (a)Thermospray Q1 mass spectrum of metabolite 10, (b) daughter ion mass spectrum obtained for *m/z* 439, the MH^+ ion of metabolite 10.

glucuronic acid. The site of the CO_2 functionality was confirmed as that shown in Fig. 1 by comparison of the chemical shifts for the α-CH_2 protons bound to nitrogen with those of SK&F 104557. The sample was also analysed by high-resolution fast atom bombardment (FAB) MS and the value for the MH^+ ion of 439.1699 corresponded well to the calculated value of 439.1717 with an error of 4.1 ppm. This confirmed that the addition of 44 a.m.u. was in fact due to CO_2.

Analysis of the human samples presented a more difficult task due to the low doses given to the human volunteers resulting in very low levels of metabolites.

The urine sample was initially analysed by LC–MS from which the selected ion traces for *m/z* 219, 235, 261 and 277 are shown in Fig. 8. By monitoring these ions all of the metabolites discussed previously, including the glucuronides, should be detected with the exception of metabolite 1 which would require a different MS–MS experiment.

From this analysis it was only possible to identify two compound related peaks, SK&F 104557 and 101468 (Fig. 8). Although there were other responses for these ions, their retention times and mass spectra did not correspond to any metabolites seen previously.

In an attempt to increase both the sensitivity and specificity it was decided to re-analyse the sample using LC–MS–MS. An experiment was created that would alternately pass *m/z* 219, 235, 261 and 277 into Q2 for collisional activation, with Q3 set for full scan to pick up the fragments.

From the MS–MS work on the monkey urine it was decided that the most important ions to look for were *m/z* 72, 160 and 176, which would identify SK&F 104557, 96990 and their conjugates, and *m/z* 114, 160 and 176 for SK&F 101468,

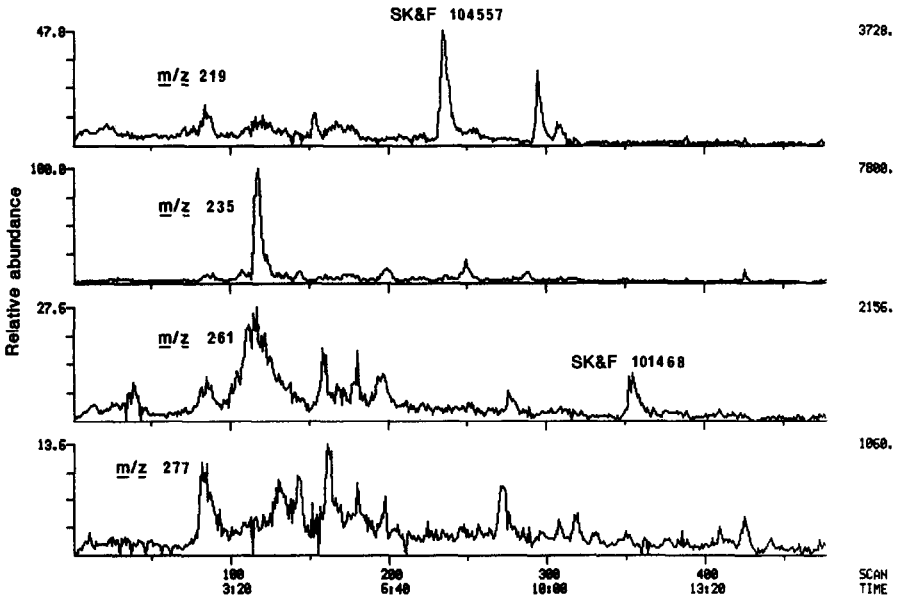

Fig. 8. Selected ion chromatogram traces of *m/z* 219, 235, 261 and 277 obtained from the LC–MS analysis of the 0–6-h human urine sample, after dosing 640 μg of SK&F 101468 p.o.

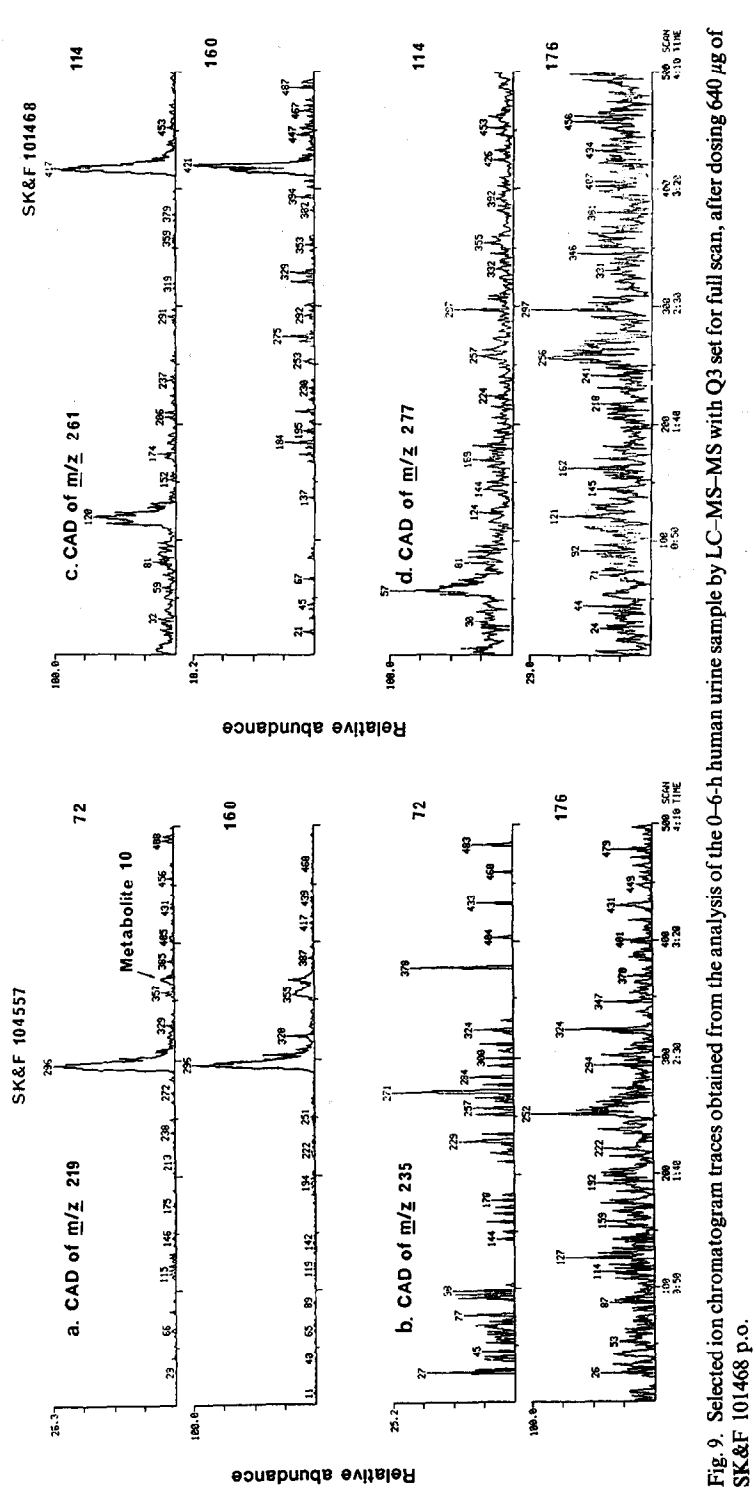

Fig. 9. Selected ion chromatogram traces obtained from the analysis of the 0–6-h human urine sample by LC–MS–MS with Q3 set for full scan, after dosing 640 μg of SK&F 101468 p.o.

SK&F 89124 and any conjugates. The results of this analysis are shown in Fig. 9. There were no responses observed for daughters of *m/z* 235 or 277 which meant that there were apparently no hydroxylated metabolites of SK&F 104557 or 101468. Two responses were observed for daughters of *m/z* 219, the major one being SK&F 104557 and the minor one corresponding to metabolite 10. There was only one response for daughters of *m/z* 261 which was attributed to SK&F 101468.

As the responses were relatively weak, an attempt was made to increase the sensitivity still further by re-analysing the sample using a similar MS–MS experiment, with the exception that Q3 was set up in MID mode to monitor the two main fragment ions of each metabolite. The results, shown in Fig. 10, were a substantial improvement over the previous results. SK&F 104557 and metabolite 10 gave a much stronger signal than before. A good response was observed for SK&F 96990 at the correct retention time. Strong responses were observed for SK&F 89124 (metabolite 9) and its O-glucuronide (metabolite 5) again at the correct retention times. There was also a substantial increase in the response for SK&F 101468 with two other possible responses which may be conjugates of SK&F 101468 which have not been seen before and their identity remains unknown. A different experiment would be required to detect metabolites 6 and 8.

The plasma sample (Fig. 11) was also analysed using this method and all of the metabolites found in the urine, were found in the plasma with the exception of metabolite 10.

CONCLUSION

The introduction of the thermospray interface has succeeded in making the coupling between HPLC and MS, in our experience, relatively routine and trouble free. It allows the direct analysis of biological samples with the minimum of sample preparation, providing structural information on drug metabolites at an early stage in the development of potential drug candidates. The technique is capable of detecting metabolites at levels of approximately 10 ng and upwards.

However, sometimes the amount of structural information obtained by thermospray LC–MS is limited as a result of the relatively "soft" nature of the ionisation method producing mainly protonated MH^+ ions. This problem can be overcome by carrying out a daughter ion MS–MS experiment in order to induce fragmentation, thus pinpointing the area of modification in the molecule.

The use of LC–MS–MS can prove invaluable for the identification of metabolites when present at levels below the detection limits of conventional LC–MS in a complex biological matrix. By switching to LC–MS–MS with Q3 on full scan the specificity of the technique is increased, as any compound related material would have to have the correct retention time, correct mass and produce structurally meaningful fragment ions. The chances of an endogenous component meeting all these requirements are remote.

However, even using this technique the sensitivity may not be sufficient, and it can be increased by setting Q3 on MID mode. The subsequent gain in sensitivity was significant and the good signal to noise ratio opened up the possibility of a quantitative LC–MS–MS assay. Although this has not yet been developed, the detection limit of the technique for SK&F 101468, when dissolved in methanol, was determined to be approximately 20 pg on column.

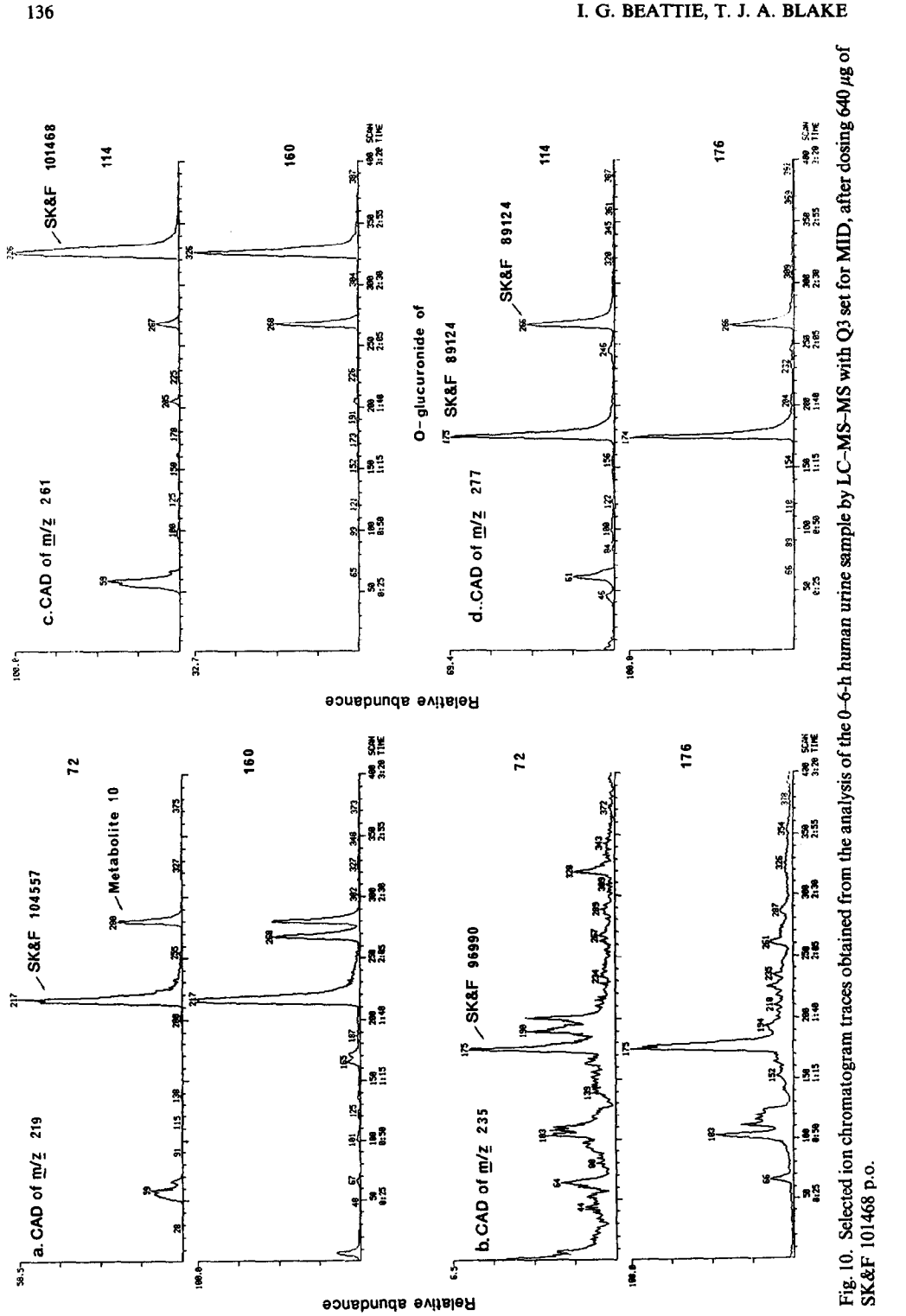

Fig. 10. Selected ion chromatogram traces obtained from the analysis of the 0–6-h human urine sample by LC–MS–MS with Q3 set for MID, after dosing 640 μg of SK&F 101468 p.o.

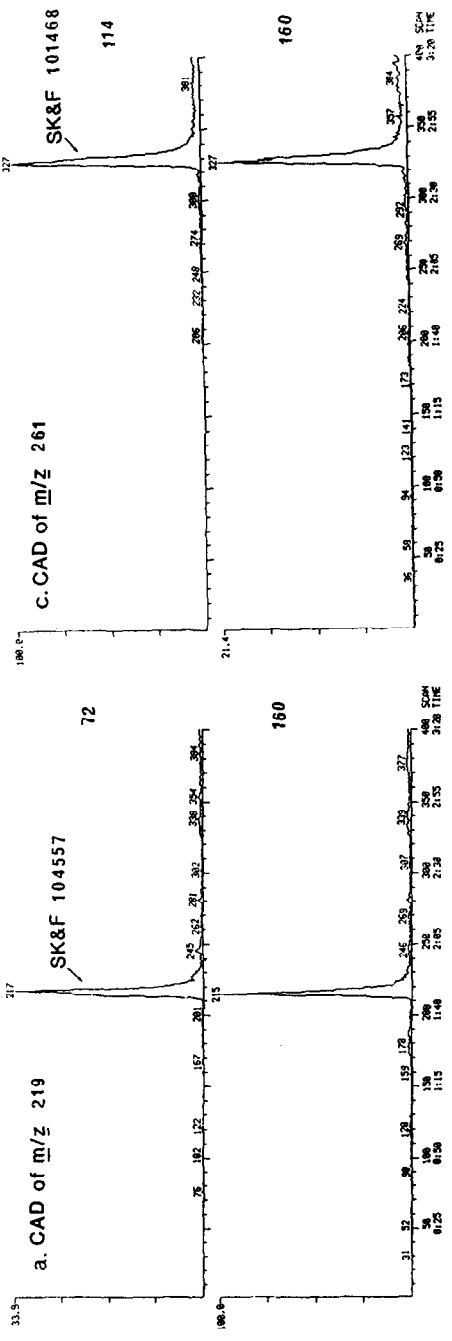

Fig. 11. Selected ion chromatogram traces obtained from the analysis of the 2-h human plasma by LC–MS–MS with Q3 set for MID, after dosing 1.25 mg of SK&F 101468 p.o.

ACKNOWLEDGEMENTS

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