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Use of thermospray liquid chromatography–mass spectrometry to aid in the identification of urinary metabolites of a novel antiepileptic drug, Lamotrigine

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ABSTRACT

The use of thermospray liquid chromatography–mass spectrometry allowed the structural elucidation of a number of urinary metabolites of Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine, formed after administering the drug to man, Cynomolgus monkey and rabbit. This data when combined with the data obtained from high-performance liquid chromatography with radiochemical detection enabled us to determine the types and amounts of unchanged drug and metabolites excreted in urine by man and a number of laboratory animal species. This technique was particularly useful as it highlighted a previously unknown fact that Lamotrigine is metabolised to form two different N-glucuronides, one of which is resistant to cleavage *in vitro* by a crude β-glucuronidase preparation from *Helix pomatia*.

INTRODUCTION

For the registration of any potential drug candidate it is essential to provide evidence that the drug's general metabolism in humans is similar to its metabolism in the animal species utilised for toxicological evaluation. To obtain this information a number of studies are performed using radiolabelled and unlabelled drug. This report shows how thermospray liquid chromatography–mass spectrometry (LC–TSP-MS) played an important role in obtaining this evidence for Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine, a novel antiepileptic drug chemically unrelated to existing therapies.

Drugs are generally metabolised by a diversity of enzymes to generate more polar compounds that can be eliminated from the body. Metabolism of drugs is normally divided into two phases: phase I, functionalization type reactions such as oxidation, reduction and hydrolysis; and phase II, conjugative reactions such as glucuronidation, methylation and acetylation [1]. As a result of the breadth of polarities that a drug and its metabolites cover, the most widely used method for their analysis is reversed-phase high-performance liquid chromatography (HPLC) utilising UV and radiochemical detectors. With the advent of LC–TSP-MS [2] it has become far easier to extend this analysis to acquire structural and molecular weight information without spending vast amounts of time and effort on sample pretreatment.

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Initial work in laboratory animals using radiolabelled Lamotrigine had shown that 95% of an orally administered dose was recovered in urine while the remaining 5% was found in the faeces. Metabolite profiling of the urine samples using HPLC with UV and radiochemical detection had suggested the following: significant interspecies variation with complex metabolite patterns; man excreted approximately 90% of the dose in urine as a polar metabolite; this polar metabolite was also excreted by Cynomolgus monkey, rat and rabbit and experiments with β-glucuronidase indicated this polar metabolite was a glucuronide of Lamotrigine. This report shows how LC–TSP-MS enabled the confirmation and extension of these findings.

MATERIALS AND METHODS

Sample pretreatment

All samples were stored at −20°C prior to analysis. Prior to examination by LC–TSP-MS all samples were filtered through 25 mm Millex 0.45-μl filters obtained from Millipore, UK.

Treatment of samples with β-glucuronidase

A 10-ml sample of urine was mixed with 700 μl of 1 *M* sodium acetate buffer, pH 5, and 500 μl of a crude solution of β-glucuronidase, *Helix pomatia* (G0876, Sigma). This mixture was incubated overnight (16 h) at 37°C.

Instrumentation

HPLC was performed on a Hewlett-Packard 1090L DR5 ternary pumping system with a Waters 490 programmable multiwavelength detector monitoring at 308, 304, 295 or 257 nm. The first ^{14}C human urine separations were carried out on a 5 μm Zorbax C_8 column (250 mm × 4.6 mm I.D.) protected by a 5-μm Zorbax C_8 guard column (12.5 mm × 4 mm I.D.) with a column temperature of 45°C. The mobile phase consisted of acetonitrile–aqueous 0.1 *M* ammonium acetate which was programmed from 5:95 to 95:5 (v/v) over 0.5 h with a flow of 1.5 or 2.0 ml/min. All subsequent work was performed on a 5-μm ChromSpher C_8 glass cartridge column (100 mm × 3 mm I.D.) protected by a 30–40 μm pellicular reversed-phase guard column (10 mm × 2.1 mm I.D.) with a column temperature of 40°C. The mobile phase was identical to that used with the Zorbax C_8 column except for the examination of component R, for which the mobile phase was acetonitrile–aqueous 0.1 *M* ammonium acetate (7.5:92.5). The flow-rate was 0.5 ml/min with a make-up solvent consisting of 0.05 *M* ammonium acetate in acetonitrile–water (30:70) at a flow-rate of 1.5 ml/min. The make-up solvent was added to the column effluent prior to UV detection.

The mass spectral data was acquired on a Finnigan MAT TSQ70 mass spectrometer equipped with a Finnigan MAT TSP 1 interface. The aerosol temperature varied from 250 to 300°C, the vaporiser temperature varied from 98 to 115°C and the repeller voltage varied from 60 to 80 V. Prior to use the system was optimised to solvent background ions, particularly an ion at m/z 433. Collisional dissociation experiments provided no additional data.

RESULTS

^{14}C labelled study in man

The first urine samples examined by LC–TSP-MS were from the oral administration to man of 240 mg (*ca.* 3.0 mg/kg) Lamotrigine. This dose administered as a gelatin capsule contained 15 μCi of ^{14}C material {3,5-diamino-6-(2,3-dichlorophenyl)-[5-^{14}C-]1,2,4-triazine} prepared by co-precipitation of [^{14}C]Lamotrigine (54.5 μCi/mg) and Lamotrigine from a methanolic solution. The urine samples analysed were pooled samples collected during the following time periods: 0–24 h and 24–48 h. Examples of the total and selected ion chromatograms obtained before and after incubation with β-glucuronidase are shown in Fig. 1. The mass spectra of com-

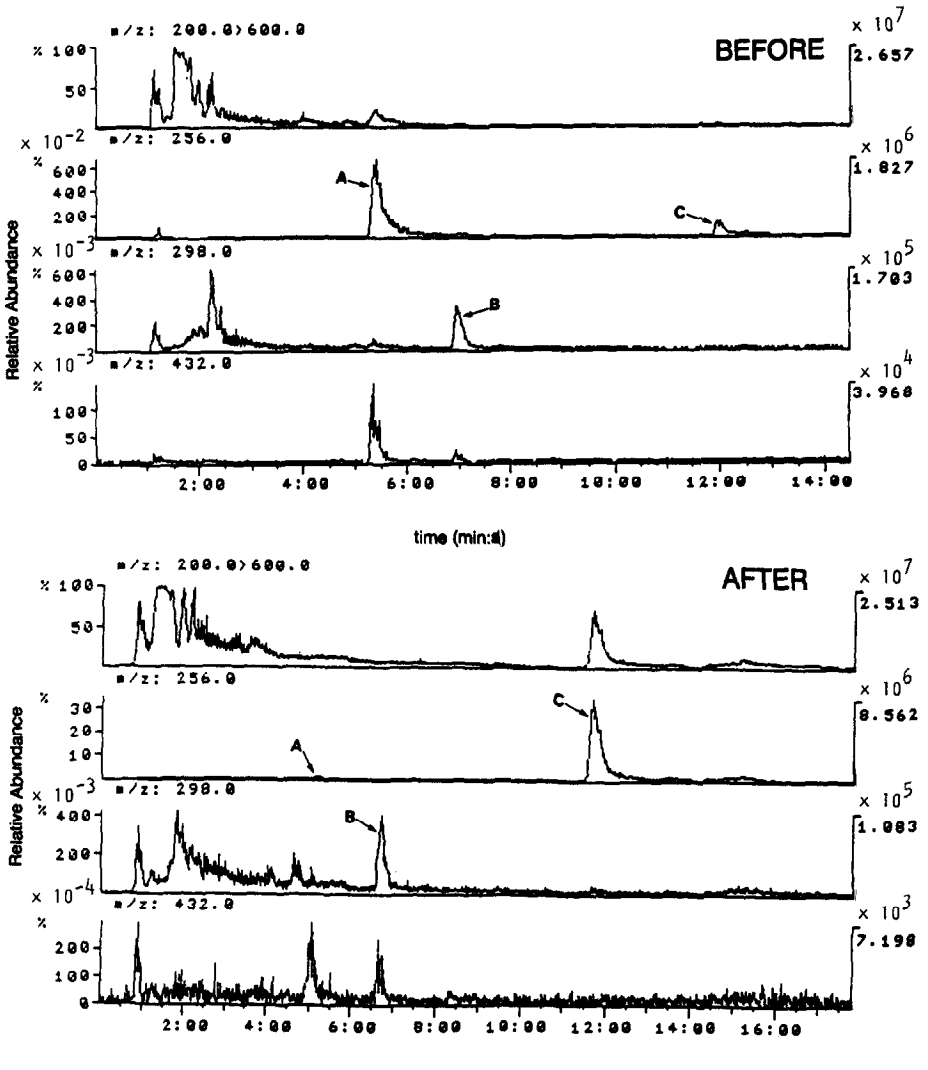

Fig. 1. Total and selected ion chromatograms obtained from LC–TSP-MS of 100-μl injections of human urine before and after treatment with β-glucuronidase.

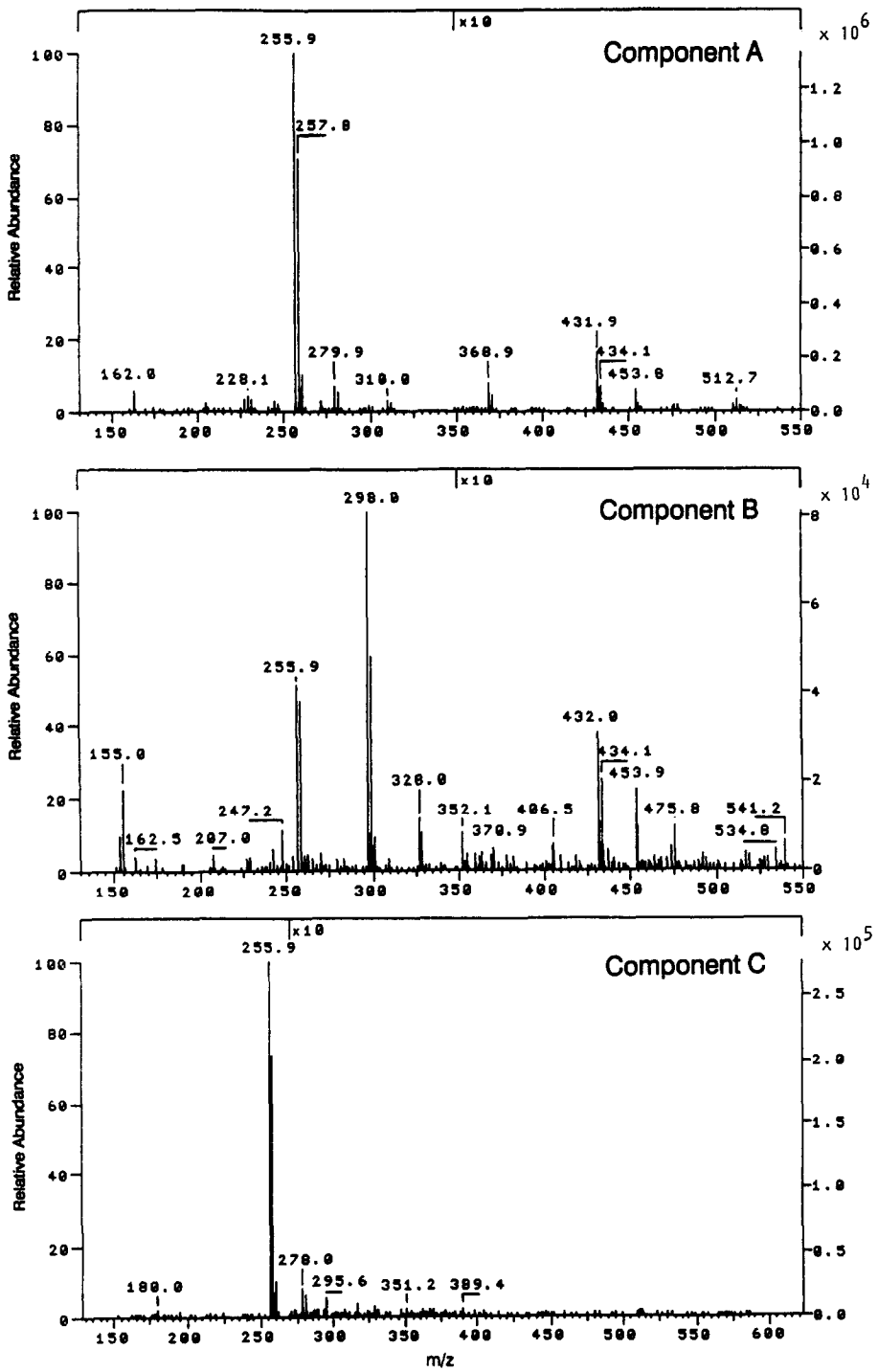

Fig. 2. Mass spectra of the components labelled A, B and C in Fig. 1 obtained by LC–TSP-MS after injection of 100 μl of human urine.

ponents A, B and C are shown in Fig. 2. The spectrum of component C consists essentially of a single ion at *m/z* 256 with the expected characteristic isotope pattern for a molecule containing two atoms of chlorine. The mass spectral data plus the results obtained after incubation with β-glucuronidase indicate that component C is the unchanged drug, Lamotrigine. The spectrum of component A has *m/z* 256 as the base peak and an ion at *m/z* 432, the expected protonated molecular ion for a glucuronide of Lamotrigine, and fragment ions at *m/z* 280 and 310 which are likely to be produced by cleavage across the glucuronic acid ring with concomitant loss of water. The spectrum of component B also contains a peak at *m/z* 432 but in this case the ion at *m/z* 256 due to the protonated aglycone is no longer the base peak. The base peak at *m/z* 298 and the ion at *m/z* 328 could again be formed by cleavage across the glucuronic acid ring but in this case without the loss of water. These results, together with the results obtained from the enzyme work with β-glucuronidase and radiochemical detection indicated that man produces two N-glucuronides of Lamotrigine of which only the major one is cleaved by the action of β-glucuronidase. Evidence of an N-oxide and a methylated metabolite were also observed in some of these urine samples but this represented less than 5% of the total radioactivity injected on to the liquid chromatograph and was only positively assigned when the results were compared with those obtained from the urines of laboratory animals.

^{14}C labelled studies in laboratory animals

Cynomolgus monkeys were dosed orally at 10 mg/kg and the 0–6-h, 6–24-h and 24–48-h urine samples were analysed.

The total and selected ion chromatograms obtained from a 100-μl injection from a 6–24-h urine sample are shown in Fig. 3. The spectra obtained for components A, B and C were the same as those obtained from human urine and represented the presence of two different N-glucoronides and unchanged Lamotrigine. The spectrum obtained for component D is shown in Fig. 4. Its HPLC retention time and LC–TSP-MS spectrum were identical to the data obtained from the chemically synthesized 2-N-oxide of Lamotrigine. Component E was only just detectable by the presence of an ion at *m/z* 270 and a large isotope peak at *m/z* 272. The presence of these ions and the comparison of its retention time with an authentic standard indicated that it was the 2-N-methyl analogue of Lamotrigine. Rabbits were orally dosed with 30 mg/kg of Lamotrigine. When 100 μl of a 0–24 h sample of urine was examined by LC–TSP-MS a large broad peak was observed in the *m/z* 256 selected ion chromatogram with a retention time equivalent to components A and B. Comparison of the *m/z* 272 and 274 ion chromatograms indicated the presence of a small amount of the 2-N-oxide of Lamotrigine. When 10 μl of rabbit urine was analysed using a chromatographic system in which the 0.05 *M* ammonium acetate was replaced by a 0.1-*M* solution a good chromatographic peak was obtained. The retention time and mass spectrum obtained from this peak indicated that the major drug related component excreted in urine by rabbits was the N-glucuronide of Lamotrigine that is resistant to hydrolysis by β-glucuronidase.

Further studies with human urine

Our American colleagues reported [3] that when certain clinical urine samples were kept at room temperature the major metabolite, component A, appeared to

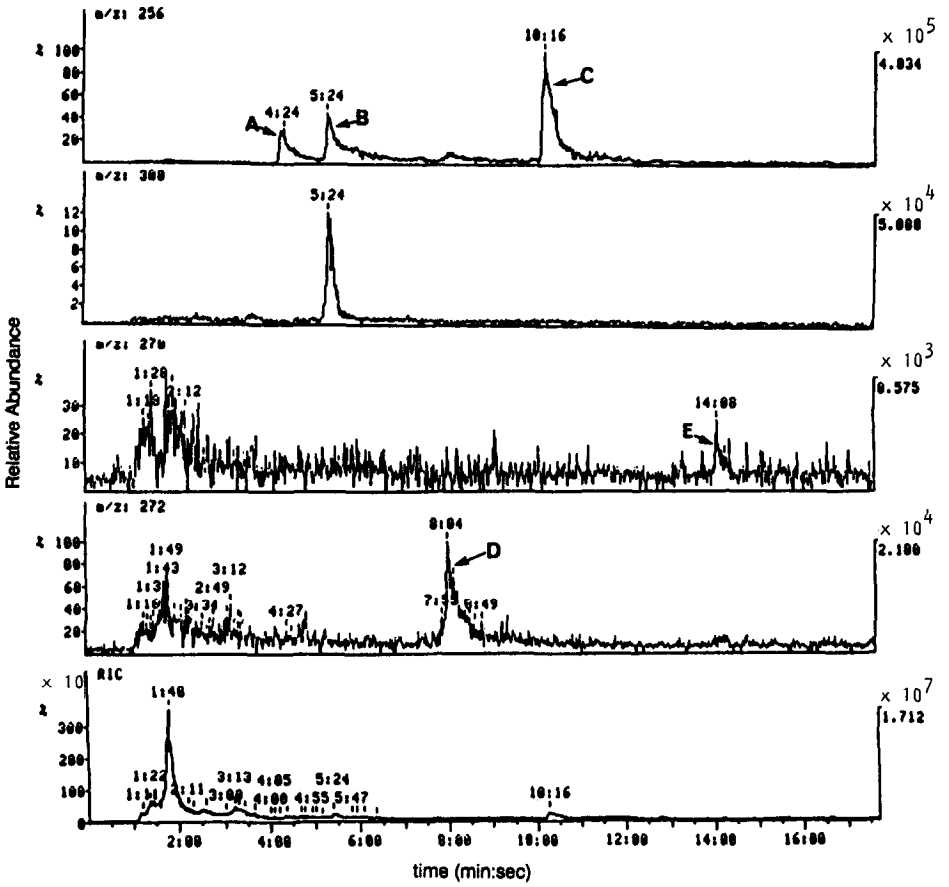

Fig. 3. Total and selected ion chromatograms obtained from an injection of 100 μl of Cynomolgus monkey urine.

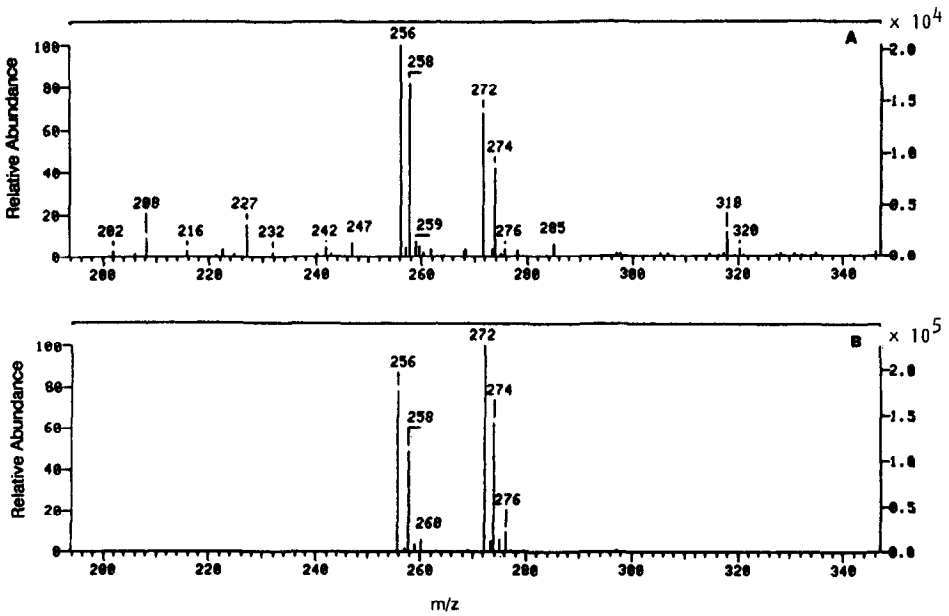

Fig. 4. Spectra obtained from (A) component D in Cynomolgus monkey urine and (B) authentic 2-N-oxide of Lamotrigine.

slowly convert to another component R, which was resistant to hydrolysis by β-glucuronidase. When we kept a sample of one of these urines (pH 7.6) at 37°C we also observed the formation of R. The spectrum of component R (Fig. 5) is consistent with a compound of molecular weight 432 formed by hydrolytic deamination of component A. The ions at m/z 433, 455 and 477 are $(M + H)^+$, $(M + Na)^+$ and $(M + 2Na - H)^+$, respectively. Two samples of urine obtained from volunteers dosed with Lamotrigine and shown to contain component A were also studied. When these urines, pH 5.5 and 6.5 respectively, were stored under identical conditions to the clinical samples no component R was formed.

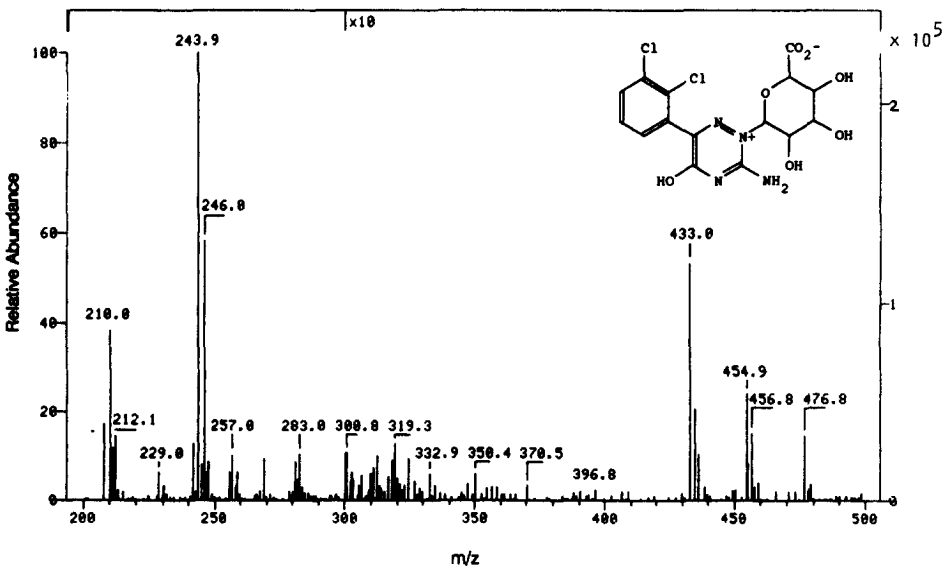

Fig. 5. The spectrum obtained from component R and its suggested structure. Component R was formed from component A when human urine with a pH 7.6 was left at room temperature.

CONCLUSIONS

The application of LC–TSP-MS allowed us to identify all the major Lamotrigine components in urine as well as many, if not all of the minor ones. Full mass spectra were obtained from samples containing approximately 100 ng of drug related material. This is important since the minor metabolites of a drug can be overlooked due to the limit on the radioactive dose that can be administered to humans and the lack of sensitivity of HPLC radiochemical detectors.

Our results, when correlated with the quantitative data obtained by reversed-phase HPLC with radiochemical detection enabled us to summarise the metabolism of Lamotrigine by man and laboratory animal species as shown in Fig. 6. Utilisation of these LC–TSP-MS conditions and facilities helped our colleagues in the Department of Physical Sciences [4] to optimise synthetic conditions for the chemical syn-

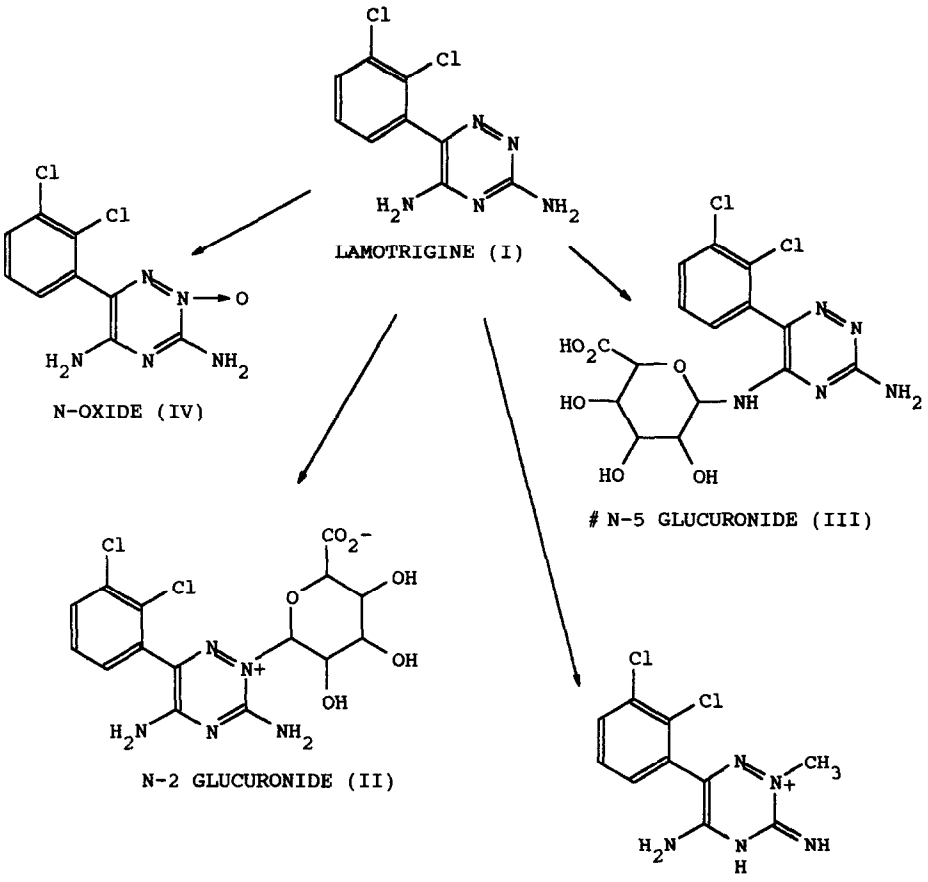

Fig. 6. A summary of the metabolism of Lamotrigine in man and laboratory animal species. Percentage of radioactivity in 0–24-h urine sample for (I) Lamotrigine: primates/man 7–30%, rabbit 3%, dog 2%, rat 50–60%; (II) N-2 glucuronide: man 80–90%, monkey 20–30%, rabbit 20–30%, rat 5–10%; (III) N-5 glucuronide: man 10%, monkey 30–40%, rabbit 40–70%, rat 5–10%; (IV) N-oxide: man 0–5%, monkey 5%, rat 10–25%; (V) methylated metabolite' dog 77%, other species 0–5%. # = Site of glucuronidation uncertain.

thesis of the N-2 glucuronide of Lamotrigine; to isolate, purify and characterise component A in human urine as the N-2 glucuronide of Lamotrigine; and to isolate, purify and tentatively conclude that the most likely structure of component B found in human and rabbit urine is an N-5 glucuronide of Lamotrigine. As we found with rabbit urine the chromatographic behaviour of component B greatly hampered its isolation, purification and characterisation and thus its absolute structural assignment is tentative rather than conclusive.

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