J. Pharm. Pharmac., 1978, 30, 547–553 Received March 20, 1978

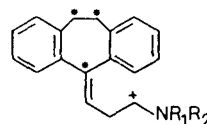
The use of ^{13}C-nmr spectroscopy for the detection and identification of metabolites of carbon-13 labelled amitriptyline

D. R. HAWKINS* AND I. MIDGLEY

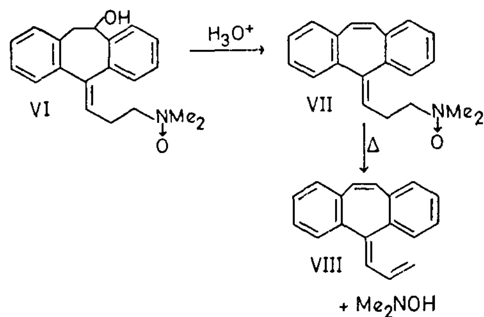
Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre, Huntingdon, PE18 6ES, U.K.

The antidepressant drug amitriptyline and two of its metabolites, nortriptyline and desmethylnortriptyline, each containing two ^{13}C atoms, have been used to determine the sensitivity and selectivity of ^{13}C-nmr spectroscopy for the detection of unchanged amitriptyline and *N*-desmethyl metabolites in the urine of animals dosed orally with the labelled drug. The resonance signals from the ^{13}C atoms detected in the ^{13}C-nmr spectrum of the entire extract from a control 12 h rat urine sample to which 1 mg of each labelled compound had been added were easily detected, using an instrument accumulation time of 1 h. The ^{13}C-nmr spectrum of an extract of hydrolysed urine from a dog that had received an oral dose of [$^{13}C_2$]amitriptyline (30 mg) exhibited signals that could be assigned to metabolites resulting from *N*-dealkylation and *N*-oxidation, as well as those bearing the intact amitriptyline side-chain. These assignments were confirmed by analysis of the same extract by g.c.–ms and h.p.l.c.

The use of radioisotopes as tracers for studying the metabolism and pharmacokinetics of drugs is the preferred technique for obtaining the maximum amount of comprehensive information (Hawkins, 1976). However, their use in man is limited and may be precluded due to unacceptable radiological hazards. Stable isotope labelled compounds do not readily provide the same information as radioisotopes, but can be exploited to provide complementary information (Hawkins, 1977). Compounds labelled with stable isotopes provide the ideal internal standards for use in the development of sensitive and specific analytical methods for drugs and metabolites. We have reported the development of such a method for the analysis of amitriptyline and its *N*-dealkylated metabolites, nortriptyline and desmethylnortriptyline using ^{13}C-labelled analogues as internal standards (Biggs, Brodie & others, 1977). We have now used the same compounds to investigate the potential of ^{13}C-nuclear magnetic resonance spectroscopy (cmr) as a technique for the detection and identification of metabolites of amitriptyline.



I (• = ^{14}C, + = ^{12}C; $R_1 = R_2$ = Me)
II (• = + = ^{13}C; $R_1 = R_2$ = Me)
III (• = + = ^{13}C; R_1 = H, R_2 = Me)
IV (• = + = ^{13}C; $R_1 = R_2$ = H)
V (• = ^{13}C, + = ^{12}C; N→O, $R_1 = R_2$ = Me)



MATERIALS AND METHODS

Materials. [$^{14}C_1$]Amitriptyline (I), [$^{13}C_2$]amitriptyline (II), [$^{13}C_2$]nortriptyline (III), [$^{13}C_2$]desmethylnortriptyline (IV), [$^{13}C_1$]amitriptyline *N*-oxide (V) and the corresponding unlabelled compounds were prepared in the authors' laboratories (Midgley, Pryor & Hawkins, 1978).

Because of the nature of the synthetic routes used, the label (*) in the singly-labelled compounds (I and V) was distributed between positions 5, 10 and 11 in the ratio 50 : 25 : 25%, respectively, whereas in the doubly-labelled compounds (II, III and IV) the second label (+) was located exclusively at the γ-position of the side-chain. The single ^{14}C atom

* Correspondence.

and first ^{13}C atom (*) were introduced using appropriately labelled barium carbonate, and the second ^{13}C atom (+) was introduced using labelled sodium cyanide. The isotopic purities of $Ba^{13}CO_3$ and Na ^{13}CN were 90 and 95%, respectively.

Animal experiments. $^{13}C_2$-Labelled amitriptyline, nortriptyline and desmethylnortriptyline hydrochlorides (1 mg of each) were added to control rat urine (10 ml). This was added to a column (20 × 2 cm) of Amberlite XAD-2 resin, which was eluted successively with water and methanol (100 ml of each). The methanol eluate was evaporated to dryness under vacuum, and the total residue used for cmr analysis. A recovery experiment using [^{14}C]-amitriptyline showed that 85–90% of the radioactivity added to urine was recovered in the methanol eluate.

For the *in vivo* experiments, a solution of [^{14}C]-amitriptyline hydrochloride (30 mg) in distilled water (1 ml) contained in a gelatin capsule was administered orally to a male beagle dog (12·4 kg). Urine was collected for 24 h at −60° (CO_2) and stored at −20° until used for analysis. About one month later, the same dog (13·7 kg) was similarly dosed with [$^{13}C_2$]amitriptyline hydrochloride (30 mg). Urine was collected and stored as above. A sample (200 ml), representing about one-half of the urine collected during the ^{13}C experiment, was hydrolysed by acidifying with conc. HCl (5 ml) and stirring at 100° for 1 h. The cooled solution was extracted with ethyl acetate (2 × 100 ml), basified with 40% NaOH solution and re-extracted with ethyl acetate (2 × 100 ml). The combined basic organic extracts were washed with water, dried ($MgSO_4$) and evaporated to dryness under vacuum. The residual brown gum was used directly for cmr, and portions of the solution used for cmr were also examined by g.c.–ms. Under the above conditions glucuronide conjugates are hydrolysed to the aglycones and 10(11)-hydroxylated metabolites of amitriptyline are dehydrated to the corresponding 10,11-dehydro (Δ^{10})-derivatives (Borga & Garle, 1972). Since it is known that 10,11-unsaturated metabolites of nortriptyline account for less than 1% of an oral dose excreted in human urine (Bertilsson & Alexanderson, 1974), identification of a Δ^{10}-metabolite indicates that it was originally present as the 10-hydroxy derivative (free or conjugated).

A small sample of the urine from the ^{14}C experiment was similarly treated. The results showed that about 60% of the administered radioactivity was recovered in the 0–24 h urine, and about three-quarters of this was extractable with ethyl acetate at pH 11 after acid hydrolysis. This extract therefore contained about 45% of the total administered dose. A sample of the extract was also examined by t.l.c.

Analysis of radioactive samples. Radioactivity was measured using a Philips liquid scintillation analyser with automatic external standard quench correction.

Thin layer chromatography (t.l.c.) was on silica gel F_{254} plates (Merck) of layer thickness 0·25 mm with chloroform–methanol–ammonia (60 : 8 : 1, v/v). Radioactive components on t.l.c. plates were detected using a Berthold Mark II scanner or by exposure of the plates to Kodak Kodirex X-ray film. Non-radioactive reference compounds were detected by their quenching of fluorescence at 254 nm.

Nuclear magnetic resonance spectroscopy. Fourier transform proton-noise decoupled carbon-13 nmr (cmr) spectra were recorded on a Bruker HX 90E spectrometer with a Nicolet 1080 computer and Kennedy magnetic tape store by the P.C.M.U., Aldermaston. The sweep width was 6000 Hz (265 ppm) and the FID data accumulation was 8 K points with an accumulation time of 1 h for all samples. Spectra were recorded on samples dissolved in [$^2H_4$]methanol or [^2H]chloroform (2 ml), and chemical shifts (δ) were measured as ppm relative to the internal standard, tetramethylsilane.

Mass spectrometry. Gas chromatography-mass spectrometry (g.c.–ms) was performed on a VG-Micromass 16F instrument, with a Pye 104 chromatograph interfaced to the spectrometer via a single stage jet separator. The g.c. separation was accomplished on a 150 × 0·4 cm (i.d.) glass column packed with 3% OV-17 on Chromosorb W(HP); carrier gas helium, flow rate 30 ml min^{-1} (6·9 MPa); column and separator temperatures 225°; ion source temperature 200°.

Chemical ionization mass spectra were obtained using isobutane as reagent gas, an electron beam energy of 50 eV and an emission current of 500 μA. Components eluting from the chromatograph were detected by the integrated ion current technique over the range *m/e* 200–300 or by mass fragmentography (selected ion monitoring). In the latter mode of operation, quasimolecular $(M+1)^+$ ions were recorded by monitoring *m/e* 280 (II), 266 (III), 252 (IV), 235 (VIII, *vide infra*) and those due to the corresponding Δ^{10}-derivatives—*m/e* 278, 264, 250 and 233, respectively.

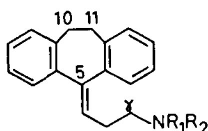
High pressure liquid chromatography. A portion of dog urine from the ^{13}C experiment was analysed qualitatively for amitriptyline metabolites by h.p.l.c. (for further details, see Biggs, Chasseaud, Hawkins & Midgley, unpublished). Thus, urine (4 ml) was

acidified with 4 M HCl (1 ml) and heated at 100° for 1 h. After cooling, the solution was basified with 4 M NaOH (1 ml) and extracted with ether (10 ml). The ether layer was separated, extracted with 0·1 M HCl (2 ml) and discarded. The aqueous phase was basified with 0·25 M NaOH (1 ml) and extracted with hexane (5 ml). Trifluoroacetic anhydride (0·2 ml) was added to the separated hexane layer, allowed to stand at room temperature (20°) for 30 min and evaporated to dryness. The residue was dissolved in methanol (50 μl) and a sample injected into a Waters Model AL202 liquid chromatograph, which used a fixed wavelength ultraviolet detector operating at 254 nm. The separation was achieved at a pressure of 2000 lb in^{-2} (137·8 MPa) using a stainless steel column (30 × 0·4 cm i.d.) packed with C-18 μBondapak. The elution solvent consisted of methanol and 0·4% aqueous KH_2PO_4 buffer (pH 3·5) programmed linearly from 45 to 90% (v/v) during 20 min, at a flow rate of 2 ml min^{-1}.

RESULTS

The ^{13}C signals in the proton-decoupled cmr spectra of the labelled reference compounds (Table 1) occur as singlets with chemical shifts characteristic of the

Table 1. *The chemical shifts of carbon*-13 *resonances in the cmr spectra of labelled amitriptyline and synthetic reference metabolites*. Figures in parentheses give the relative percentage intensities.



R_1, R_2	Compound	Solvent	Chemical shift (ppm) C-10	(11)	C-5	C-γ
$R_1=R_2=CH_3$	Amitriptyline	CD_3OD	33·0 (20·9)	34·8 (21·2)	148·5 (10·4)	58·3 (100)
$R_1=CH_3, R_2=H$	Nortriptyline	CD_3OD	33·0	34·8	148·3	50·0
$R_1=R_2=H$	Desmethylnortriptyline	CD_3OD	33·0 (22·8)	34·8 (23·6)	148·2 (9·7)	40·6 (100)
$R_1=R_2=CH_3$ N → 0	Amitriptyline *N*-oxide	$CDCl_3$	32·0 (74·1)	33·7 (100)	146·4 (83·3)	*

* This carbon was not labelled.

environment of each carbon atom. The spectra of the three amines (II–IV) each show four ^{13}C resonance signals, which were assigned to C-5, 10 and 11 in the tricyclic ring and the γ-carbon of the side-chain. The 10 and 11 carbon atoms can be individually distinguished due to their different spatial orientation relative to the side-chain. Molecular models of amitriptyline show that the seven-membered ring can adopt a twist-boat conformation in which one of these bridgehead carbons is in closer proximity to the exocyclic double bond than the other. The chemical shifts of these carbon atoms (33·0 and 34·8 ppm) were identical for the primary (IV), secondary (III) and tertiary (II) amines. Similarly the signals due to C-5 were almost identical, occurring at 148·2–148·5 ppm. The γ side-chain carbons exhibited the predicted upfield shift in the series $NMe_2 \rightarrow NHMe \rightarrow NH_2$, occurring at 58·3, 50·0 and 40·6 ppm, respectively. The relative intensities of the ^{13}C signals were consistent with the isotopic incorporation at each labelled position and the hybridization state of the carbon atom. Thus, the γ-carbon produced the most intense signal, while the signals due to the sp^3 C-10 and 11 atoms were greater than those due to the sp^2 C-5 atom, despite the greater incorporation at the latter position. The spectrum of [$^{13}C_1$]amitriptyline *N*-oxide (V) showed small upfield shifts in the C-5, 10 and 11 signals, but there was a notable difference in the relative intensities of these signals compared to the corresponding amines (II–IV).

The cmr spectrum of the extract of control rat urine to which 1 mg each of $^{13}C_2$-labelled amitriptyline, nortriptyline and desmethylnortriptyline hydrochlorides had been added is depicted in Fig. 1. Taking into account the recovery during extraction, the sample probably contained about 800 μg of each compound. The signals due to the 10, 11 and γ-carbons are clearly visible, although the γ signal of nortriptyline is partially obscured by the solvent. There is essentially no background interference from endogenous urine components in the crude extract, which probably contains most of the normal constituents of urine except salts and urea. This demonstrates the potential sensitivity of cmr for the detection of metabolites of ^{13}C-labelled drugs in urine using only the minimum isolation and purification procedures.

The cmr spectrum of the urine extract of a dog dosed orally with [$^{13}C_2$]amitriptyline hydrochloride (30 mg) is shown in Fig. 2. It shows a complex series of signals, consistent with the large number of metabolites detected by t.l.c. of the urine extract from the radioactive experiment. The most intense signal occurs at 58·0 ppm and corresponds to the γ-carbon of amitriptyline or other metabolites bearing the intact $CH_2CH_2NMe_2$ side-chain. The low intensity signals at 41·3 and 39·3 ppm correspond

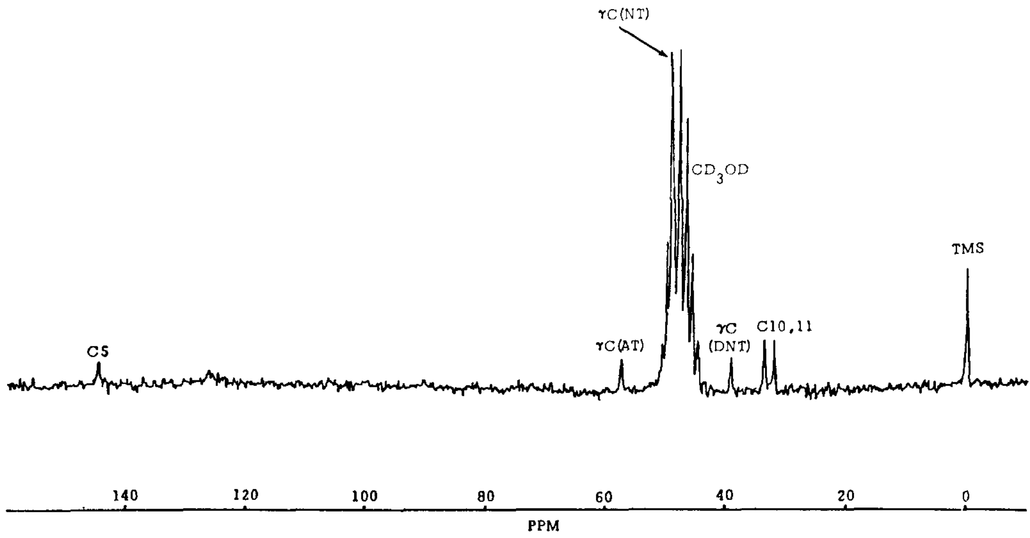


FIG. 1. Cmr spectrum of a 0–24 h rat urine extract containing $^{13}C_2$-labelled amitriptyline (AT), nortriptyline (NT) and desmethylnortriptyline (DNT) (about 1 mg of each).

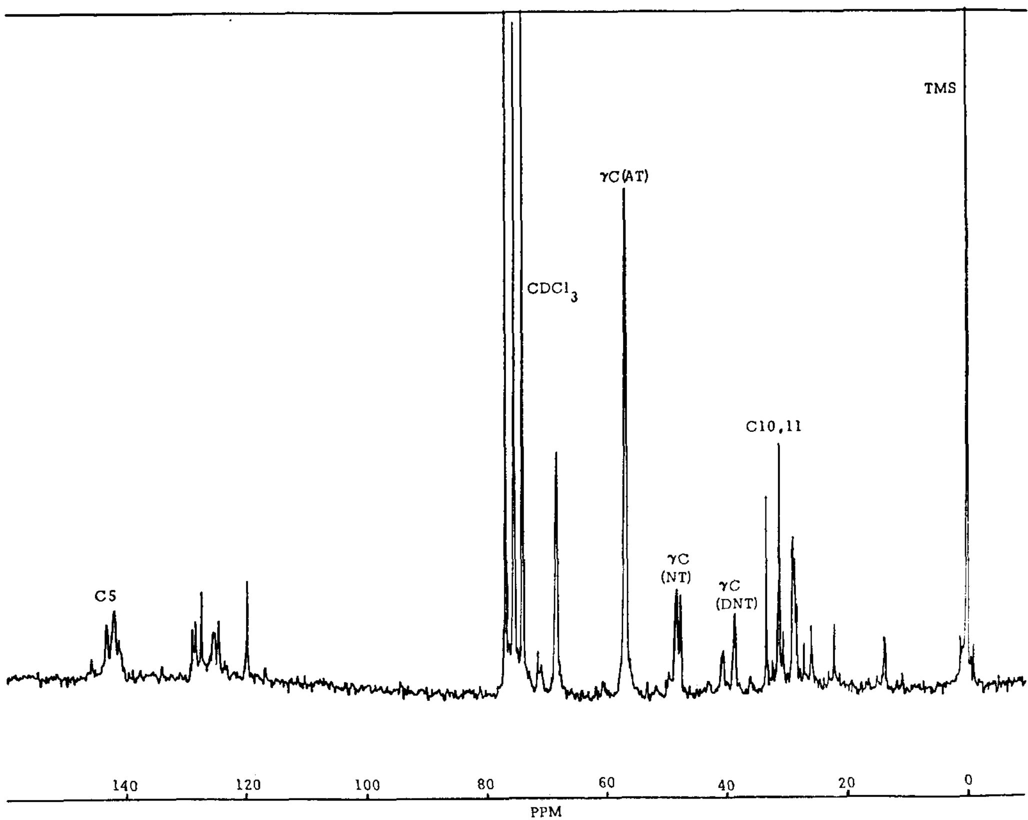


FIG. 2. Cmr spectrum of an extract of 0–24 h dog urine collected after oral administration of 30 mg of $^{13}C_2$-amitriptyline hydrochloride. Abbreviations as for Fig. 1.

to the γ-carbons of nortriptyline and desmethylnortriptyline, respectively, and several other signals in this region probably represent metabolites also containing the CH_2CH_2NHMe and $CH_2CH_2NH_2$ side-chains. The position of the second most abundant peak (69·7 ppm) indicates that it arose from the γ-carbon atom of a biotransformation product whose nitrogen substituent was capable of exerting a strong deshielding effect, such as an *N*-oxidized metabolite. The deshielding of the protons on a methylene group adjacent to an *N*-oxide group, for example, has been observed in a ^{1}H nmr spectrum (Kirkpatrick, Weston & others, 1978). Two signals at 34·0 and 31·8 ppm must represent C-10 and 11, but the origin of the other signals in this region of the spectrum is unclear. There are also two other distinct groups of signals: those at 144 ppm, which must represent C-5 carbons, and several at 120–130 ppm, which are probably due to carbons 10 and 11 of the dehydro (Δ^{10}) compounds.

G.c.–ms analysis of the same urine extract, using conventional integrated ion current detection, demonstrated the presence of a major component which was identified as 10-hydroxyamitriptyline *N*-oxide (VI). This compound, which has also been identified as an important metabolite of amitriptyline *N*-oxide in human urine (Hawkins, Midgley & Chasseaud, 1978), was dehydrated during the acid hydrolysis treatment to the Δ^{10}-analogue (VII) and thermally degraded via the Cope elimination during gas chromatography to the olefin VIII ($^{13}C_2$; mol. wt = 234) and dimethylhydroxylamine.

A chemical ionization mass fragmentogram of this urine extract is shown in Fig. 3, where the quasi-molecular $(M + 1)^+$ ions of the $^{13}C_2$-labelled reference compounds were monitored. The retention times of authentic reference compounds are indicated. Two components with retention times of 4 and 5 min showed a response to the ions *m/e* 235 and 233, respectively, but no significant response to the other ions monitored. These retention times corresponded to the Cope elimination products of amitriptyline *N*-oxide and its Δ^{10}-analogue, respectively, and their respective peak heights indicate that the dehydro metabolite was considerbly more abundant than the other. Two other components corresponded to Δ^{10}-amitriptyline (Rt = 10 min) and Δ^{10}-nortriptyline (Rt = 11·4 min), but there were only insignificant amounts of amitriptyline, nortriptyline and desmethylnortriptyline. The mass spectrometry results indicate, therefore, that the most intense signal in the cmr spectrum of the urine extract was due principally to Δ^{10}-amitriptyline, and that the second most intense signal was due mainly to Δ^{10}-amitriptyline *N*-oxide, but with a small contribution from amitriptyline *N*-oxide.

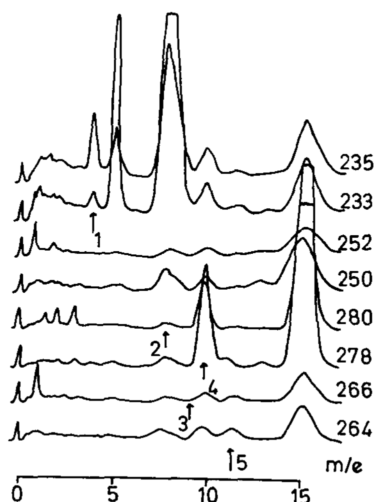


FIG. 3. A mass fragmentogram of the dog urine extract used for cmr (Fig. 2). Key: (1) amitriptyline *N*-oxide (2) amitriptyline (3) nortriptyline (4) Δ^{10}-amitriptyline (5) Δ^{10}-nortriptyline abscissa: time (min).

Additional confirmation of the presence of Δ^{10}-amitriptyline, Δ^{10}-nortriptyline and Δ^{10}-desmethylnortriptyline in dog urine was obtained by h.p.l.c. (Fig. 4). The extraction procedure used for this analytical method was different to that used for nmr and g.c.–ms (see Methods) and did not permit extraction of *N*-oxide metabolites, but the chromatogram clearly illustrates the presence of these Δ^{10}-

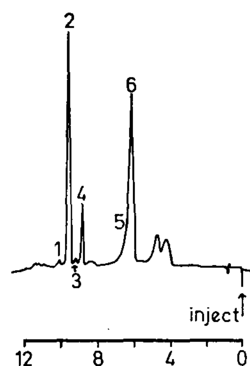


FIG. 4. An h.p.l.c. chromatogram of a dog urine extract similar to that used for cmr (Fig. 2). Key: (1) Nortriptyline (2) Δ^{10}-nortriptyline (3) desmethylnortriptyline (4) Δ^{10}-desmethylnortriptyline (5) amitriptyline (6) Δ^{10}-amitriptyline abscissa: time (min).

amines with retention times of 6, 9·5 and 8·8 min respectively.

The extract of dog urine from the ^{14}C experiment prepared in the same manner used for the ^{13}C experiment was analysed by t.l.c., although this technique was not able to resolve the Δ^{10} compounds from the corresponding saturated derivatives. Radiochromatogram scanning showed the presence of three radioactive compounents with R_F values (0·87, 0·74 and 0·48) which corresponded to amitriptyline, nortriptyline and amitriptyline *N*-oxide and/or the corresponding Δ^{10} compounds, respectively, providing further evidence of the identities of these metabolites.

DISCUSSION

The technique of cmr is usually regarded as relatively insensitive compared with proton nmr, due in part to the low natural isotopic abundance (1·1%) of ^{13}C. However, the use of synthetic isotope labelling —which permits ^{13}C incorporation of over 90 atom % —increases the sensitivity by almost two orders of magnitude. There is also the advantage that the spectra of such labelled compounds can be recorded under conditions where only the signals from isotopically enriched carbons are observed. Consequently, it should be possible to obtain spectra from enriched compounds in the presence of relatively large amounts of other compounds containing only the natural abundance of ^{13}C, such as the natural components of biological fluids.

The known pathways of amitriptyline metabolism in animals and man include *N*-demethylation and hydroxylation at position 10(11) (Gram, 1974). *In vivo* formation of the *N*-oxide has not been reported in animals and the *N*-oxide has been shown to be only a minor urine metabolite in man (Santagostino, Facino & Pirillo, 1973). The use of cmr as a probe for the detection and identification of drug metabolites obviously requires the presence of a label(s) located at or adjacent to the sites of biotransformation. For this reason amitriptyline was synthesized with ^{13}C labels at positions 10(11), which would indicate metabolites resulting from oxidation at these positions, and at the γ-carbon of the side-chain, which would provide a sensitive indicator for biotransformations occurring at the adjacent nitrogen atom. Furthermore, isotope labels incorporated in these positions would be present in all the characteristic ions in the mass spectra of known amitriptyline metabolites, and hence provide useful isotopic markers for the detection and identification of metabolites by mass spectrometry.

We have demonstrated that cmr spectra of isotopically enriched compounds can be obtained on less than 1 mg samples, using an instrument accumulation time of 1 h, when these compounds are present as metabolites in animal urine, without the need for extensive purification and with no interference from signals due to endogenous urine components.

The analysis by g.c.–ms and h.p.l.c. of extracts of dog urine containing ^{13}C-labelled amitriptyline metabolites confirmed the general conclusions about the nature of these metabolites from a consideration of the cmr spectrum of the crude urine extract. In particular, the prediction of the presence of an *N*-oxidized metabolite was confirmed. The chemical shift of the γ-carbon would be characteristic of the oxidation state of the adjacent nitrogen and provide a definitive probe for the detection of urine metabolites such as *N*-hydroxy derivatives, nitrones and oximes. Thus, cmr provides an effective, non-destructive method of detecting drug metabolites in urine with only minimal isolation and purification, at the same time giving a useful insight into the structural nature of the metabolites present.

This use of cmr also provides the possibility for investigating the presence of unstable, reactive metabolites. Another application would be in the comparison of patterns of urinary metabolites from different animal species, which could be complementary to conventional chromatographic comparisons when radioisotopes were used, for example in the study of species differences in the extent of *N*-hydroxylation. In cases where the use of radioisotopes was precluded, cmr of urine extracts could also provide a means of indicating which samples and extracts contained metabolites, and detection of metabolites would then be facilitated by the use of a mixture of ^{13}C-labelled and unlabelled drugs which would yield characteristic doublets in their mass spectra (e.g. Knapp, Gaffney & McMahon, 1972).

Acknowledgement
We are grateful to Mr S. R. Biggs for performing the g.c.–ms and h.p.l.c. analyses.

REFERENCES

Bertilsson, L. & Alexanderson, B. (1974). *Eur. J. clin. Pharmac.*, **4**, 201–205.

Biggs, S. R., Brodie, R. R., Hawkins, D. R. & Midgley, I. (1977). *Proc. Eur. Soc. Toxic.*, pp. 174–176. Amsterdam Oxford: Excerpta Medica.

BORGA, O. & GARLE, M. (1972). *J. Chromat.*, **68,** 77–88.

GRAM, L. F. (1974). *Dan. med. Bull.*, **21,** 218–231.

HAWKINS, D. R. (1976). *Chem. Br.*, **12,** 379–383.

HAWKINS, D. R. (1977). *Progress in Drug Metabolism*, Vol. 2, pp. 163–218. Editors: Bridges, J. W. & Chasseaud, L. F. London: John Wiley and Sons.

HAWKINS, D. R., MIDGLEY, I. & CHASSEAUD, L. F. (1978). *Proc. 2nd Internat. Symp. on Biological Oxidation of Organic Compounds*, pp. 479–482. Editor: Gorrod, J. W. Amsterdam: Elsevier/North-Holland Biomedical Press.

KIRKPATRICK, D., WESTON, K. T., HAWKINS, D. R., CHASSEAUD, L. F. & ELSOM, L. F. (1978). *Xenobiotica*, **7,** 747.

KNAPP, D. R., GAFFNEY, T. E. & MCMAHON, R. E. (1972). *J. Pharmac. exp. Ther.*, **180,** 784.

MIDGLEY, I., PRYOR, R. W. & HAWKINS, D. R. (1978). *J. Labell. Compd. Radiopharm.*, in the press.

SANTAGOSTINO, G., FACINO, R. M. & PIRILLO, D. (1973). *J. pharm. Sci.*, **63,** 1690–1692.