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Analytical Survey

Strategies for drug metabolic profiling in human bile after administration of non-radioactive butoprozine

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Abstract: A systematic approach to the recognition, isolation and identification of metabolites of butoprozine in human bile is described as an example of the general approach in this area. Human profiles are compared with those obtained after administration of ^{14}C-labelled drug to the dog. The screening method is based on gradient-elution high-performance liquid chromatography (HPLC). Isolation is by isocratic reversed-phase HPLC and the identification procedure is performed using ultraviolet, mass and nuclear magnetic resonance spectroscopy.

Keywords: *Butoprozine metabolism in human bile; radioactive and non-radioactive drug administration; reversed-phase HPLC; UV, MS and NMR spectroscopy.*

Introduction

Labelling of a drug in one or more essential parts of the molecule by a radioactive (RA) isotope is an important tool in screening for drug metabolites [1] and can help in selecting the most relevant biological sample(s) for metabolic analysis. In HPLC separations RA-labelling can also serve as a specific detection mode for individual metabolites. In general, a drug metabolic study is commenced using a mammalian species other than man. In such experiments there are few objections to giving the RA drug.

For a good understanding of the therapeutic and toxic action of a drug, it is clearly important to have insight into the metabolic pathway of the drug in man; however, administration of a RA drug to man can pose unacceptable risks, especially when the drug is still in the development phase. Therefore animal models can be a useful alternative, but only when extrapolation of the results to man can be achieved in a relatively simple and reliable way, as discussed recently [1–3]. Several examples have

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been reported of considerable interspecies variation. Thus, in order to obtain adequate insight into the metabolic degradation of drugs in man, suitable screening methods are required for human excretory fluids after administration of a non-RA-drug.

Hitherto such systematic methods have been few. In recent publications [4–6], the present authors have proposed an approach based on HPLC, and capable of giving a reliable impression of the whole metabolic excretion profile of a drug. Methods have also been developed for recognizing metabolites in complex biological fluids, both after administration of a RA-drug and especially after administration of a non-RA 'cold' drug.

These strategies have been applied to a study aimed at elucidating the metabolic profile of butoprozine in human bile after administration of the unlabelled drug. Butoprozine, a new drug with anti-anginal properties, is to a large extent eliminated via metabolites in the bile [7]. This case study is presented as an example of approaches which can be successfully adopted in establishing the metabolic profile of a drug in man, without administration of a RA form of the drug.

Materials and Methods

Chemicals

Butoprozine samples were gifts from Labaz (Brussels, Belgium). Before use purity was checked by thin-layer chromatography (TLC) and HPLC [8]. Water was freshly obtained from a Milli Q water purifier (Millipore Co., Bedford, MA, USA). Methanol and acetonitrile were of HPLC grade and obtained from Baker (Deventer, The Netherlands). Triethylamine (TEA) and ammonia (NH_3) used as basic modifiers were of 'pro-analysi' grade and obtained from Merck (Darmstadt, FRG). All other chemicals were obtained from Merck and of 'pro-analysi' grade.

Bile samples

Dog bile was obtained from male Beagle dogs with body weights of *ca.* 15 kg. After anaesthetizing with an i.p. dose of urethane (1.28 g/kg) the bile duct was cannulated. Blank bile was then collected during a period of 1 h. At time zero ^{12}C- or ^{14}C-butoprozine were injected into the femoral vein in a dose of 5 mg/kg body wt. Treated bile was collected as hourly fractions in ice-cooled tubes in the dark. After collection, the bile was stored at −18°C. Before analysis by HPLC, the bile was subjected to a mild clean-up procedure. Part of the endogenous bile components were precipitated by adding two volumes of methanol to one volume of bile. The supernatant was then examined by HPLC.

Human bile samples were obtained from two male patients coded as follows–1: 70 years and 70 kg; and 2: 57 years and 65 kg; both had been given a biliary drain. Butoprozine was administered i.v. in a dose of 15 mg diluted in a Baxter glucose solution during 15 min. The co-medication of patient 1 was: Cefacidal®, Maalox®, Amukin®, Valoron®, Dipidolor® and a multivitamin preparation; that of patient 2 was: Primpéran®, Tagamet®, Maalox®, Valoron®, Valium®, Amukin®, Daktarin®, Rifocine® and a multivitamin Rifocine preparation.

HPLC analysis

Separation was performed using two stainless-steel columns (150 × 4.6 mm for screening experiments, and 250 × 6.2 mm for isolation) packed with 5-μm LiChrosorb RP 8 (Merck) by means of a balanced density slurry method specially developed for the

elution system used. Continuous gradient elution was carried out using gradients of water (containing 0.05 M TEA) to methanol (no TEA) in runs of 100 min at a flow-rate of 1 ml/min [4]. Isocratic elution took place with water–methanol or water–acetonitrile mixtures containing 0.05 M TEA (for screening experiments) or 0.05 M NH_3 (for isolation) at a flow-rate of 1 ml/min or 2.5 ml/min. The role of TEA and NH_3 has been described in [4] and [6]. The chromatography apparatus consisted of two M-45 pumps, a M-720 system controller and a U6K universal injector (all from Waters Associates, Milford, MA, USA).

UV-detection was by single-wavelength detector using a M-440 interchangeable filter detector (Waters) or a SF-770 variable wavelength detector (Schoeffel, NJ, USA). Off-line radioactivity detection was performed by collection of the UV-detector effluent in 0.5 ml fractions in counting vials. To each fraction, 5 ml of Picofluor 30 was then added. After mixing until a clear solution was obtained, the radioactivity was measured by liquid scintillation counting in an Isocap or Mark 300 scintillation analyzer (Nuclear–Chicago, USA). The counting efficiency was determined by the external standard channels ratio method. Values obtained varied between 70 and 80%. The UV-signal was recorded by a BD-40 recorder (Kipp, Delft, The Netherlands).

Spectroscopic methods

UV-spectroscopy. A HP-8450A multichannel diode array UV/VIS spectrophotometer (Hewlett–Packard, Palo Alto, CA, USA) was used for recording the UV-spectra.

Mass spectrometry. A M-3300 quadrupole spectrometer with a M-6110 computer (Finnigan, Sunnyvale, CA, USA) was used to record the mass spectra. The samples for electron impact (EI-MS) were introduced with a solid probe and for chemical ionization (CI-MS; OH^-, NH_3) with a desorption probe [9].

NMR spectroscopy. ^{1}H-NMR spectra were obtained with a XL-100 spectrometer (Varian, Palo Alto, CA, USA) operated in the pulsed Fourier transform mode. The pulse duration was 10 μs, the acquisition time 2 s, the spectral width 1000 Hz. The samples were dissolved in methanol-d_4 (99.9% purity for the butoprozine spectrum and 99.95% for the metabolite spectrum). The measurements were carried out at room temperature, using a 1.7 mm o.d. sample tube.

Results and Discussion

Drug metabolic profiling can be subdivided into three phases of investigation [6]: (1) screening for unknown metabolites in the excretion fluid selected; (2) isolation of the metabolites from the biological matrix; and (3) identification of the metabolites by structure elucidation techniques.

Screening

In the development of the screening methodology, HPLC was used as a separation system for investigation of the bile samples for drug metabolites. Reversed-phase HPLC has proved to be an especially suitable technique in drug metabolic profiling [4–6]. Gradient elution was chosen as the basis of a systematic approach for obtaining a complete and reliable drug metabolic profile, with gradients running from 100% water to 100% methanol in a pH range of 10–11, established with triethylamine (TEA) or

ammonia (NH_3). In this system the eluent polarity is decreased, stepwise or continuously, so that it is possible to separate the metabolites in order of decreasing polarity. The gradient elution chromatogram can be considered as a metabolic profile which gives a first impression about the existence of metabolites.

In the present studies on butoprozine metabolism in different dogs, both RA drug and non-RA drugs have been administered. In cases where a RA-drug was administered, HPLC detection was carried out by monitoring fractions for radioactivity and by UV-detection. For the non-RA drug, UV-detection was employed. These 'cold' and 'hot' parallel animal experiments enabled a non-RA screening method with UV detection to be developed and validated for experiments in which RA administration is inadvisable, i.e. in human metabolic studies. UV-detection lacks the specificity of RA detection and the metabolites must be recognized against a complex endogenous UV-absorbing background. The authors have developed screening methods based on comparison of gradient-chromatograms and decomposition of endogenous components. The performance of these methods has been checked in dog with the aid of RA counting. In the present paper, applications in human are described.

In Fig. 1 the structure of butoprozine and its behaviour in a linear-continuous gradient of 100% water to 100% methanol in 100 min is given. Metabolites with a more polar character than the parent compound can be expected to elute in the range 0–88% methanol. In the case of dog bile, at least 10 of the metabolites could be satisfactorily separated in this region and detected both with RA and UV detection. Figure 1 also shows a gradient run of bile of patient 1 after administration of 'cold' butoprozine. The 0–3 h fraction was chosen as the initial sample, because in dog this fraction contained most of the observed RA. The parent drug is not present in this human bile sample. In order to differentiate between metabolites and background, the chromatogram of a

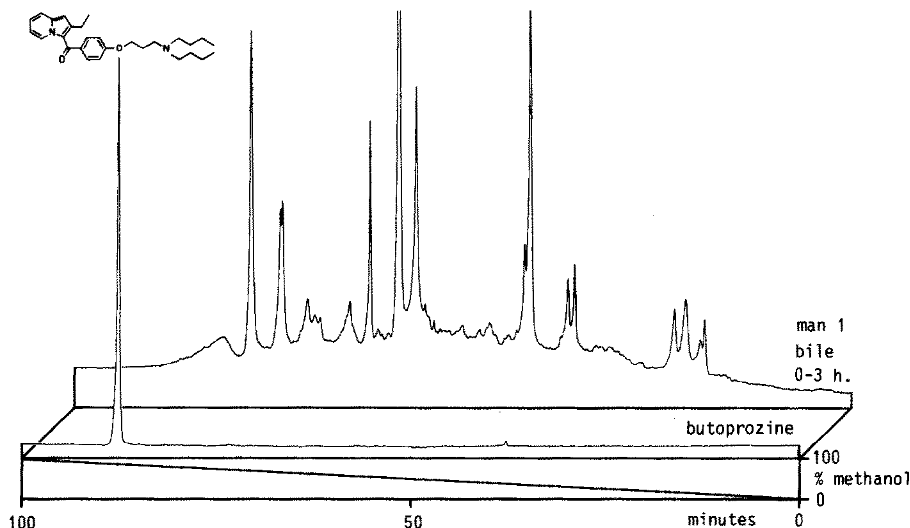

Figure 1
Continuous gradient chromatograms of butoprozine metabolites in the bile of patient 1, 0–3 h after administration of non-radioactive butoprozine. HPLC conditions: column, 150 × 4.6 mm; packing, LiChrosorb RP-8 (5 μm); eluent, 100% water (0.05 M TEA) to 100% methanol (no TEA) in 100 min; flow-rate 1 ml/min; injection volume, 30 μl; detection, UV at 405 nm, 0.05 a.u.f.s.

treated bile fraction is first compared with a chromatogram of blank bile collected before drug administration, as shown in Fig. 2 (0–3 h compared with −1 to 0 h). This permits an initial differentiation between drug metabolites and endogenous bile components to be made.

It seems likely that peak 5 is a metabolite. To obtain additional evidence it is also possible to compare chromatograms of differently treated fractions in the time. It is assumed that the background remains substantially constant. Figure 2 clearly illustrates the rise and fall of peak 5 against a fairly constant background.

With chromatograms of this kind, recorded under exactly the same conditions and facilitated by the good reproducibility of this HPLC system, it is also possible to compare biological fractions of different animals with each other. Figure 3 shows that comparison of the same fraction (0–3 h) of patients 1 and 2 gives almost the same metabolic profile. It can further be noted that the various comedications did not seem to interfere in these chromatograms (Figs 2 and 3), nor did they appear to affect the drug metabolic and endogenous profiles in these particular cases.

Figure 3 illustrates the profile of metabolism in the dog. Metabolites 1–9 were confirmed by RA detection and structure elucidation [10]. Comparison of the profiles of man and dog shows a dramatic difference in metabolism, and a remarkable correspondence in the patterns of endogenous components. Apparently, only one metabolite in the dog features also in human biliary metabolism, namely peak 5.

However, in spite of the good reproducibility of the system, it may be dangerous to simply compare retention times in different chromatograms. In order to obtain additional information it is possible to collect a metabolite fraction of one species and check its coelution with peaks in a sample of another species, as illustrated in Fig. 4, which shows two consecutive dog bile runs: one without and a second with addition of the human main metabolite. For reasons of selectivity and time isocratic conditions were used. As can be seen, the human main metabolite has the same retention time as peak 5 in the dog. This was checked in other chromatographic systems, when the human main metabolite and dog metabolite 5 appeared to have identical retention times. However, the identity can only be confirmed after isolation of both compounds and comparison of the UV, MS and NMR spectra.

During the HPLC experiments it appeared that the profile of endogenous components observed in the gradient runs changed at room temperature, since decomposition of endogenous components was apparently taking place. This led to a systematic investigation of blank bile, both for dog [7] and for human bile. Figure 5 (upper part) shows blank bile stored under the following conditions: (a) stored at −20°C in the dark and brought to room temperature for rapid HPLC analysis; and (b) stored at +5°C in the dark for 36 days. As can be seen, a notable change in the endogenous profile has taken place. This phenomenon can be exploited to differentiate between UV-absorbing metabolites and endogenous peaks, since the metabolites were stable at 5°C in the dark, as illustrated in Fig. 5 (lower part). Almost all endogenous peaks with UV-absorption at 405 nm have disappeared, whereas peak 5 appears unaffected, giving additional evidence for the metabolic character of this peak.

For reasons of simplicity only the 405 nm gradient-elution chromatograms are given. In dog and in human bile experiments, it appeared that all metabolites could be detected at 405, 280 and 235 nm. Recent structural elucidation of the dog bile metabolites showed that the butoprozine aromatic system is intact in these metabolites [10]. However in dog, metabolites 2 and 3 were rapidly converted to a decomposition product which could only

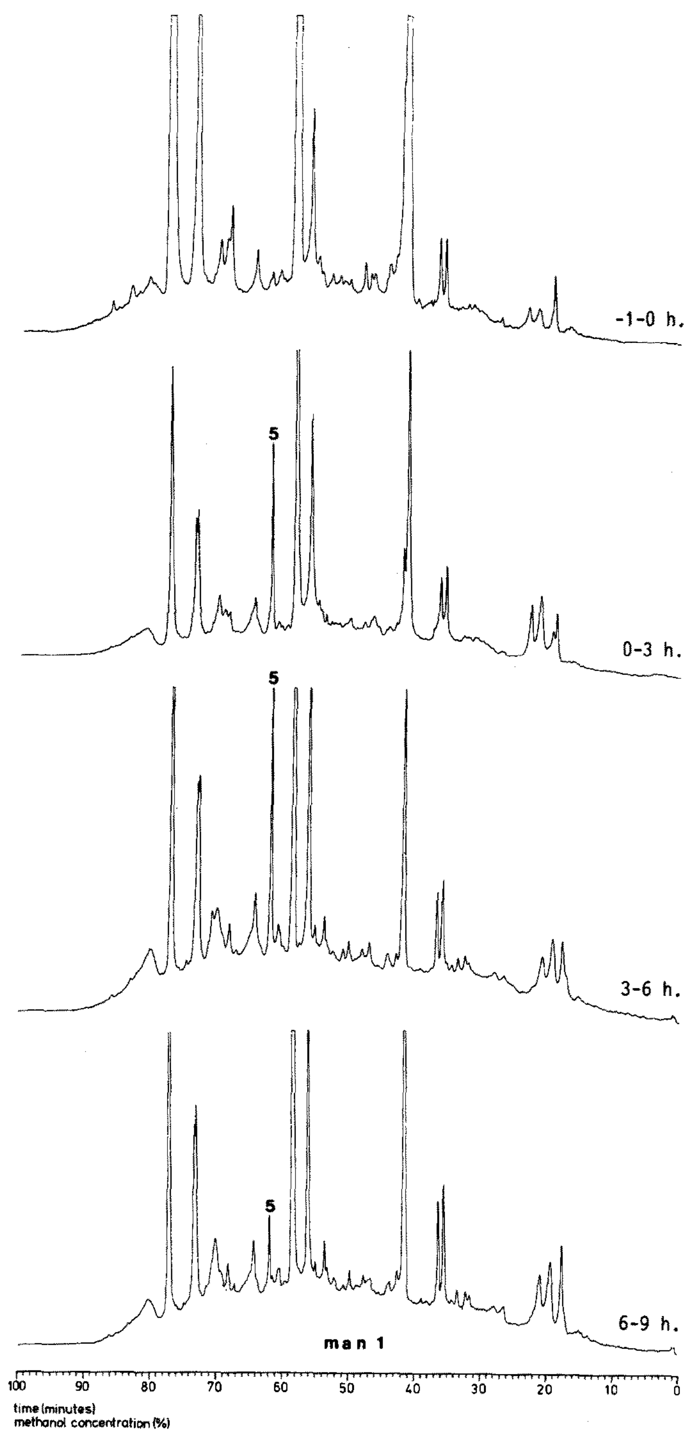

Figure 2
Gradient elution of samples of bile for patient 1 1 h before (−1 to 0 h), and 0 to 3 h, 3 to 6 h or 6 to 9 h after butoprozine administration. Peak 5 is deduced to be the human biliary metabolite in this kinetic comparison. Chromatographic conditions as in Fig. 1.

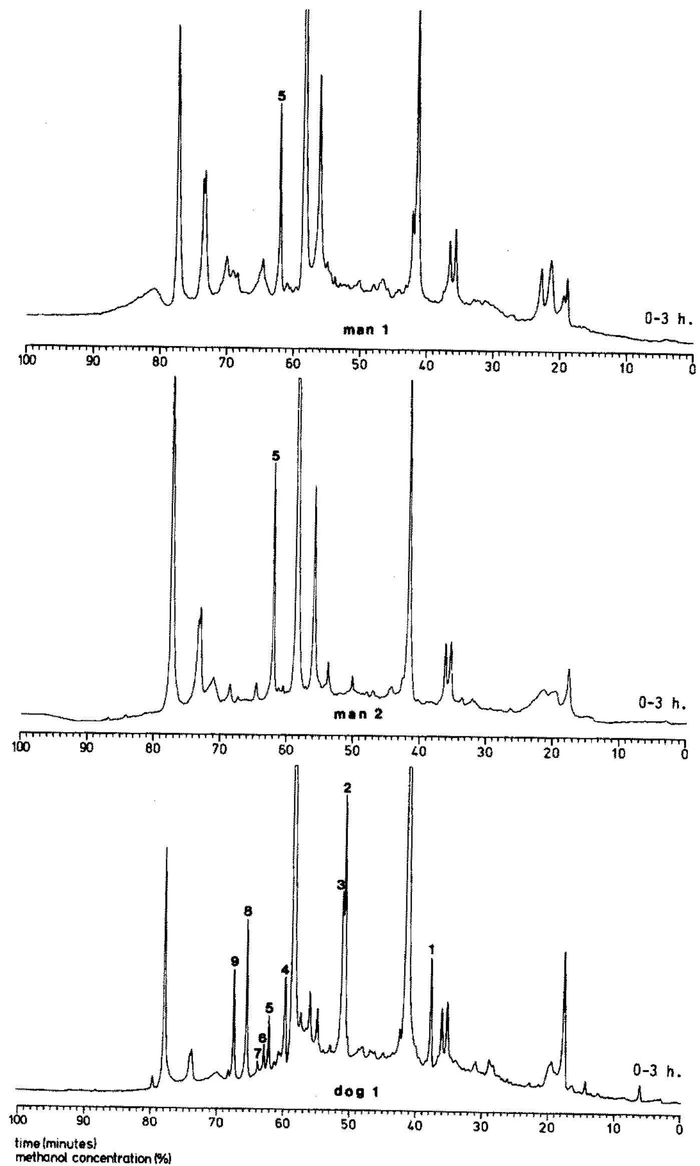

Figure 3
Additional evidence for the metabolic character of peak 5 by interindividual comparison between the biliary metabolism of patient 1 and patient 2, and interspecies comparison between patients 1 and 2 versus dog. HPLC conditions as in Fig. 1.

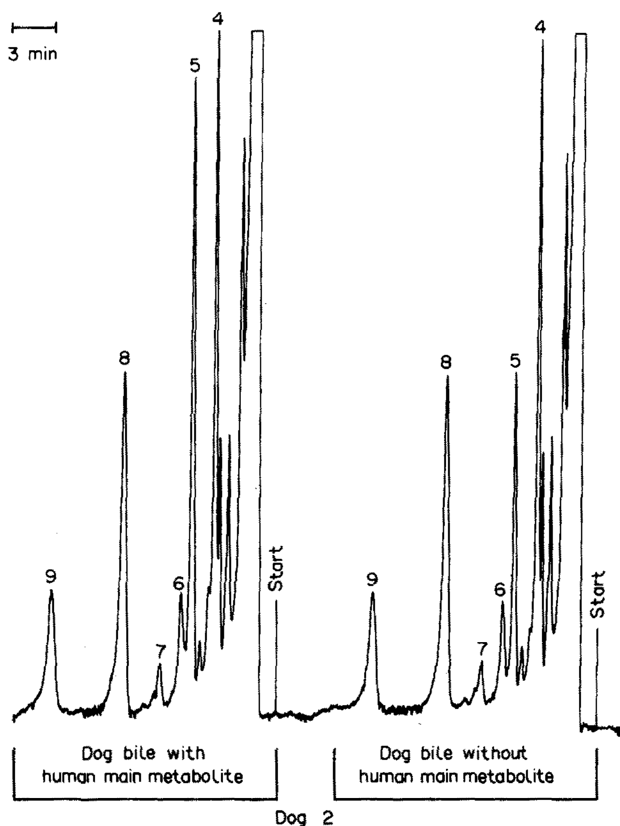

Figure 4
Isocratic chromatograms of dog bile with and without addition of peak 5 collected from the bile of patient 1, giving evidence for similar retention times for peak 5 in dog and man. Conditions as in Fig. 1, modified as follows: eluent, water (0.05 M TEA) — methanol (0.05 M TEA) (38:62, v/v); injection volume: 10 µl; UV-detection at 380 nm, 0.01 a.u.f.s.

be detected at 235 nm. In human bile studies neither of these compounds could be detected, so it can be assumed that butoprozine is converted to a single biliary main metabolite.

Isolation

Metabolic profiles of drugs can be distinguished to differing degrees of perfection. Gradient elution profiles discussed above can be considered as screening profiles for the purpose of obtaining a first impression about the existence of metabolites. However, the final aim of drug metabolic profiling is to elucidate the structure of the metabolite(s). After synthesis in the laboratory in sufficient quantity, then the physical, chemical and pharmacological characteristics of the new metabolite can be determined.

In metabolic profiling UV, MS and NMR spectroscopy are generally used for identification purposes. In order to obtain sufficient material for NMR-analysis the metabolites must be isolated from the biological fluid. In a recent publication [7] methodology was described for an isolation procedure in drug metabolic profiling, based

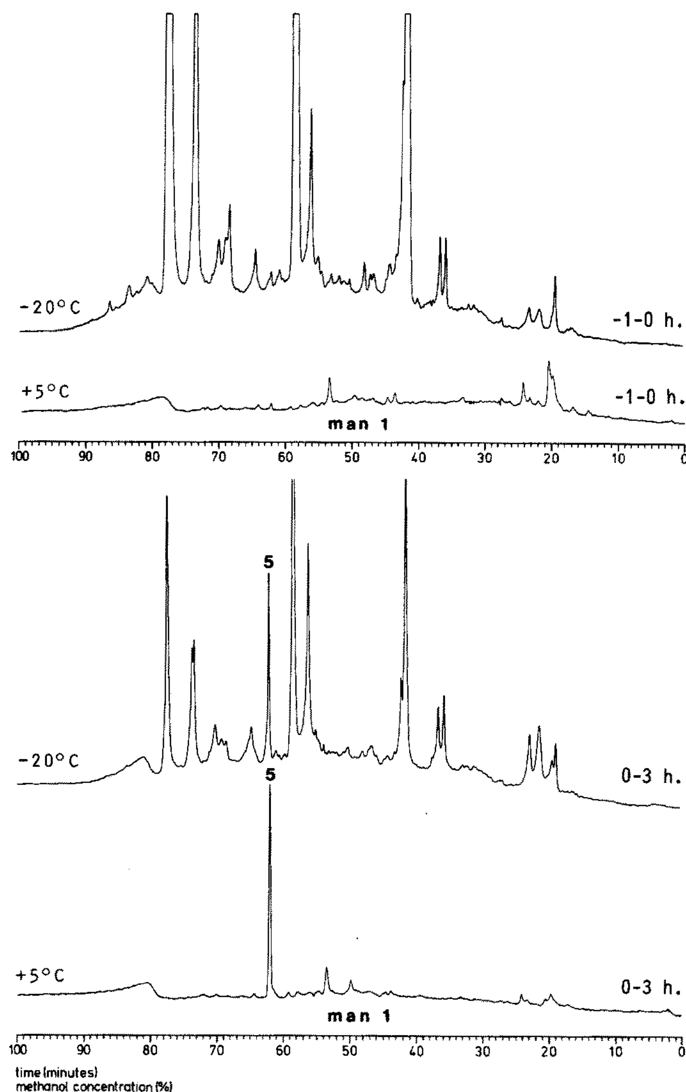

Figure 5
Recognition of the human main metabolite against the background by decomposition of endogenous bile components after storing the bile of patient 1 under different conditions: deep-frozen (−20°C) in the dark; or stored at +5°C in the dark for 36 days. Conditions as in Fig. 1.

on HPLC. The approach was illustrated with the isolation of the butoprozine metabolites (1–9) from dog bile (Fig. 3). In man isolation can be subdivided into three phases, as described below.

(a) *Collection of metabolite peaks after separation by HPLC.* Because in the case of human bile only one major metabolite needs to be isolated, isocratic elution on a column with a higher loading capacity was preferred for screening: 25 cm × 6.2 mm (instead of 15 cm × 4.6 mm). To avoid the higher flow-rate (2.5 ml/min instead of 1 ml/min) required to maintain good peak shape, leading to an unacceptable pressure increase, the

methanol in the eluent was replaced by acetonitrile, a constituent with lower viscosity in mixtures with water. In this system, volumes up to 1 ml could be injected onto the column.

In Fig. 6A a chromatogram is shown representing the collection of metabolite 5. After such an isocratic run the column was cleaned with pure acetonitrile to remove less polar endogenous solutes. Then, the run was repeated until the metabolic material from the bile of patient 1 had been collected (about 30 runs). In the authors' experience, at least 200 μg is required for suitable NMR analysis. However, at this stage, it is always difficult to assess the quantity of metabolite during collection of the fractions. Structure and molar extinction coefficient are still unknown, so that the relation between peak-height and mass of the compound cannot be established.

(b) *Work-up of collected fractions.* A metabolite collected by HPLC is present in the eluent employed. For UV-spectroscopy this poses no major problem. However, for MS and NMR the eluent must be removed, for which several procedures may be used [7]. For the human main metabolite freeze-drying was selected, after evaporation of the

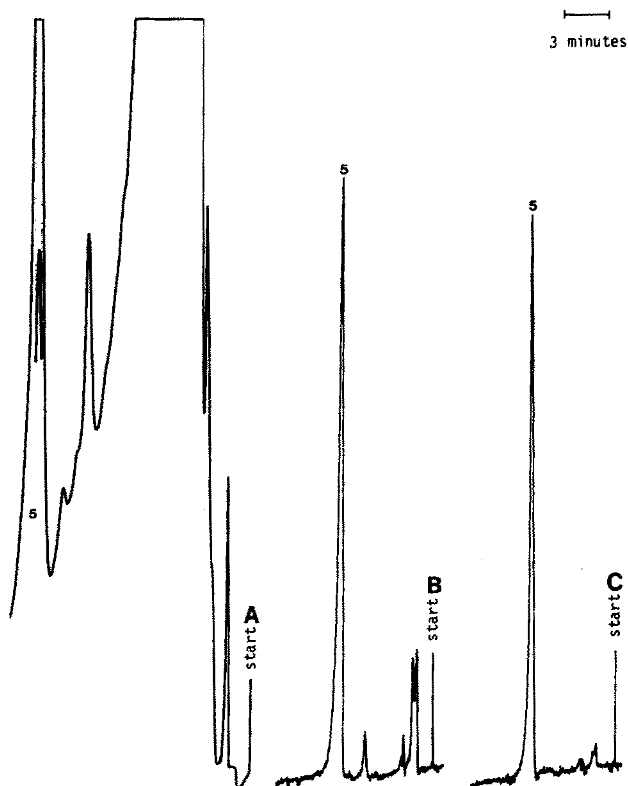

Figure 6
Isolation of the main biliary metabolite of patient 1. A: Collection of peak 5 in an isocratic run; conditions as in Fig. 1, modified as follows: column, 25 cm × 6.2 mm; eluent, water (0.05 M NH_3)–acetonitrile (0.05 M NH_3) (70:35, v/v); flow-rate, 2.5 ml/min; injection volume, 1000 μl; UV-detection at 380 nm, 1.0 a.u.f.s. (peak-top: 2.0 a.u.f.s.). B and C: purity check of the collected metabolite after removal of eluent before (B) and after (C) purification. Conditions as in Fig. 1, modified as follows: eluent, water (0.05 M NH_3)–methanol (0.05 M NH_3) (36:64, v/v); injection volume: 10 μl; UV-detection at 380 nm, 0.01 a.u.f.s.

acetonitrile. Freeze-drying is rather time consuming, but the low temperature during the removal of water avoids decomposition of the metabolite. After this stage, it is possible to assess the quantity of the dried metabolite by weighing directly.

(c) *Purity analysis of metabolites.* To avoid incorrect conclusions in the identification based on spectrical data, knowledge of the purity of a metabolite is necessary. Separation by HPLC with detection at 235 nm permits more endogenous compounds to be detected than at 405 nm, thus providing a better estimate of the purity of a fraction.

Figures 6B and 6C show two chromatograms recorded before and after purification. Figure 6B shows that some impurities are present just before peak 5 and near the solvent front. To remove these peaks, purification was performed under the conditions used for the purity check, but with a lower detector sensitivity (2.0 a.u.f.s.) and injection of 500 μl of the concentrated samples.

Figure 6C shows a chromatogram, recorded under exactly the same conditions as Fig. 6B, representing the UV-purity after the purification step. As can be seen, purification could be achieved with little loss of material. However, if only HPLC–UV detection is used for purity control, components with no UV-absorption can remain unnoticed. Though thin-layer and gas chromatography offer additional possibilities for detection, spectroscopic data should first be obtained, to decide if additional work-up is required, thus saving material. In this case UV, MS and NMR analyses were performed directly on the material obtained after purification.

Identification

For identification purposes various analytical techniques and methods are available. However, in drug metabolic profiling the main problem is the amount of material that can be obtained from the biological fluid. This means that in practice only spectroscopic techniques such as UV, MS and NMR can be used. In addition the HPLC-behaviour should be taken into account.

HPLC. Looking at the behaviour of metabolite 5 with respect to the character of butoprozine in the gradient elution chromatogram, it can be concluded that this metabolite is rather more polar than butoprozine.

UV-spectroscopy. Comparison of the UV-spectrum of the human main metabolite with butoprozine (Fig. 7) shows that the two spectra are almost identical, there being

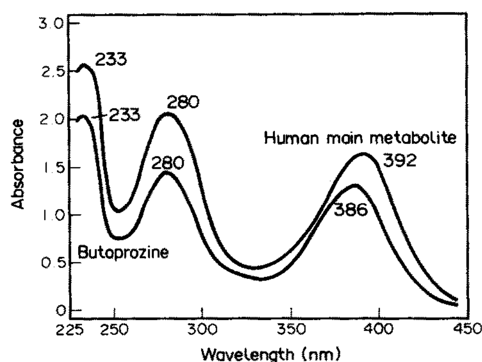

Figure 7
UV spectra of purified human main metabolite and butoprozine.

only a slight shift in one of the maxima. Because both indolizine and 4-[3-(*N,N*-dibutylamino)propoxy]-benzoic acid give quite different spectra from those in Fig. 7 and because the purified metabolite is also coloured yellow, similar to butoprozine, an intact indolizine-carboxy-phenyl aromatic system can be assumed. The introduction of substituents in this ring system would be expected to lead to the slight shift in absorption maximum.

MS data. By comparison with UV spectroscopy, a mass spectrum can provide considerably more information about the structure of a molecule. With respect to NMR, MS has the advantage that very small quantities can give an interpretable spectrum, typically *ca.* 1 μg versus *ca.* 200 μg in NMR. Figure 8 shows three mass spectra of the human main metabolite: an electron-impact (EI) spectrum, a chemical ionization (CI)–positive ion–ammonia spectrum and a CI–negative ion–hydroxide spectrum. Especially from *m/z* 451 = $(M+1)^+$ and *m/z* 449 = $(M-1)^-$ a molecular weight of 450 can easily be deduced. Taking into account the molecular weight of butoprozine (434), a difference of 16 mass units is evident. This indicates the introduction of one oxygen atom in the parent molecule.

The fragmentation pattern in the EI spectrum can now give additional information about the structure of the metabolite. The fragment ions *m/z* 100, 142 and 407 are especially interesting when considered with respect to the EI spectrum of butoprozine

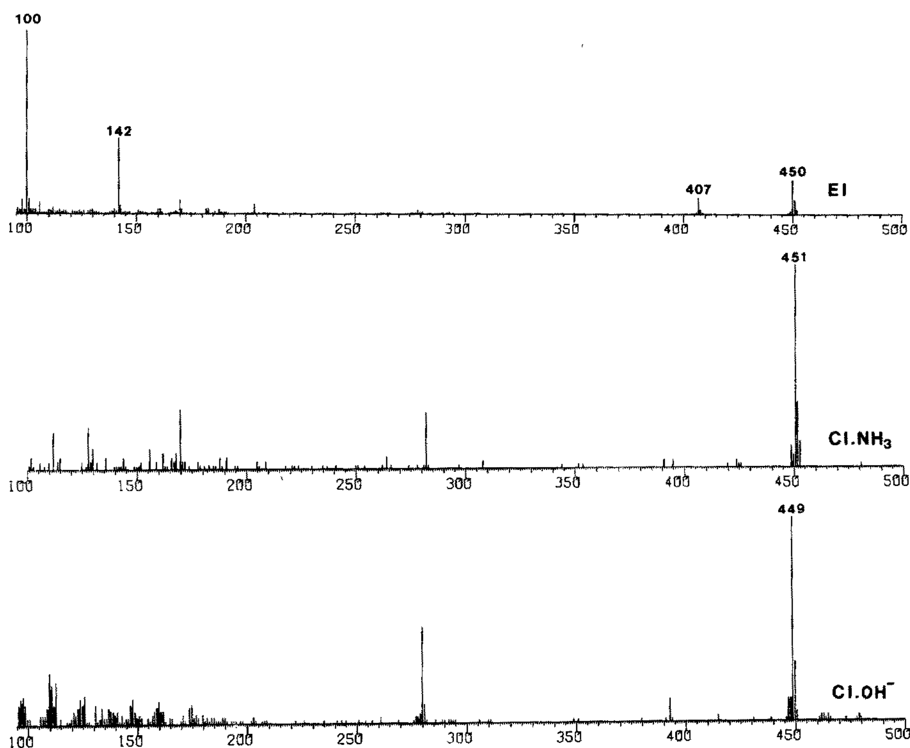

Figure 8
Mass spectra of human main metabolite: electron impact; chemical ionization–positive ion–ammonia; and chemical ionization–negative ion–OH^-.

itself. In the latter spectrum these fragments are also present and can be ascribed to the trialkyl-amino-moiety of the molecule [9]. Because these fragments are also present in the metabolite, it may be assumed that this trialkyl-amino-moiety has not been altered in the metabolic transformation. Thus, it can be concluded that the introduction of the oxygen atom must have taken place in the aromatic part of the molecule. Unfortunately, MS could not be used to obtain further information on the exact position of substitution, so that NMR was employed.

NMR spectroscopy. For the human main metabolite, the question arises as to which proton, with respect to butoprozine, is absent and replaced by a hydroxy group. Figure 9 shows the 'aromatic part' of the proton spectra of butoprozine and of the metabolite. As can be seen, the signals of the metabolite are quite small as compared to those of butoprozine, the latter being available in larger quantities. However, the following observations and conclusions can be made:

doublet H_{13} due to ortho-coupling between $H_{12} \rightarrow H_{13}$; H_{12} present;
doublet H_{12} due to ortho-coupling between $H_{13} \rightarrow H_{12}$; H_{13} present;
doublet H_5 due to ortho-coupling between $H_6 \rightarrow H_5$; H_6 present;
doublet H_8 due to ortho-coupling between $H_7 \rightarrow H_8$; H_7 present;
double doublet H_6 due to ortho-coupling with H_5 and $H_7 \rightarrow H_6$; H_5 and H_7 present;
double doublet H_7 due to ortho-coupling with H_6 and $H_8 \rightarrow H_7$; H_6 and H_8 present;
triplet H_{15} due to ortho-coupling with $H_{16} \rightarrow H_{15}$; H_{16} present;
no signal for H_1; H_1 absent.

All the evidence points to the following conclusions:

the presence of H_{13}, H_{12}, H_5, H_6, H_7 and H_8 in the aromatic system; and
the absence of H_1.

Thus, it can be concluded that the introduction of the hydroxy group has taken place at the C_1-position of the molecule and that the human main metabolite can be named 1-hydroxy-butoprozine. To obtain absolute evidence about a new structure, it would be necessary to synthesize this compound. After that, comparison of mass and NMR spectra and of other properties would indicate whether or not the biological compound was identical to the synthetic compound. Unfortunately laboratory methods presently available seem inferior to the *in vivo* capabilities of man, since it has not yet been possible to synthesize 1-hydroxy-butoprozine. This was also shown to be the structure of metabolite 5 in the dog.

Conclusions

This survey is intended to show that drug metabolic studies in man can be completed without the administration of radioactive drug. However, the use of radioactivity experiments in one or more animal models is important for validating the system and methods to be used. A systematic approach is also shown to be necessary for successful completion of such studies. The advantage of the HPLC system presented as the basis for screening and isolation is that it can be easily adapted to the demands of each investigation step. Thus the gradient mode is used for the separation of as many metabolites as possible; while the isocratic mode is employed for collection, purity control and, if required, purification of the metabolites. During the identification process

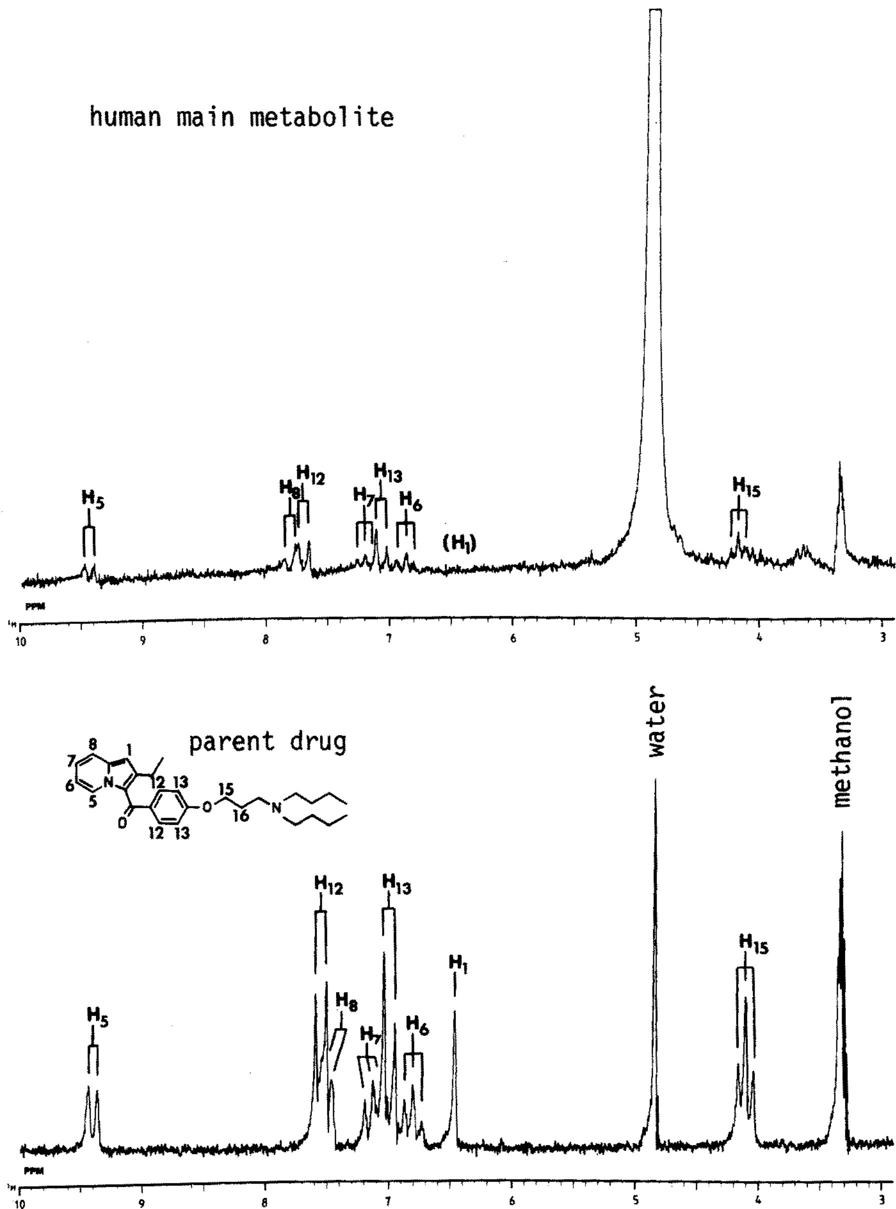

Figure 9
Comparison of the proton-NMR spectra of butoprozine and the main human metabolite.

it has been shown that a careful combination of spectroscopic techniques is of great importance. The approach outlined can be successfully applied to a number of other drugs whose metabolic pathways are of interest.

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