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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ELUCIDATION OF THE METABOLIC FATE OF BUTOPROZINE

SCREENING FOR UNKNOWN METABOLITES IN THE RAT

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SUMMARY

A systematic approach to the investigation of metabolic degradation of drugs is presented. Extraction of the biological sample is avoided by direct injection of bile or urine in a reversed-phase high-performance liquid chromatography system. Good separation of the various metabolites is obtained by changing the composition of the mobile phase so that all components become separated from the solvent front and are eluted from the column. The metabolites can be detected by measuring the amount of radioactivity in the column effluent, which also serves as a check on the overall recovery. Simultaneous registration of the UV absorbance permits certain conclusions to be drawn with regard to the nature of the metabolites.

This approach is illustrated in the screening for metabolites of Butoprozine, a new anti-anginal drug, in rat bile. Essentially, all metabolites could be followed by suitable adaptations of the mobile phase, thus providing a metabolic profile with a recovery of 95% of the radioactivity.

INTRODUCTION

In the development of a new drug, the elucidation of its metabolic fate is required in order to obtain adequate pharmacological and toxicological data. Radio-thin-layer chromatography is often used for the detection of drug metabolites. However, problems may arise with hydrophilic compounds that are difficult to extract and which often cannot be separated without some sample pretreatment, *e.g.*, hydrolysis of conjugates. In our opinion reversed-phase high-performance liquid chromatography (HPLC) is more suitable for such separations, especially when a large number of metabolites of widely different hydrophilic character are present. Moreover, with reversed-phase HPLC the extraction problem can be circumvented since the biological fluid can be injected directly.

In this paper we report the application of reversed-phase HPLC to the screening for metabolites of Butoprozine, {4-[3-(dibutylamino)propoxy]phenyl} (2-ethyl-3-indolizinyl)methanone. Fig. 1 shows the structure of this indolizine derivative,

a new anti-anginal drug. The synthesis and pharmacological properties have been described elsewhere[1–3].

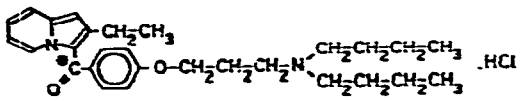

Fig. 1. Structure of Butoprozine hydrochloride. The asterisk indicates the location of the ^{14}C label in the radioactive molecule.

EXPERIMENTAL

Compounds

Butoprozine and [^{14}C]Butoprozine were gifts from Labaz (Brussels, Belgium). The purity control and purification of the crude products were done by analytical and semipreparative HPLC, and will be described elsewhere.

The radiochemical impurities in [^{14}C]Butoprozine amounted to 2%. After dilution with non-radioactive Butoprozine the chemical purity was *ca.* 98%. The specific activity after dilution was 5.7 μCi/mg.

Chemicals and reagents

Urethane (Dutch Pharmacopoeia VI) was obtained from Lamers & Indemans ('s Hertogenbosch, The Netherlands). Diethylamine (synthetic grade) was obtained from E. Merck (Darmstadt, G.F.R.). Picofluor 30 (Packard, Brussels, Belgium) was used as scintillation cocktail. All other chemicals and solvents were analytical reagent grade and obtained from E. Merck.

Biological samples

Adult male Wistar rats (body weight *ca.* 300 g) were used. They were anaesthetized with an i.p. dose of urethane (1.28 g/kg) after which the *vena jugularis* was cannulated. A 1-ml volume of Krebs-bicarbonate solution was administered via this cannula every hour. The biliary duct was cannulated and the bile was collected for 6–8 h. During the collection the bile was cooled with ice. After the experiments the bile was frozen (−18°) until analyzed. Butoprozine (10 mg/kg) was given by i.v. injection into the *vena femoralis*.

Blank bile was obtained from rats injected with water instead of Butoprozine solution. Radioactive bile samples were gifts from Labaz.

High-performance liquid chromatography

A Spectra-Physics (Berkeley, Calif., U.S.A.) Model 3500 B liquid chromatograph was used with a Model SF 770 variable wavelength UV detector (Schoeffel). The wavelength used was 380 nm, corresponding to the UV absorption maximum of Butoprozine.

The samples were injected with a Valco CV-6-UHPa injection valve fitted with a 90-μl sample loop. In all experiments a stainless-steel column (15 cm × 4.6 mm I.D.) was used. The column was packed with LiChrosorb RP-8 (mean particle size, 5 μm: E. Merck) using a balanced density slurry.

The mobile phases consisted of mixtures of methanol and distilled water, to which 0.5% (v/v) diethylamine was added. Before mixing, the components were filtered through a 0.45-μm membrane filter. The flow-rate was 1 ml/min.

When fractions were collected for liquid scintillation counting, 1-min fractions were taken during 30 min after injection. In the chromatograms the UV trace is shown together with the radioactivity values obtained in the same run.

Measurement of radioactivity

The 1-min HPLC fractions were collected in counting vials. A 3-ml volume of Picofluor 30 was added when the HPLC mobile phase contained more than 15% methanol. When the mobile phase contained less than 15% methanol, 6 ml Picofluor 30 were added. In this way after mixing always a clear solution was obtained.

Radioactivity was measured by liquid scintillation counting in a Isocap or Mark 300 scintillation analyzer (Nuclear-Chicago). Efficiency was determined using the external standard channels ratio method. Values obtained varied between 70 and 80%.

Recovery

Recoveries were calculated by comparison of the amount of radioactivity eluted from the column (A_1) with the amount of radioactivity injected (A_2):

$$\text{Recovery (\%)} = \frac{A_1}{A_2} \cdot 100.$$

The amount eluted was determined by collecting during 30 min. The amount injected was determined by injecting a second sample from the same solution without the column being attached to the injection valve, and collecting during a similar period.

RESULTS AND DISCUSSION

In order to avoid any losses or changes due to pretreatment, no prior extraction or other clean-up was performed. Bile was chosen because it is the major excretion route in the rat[4].

The mobile phases were chosen according to the principles of "solvophobic chromatography"[5,6]. By choosing an alkaline pH in the mobile phase, conditions can be obtained in which the metabolites are expected to be eluted faster than the parent compound.

When using a non-aqueous mobile phase, all the radioactivity was found in the solvent front. By decreasing the methanol content in the subsequent runs, the degree of interaction between the solute and the stationary phase is increased. Figs. 2–4 represent subsequent isocratic runs and show the increase in retention time for the same compounds using different percentages of methanol in the mobile phase. The first major radioactive peaks appeared when using *ca.* 60–65% methanol in the mobile phase. Fig. 5 shows the relation between the retention times of the radioactive peaks and the methanol content of the corresponding mobile phases. As can be seen, all radioactive compounds are eluted at lower methanol percentages than unchanged Butoprozine.

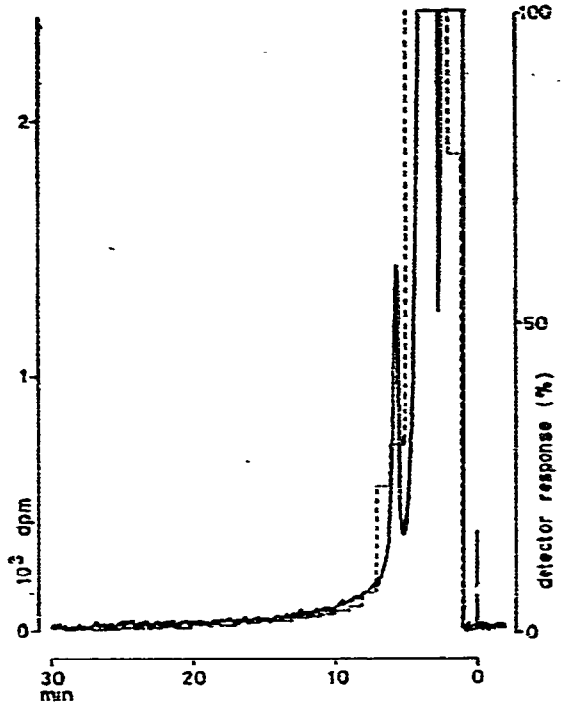

Fig. 2. Chromatogram of total bile. Mobile phase: methanol–water–diethylamine (65:35:0.5). ——, UV 380 nm, 0.01 a.u.f.s.; – – – –, radioactivity (dpm) in the fractions.

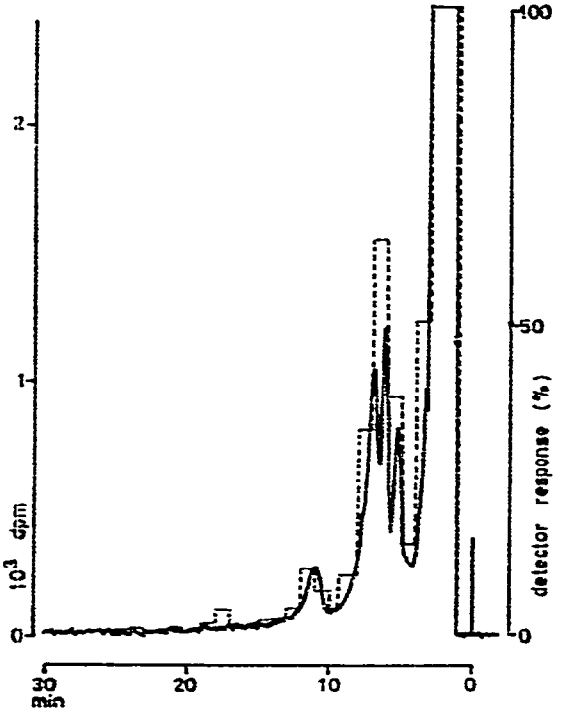

Fig. 3. Chromatogram of total bile. Mobile phase: methanol–water–diethylamine (60:40:0.5). Other conditions as in Fig. 2.

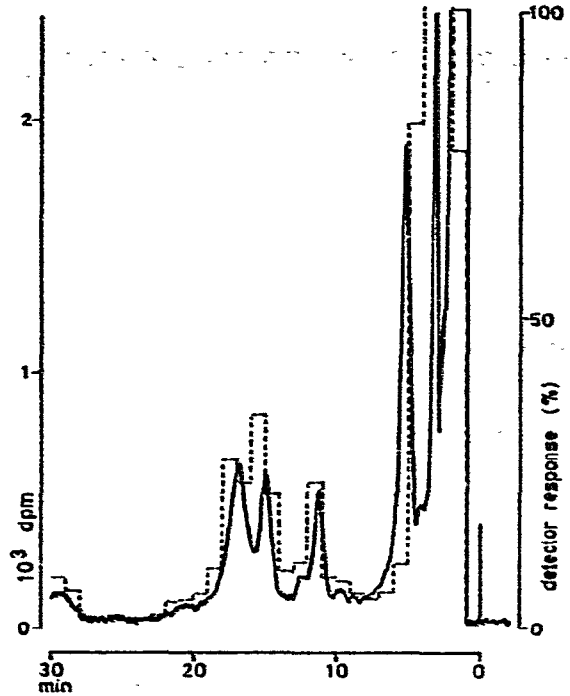

Fig. 4. Chromatogram of total bile. Mobile phase: methanol–water–diethylamine (55:45:0.5). Other conditions as in Fig. 2.

Table I gives the fractions of radioactivity eluted in 30 min at various compositions of the mobile phase. When using a mobile phase without methanol, *ca.* 5% of the amount of radioactivity injected was still left in the solvent front. This means that almost all of the radioactive metabolites can be followed.

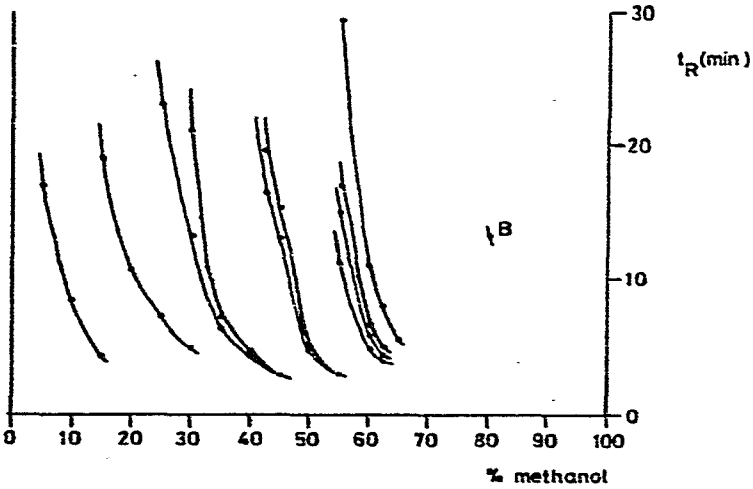

Fig. 5. Relation between the retention times of the radioactive peaks and the methanol content of the corresponding mobile phases. B = Unchanged Butoprozine.

TABLE I

RECOVERY OF ^{14}C FROM RAT BILE WHEN USING DIFFERENT MOBILE PHASES

Amount of methanol in mobile phase (%)	*Recovery (%)*
65	92
50	70
40	51
25	28
10	11

Cross-contamination in the succeeding runs was avoided by cleaning the column after each chromatographic run using methanol with 0.5% diethylamine as a mobile phase for 30 min. Even after 3 months, no increase in background counting rate was observed using this cleaning procedure.

The above results clearly indicate that the present approach is highly promising for this type of metabolic study. In the case of Butoprozine, almost all of the radioactive metabolites could be followed. The presence of about ten different metabolites is readily apparent. Furthermore, the recovery of the radioactive metabolites was almost 100% thus indicating that a complete metabolic profile is being obtained in the bile. It should be noted that the probability of overlooking non-radioactive metabolites is relatively small due to the position of the radioactive label.

The large number of metabolites and their elution behaviour as shown in Fig. 5 indicate that it will be impossible to select isocratic conditions allowing chromatographic separation in a single run. Yet the data in Fig. 5 can be used to establish a suitable system for gradient elution (continuous or stepwise)[7–9].

We are presently applying a stepwise elution system to analytical as well as preparative HPLC investigations of rat bile collected after administration of non-radioactive Butoprozine. The structures of the metabolites thus collected are then examined. The results of the latter studies will be described elsewhere.

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