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Study of the biotransformation of a potential benzo[*c*]fluorene antineoplastic using high-performance liquid chromatography with high-speed-scanning ultraviolet detection

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Abstract

As the sum of benfluron metabolites found was only a part of the total amount applied, a search for undiscovered metabolites was undertaken in the extracts from isolated rat hepatocytes and in the bile and perfusate in the experiments with an isolated perfused rat liver. To identify the metabolites, high-performance liquid chromatography with UV spectral analysis was used, as benfluron derivatives exhibit characteristic absorption spectra. Administration of known metabolites to experimental animals and selective induction of certain metabolic pathways led to the finding of new metabolites and of the respective conjugates. Fast atom bombardment–mass spectrometry analysis was used to identify the newly found metabolites and conjugates.

Keywords: Benfluron; Hepatocytes; Liver, perfused; Ultraviolet detection, high-speed scanning

1. Introduction

A potential benzo[*c*]fluorene antineoplastic agent, benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride, see Fig. 1, hydrochloride of compound **10**] was prepared ten years ago in the Research Institute of Pharmacy and Biochemistry in Prague [1,2]. An interesting spectrum of its pharmacodynamic properties was found in experiments carried out in vitro (with animal and human cells) and in vivo [2–7]. Disposition of the compound in animals was followed and pharmacokinetic parameters were calculated on the basis of TLC–densitometric analyses of serum extracts after oral and intravenous administration of benfluron in four animal species [8]. The absorption, distribution and elimination of [^{3}H]-benfluron following its oral and intravenous administration to rats was also studied [9].

Biotransformation of benfluron has been investi-

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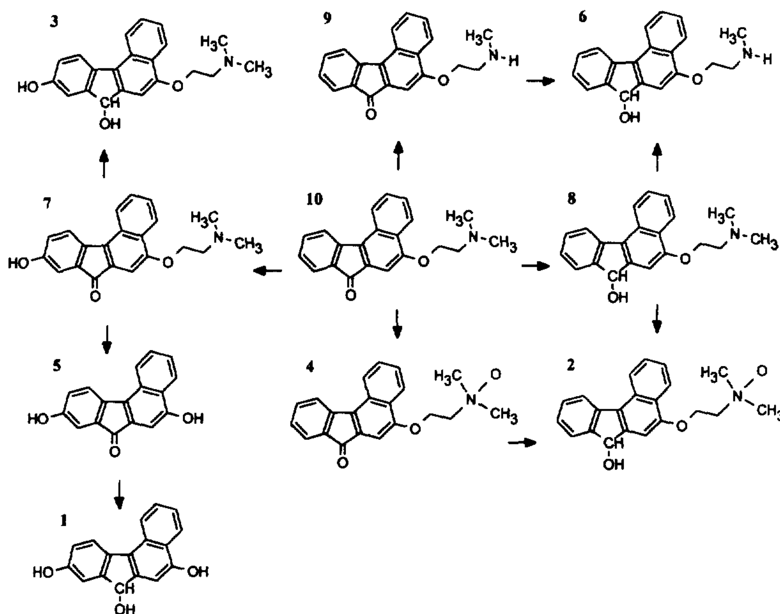

Fig. 1. Survey of the biotransformation of benfluron (compound **10**). The arrows refer to hypothetical pathways.

gated in vitro and in vivo [10–13] and chemical structures of nine metabolites were elucidated using infrared, FT-NMR and mass spectrometry of isolated metabolites and their synthetic standards [10,14–16]. A high-performance liquid chromatographic method for determination of benfluron and its metabolites in extracts from biological samples was developed [17,18].

Previous studies on benfluron biotransformation revealed significant differences between the total amount of benfluron given to the rat and the sum of the nine metabolites found in faeces and in urine over eleven days after oral administration of the parent compound [13]. This fact could be explained in several ways: (A) The oral administration of benfluron has been shown to lead to very slow absorption due to strong affinity of this compound for rat stomach wall [8,9]. (B) Benfluron may form metabolites and their derivatives which are poorly extracted and/or are hardly detectable because of their changed structure and polarity (e.g. polar metabolites or conjugates as products of Phase II reactions).

This is why this study is focused on finding and identifying further metabolites of the Phase I and II biotransformation of benfluron by HPLC with UV detection and, subsequently, on extending a theoretical scheme of benfluron biotransformation in the organism (Fig. 1).

2. Experimental

2.1. Laboratory animals

Male rats (*Rattus norvegicus* var. *alba*, Wistar type) from the Konárovice breeding station (Research Institute for Pharmacy and Biochemistry, Prague, Czech Republic) were used. They weighed around 250 g and were allowed access to water and food pellets *ad libitum* before and during the experiments. The experiments were approved by a local ethics committee.

2.2. Chemicals, solutions and materials

The following benzo[*c*]fluorene derivatives were available as the standards of benfluron metabolites

(see Fig. 1): 5,7,9-trihydroxy-7*H*-benzo[*c*]fluorene (compound **1**), 5-(2-dimethylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene N-oxide (**2**), 5-(2-dimethylaminoethoxy)-7,9-dihydroxy-7*H*-benzo[*c*]fluorene (**3**), 5-(2-methylaminoethoxy)-7,9-dihydroxy-7*H*-benzo[*c*]fluorene (**3a**), 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene N-oxide (**4**), 5,9-dihydroxy-7-oxo-7*H*-benzo[*c*]fluorene (**5**), 5-(2-methylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene (**6**), 5-(2-dimethylaminoethoxy)-9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene (**7**), 5-(2-methylaminoethoxy)-9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene (**7a**), 5-(2-dimethylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene (**8**), 5-(2-methylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene (**9**), 5-(2-aminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene (**9a**), 7-oxo-7*H*-benzo[*c*]-5-fluorenoxy-acetic acid (**9b**), 5-(2-hydroxyethoxy)-7-oxo-7*H*-benzo[*c*]fluorene (**9c**) and 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene (**10**) (for methods of preparation see [14] and [16]). The compounds **3**, **3a**, **6**, **7**, **7a**, **9**, **9a** and **10** were in the form of hydrochlorides. Acetonitrile (HPLC grade, Merck, Darmstadt, Germany), nonylamine (purum, Fluka, Buchs, Switzerland), chloroform, methanol, 2-propanol, phosphoric acid (85%), aqueous ammonia (26%), triethylamine and ethyl acetate (all of analytical grade, Lachema, Brno, Czech Republic) were used for sample preparation, TLC and HPLC.

Nonylamine buffer for the mobile phase was prepared by mixing 2 ml of nonylamine with 990 ml of redistilled water, adjusting to pH 7.4 with a solution of phosphoric acid (2 mol l^{-1}) and making up to 1000 ml with water.

A standard mixture (10^{-4} *M*) of five principal benzo[*c*]fluorenes (**4**, **7**, **8**, **9** and **10**) was prepared by mixing 5 ml of stock solutions of each of the compounds (10^{-3} *M* in the mobile phase) and making up to 50 ml with mobile phase.

Kieselgel 60 H (Merck) was used for the preparation of TLC-layers. Sodium chloride, potassium chloride, calcium chloride, potassium dihydrogen phosphate, magnesium sulfate, sodium hydrogen carbonate, glucose and polyvinylpyrrolidone K 25 (all of analytical grade, Fluka) were used for preparing the Krebs–Henseleit bicarbonate buffer used in both isolated hepatocytes and isolated perfused liver and they were also used in the preparation of a modified Hanks buffer, pH 7.4, used in the preparation of isolated hepatocytes [19].

Collagenase (type IV), trypan blue, EGTA [ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid], HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) and bovine albumin (fraction V) (all from Sigma, St. Louis, MO, USA) were used for the preparation of isolated hepatocytes.

Phenobarbitalum natricum (ČsL3, Farmakon, Olomouc, Czech Republic), β-naphthoflavone (analytical grade, Sigma) and ethanol (ČsL4, Lachema) were used for induction of enzymes.

2.3. Induction of liver enzymes

Induction of the liver cytochrome P-450s (2B, 1A and 2E1 isoforms) was performed as follows:

Induction of the P-450 2B isoform: a 0.1% (w/v) aqueous solution of phenobarbital was administered (p.o., *ad libitum*) in drinking water for one week prior to the isolation of hepatocytes.

Induction of the P-450 1A isoform: a 1% solution of β-naphthoflavone in *oleum helianthi* was administered i.p. 24 h before isolation of hepatocytes (40 mg β-naphthoflavone per kg).

Induction of the P-450 2E1: a 10% (v/v) solution of ethanol in drinking water was administered (p.o., *ad libitum*) two weeks before the isolation of hepatocytes.

2.4. Preparation and incubation of isolated hepatocytes

Hepatocyte isolation was performed using the collagenase perfusion method [19]. The cells were counted and their viability (>85%) was determined by trypan blue exclusion. Incubation was performed for 60 min at 37°C in Krebs–Henseleit buffer containing 12.5 m*M* HEPES and 15.3 μ*M* substrate (benfluron or one of its metabolites), at a cell concentration of $1 \cdot 10^6$ cells/ml.

2.5. Preparation of isolated perfused liver and conditions for biotransformation [20]

Wistar Han II rats were used to obtain livers. A recirculating system was used for liver perfusion.

The rat liver was isolated from the body and perfused through a cannulated portal vein with oxygenized red blood cell-free Krebs–Henseleit buffer (37°C, pH 7.4) with glucose (0.1%) and polyvinylpyrrolidone (3.5%). Bile flow and oxygen consumption were determined during the perfusion as metabolic characteristics. Benfluron was added to the perfusion medium in the total dose of 1 mg benfluron. Samples of perfusion medium and bile were withdrawn every 15 min for 2 h of perfusion.

2.6. Sample preparation

The suspension of isolated hepatocytes, after incubation with a benzo[*c*]fluorene compound of interest, was alkalinized with the same volume of 15% aqueous ammonia to pH 9–10 and repeatedly extracted (three times) with 10 ml of ethyl acetate. Ethyl acetate extracts were evaporated in vacuo (max. 40°C) to dryness. The residues were dissolved in a known volume (usually 1–2 ml) of the mobile phase, to be used in HPLC.

The perfusate medium was worked up in the same way. The collected bile was only diluted with the mobile phase used in HPLC or in methanol for preparative TLC.

2.7. Chromatography

A Spectra Physics (now Thermo Separation Products) chromatograph was used. The configuration used was as follows: a solvent degasser SCM400, quaternary gradient pump P4000, autosampler AS3500 with a 100-μl sample loop, Spectra FOCUS high speed scanning UV detector, system controller SN4000, computer Spectra 386 E with PC1000 analytical software working under OS-2. Analyses were performed on an HPLC column LiChroCART 125 × 4 mm I.D. with precolumn LiChroCART 4 × 4 mm I.D., containing LiChrospher 100 RP-18 (5 μm, all from Merck) Mobile phase A consisted of nonylamine buffer (see Section 2.2), acetonitrile and 2-propanol (2:2:1, v/v) and it was used for the separation of the metabolites of phase I of biotransformation. Mobile phase B consisted of the same nonylamine buffer and acetonitrile (3:2, v/v) and it was used for the resolution of the two main conjugates found in bile samples. The flow-rate of the mobile phase was 0.9 ml min^{-1}. Detection was performed in dual wavelength mode (295 and 340 nm) or in high-speed scanning mode (range 195–365 nm with 5 nm distance).

A preparative TLC was used for the isolation of the newly found metabolites and their conjugates in bile. Layers of Kieselgel 60H (0.3 mm thick on 20 × 20 cm glass plates) were prepared from a suspension in triethylamine–methanol (1:1, v/v) using the Camag coating apparatus and left to dry (in fume chamber) for about 20 min. The extract from incubated hepatocytes or diluted bile was deposited as a line on plate(s). Chromatography was performed in a chloroform–methanol–triethylamine mixture [80:10:5 (v/v) in case of isolation of the metabolites of phase I of biotransformation or 55:25:5 (v/v) in case of isolation of benzo[*c*]fluorene conjugates]. After chromatography the individual bands of separated benzo[*c*]fluorene metabolites were detected according to their colour or fluorescence and scraped off the silica gel. The metabolites were eluted with methanol from the silica gel on a No. 4 sintered-glass filter and evaporated to dryness.

2.8. Fast atom bombardment–mass spectrometry (FAB–MS)

The positive and negative FAB spectra were recorded on a ZAB-EQ mass spectrometer (VG Organic, Manchester, UK) operating at 8 kV and equipped with a VG11-250J data system. The saddle-field gun (IonTech, UK) was operated at 8 kV using xenon gas. A mixture of glycerol and thioglycerol (1:3, v/v) was used as a matrix.

3. Results and discussion

The absorption spectra (namely in the near ultraviolet and visible region, 200–750 nm) do not generally give much information about the chemical structure of analysed compounds. However, in the case of benzo[*c*]fluorene derivatives, the combination of HPLC with high-speed scanning UV detection represented a reliable tool for the fast on-line identification of benflurone metabolites in analysed mixtures.

Benfluron contains in its molecule a large and well

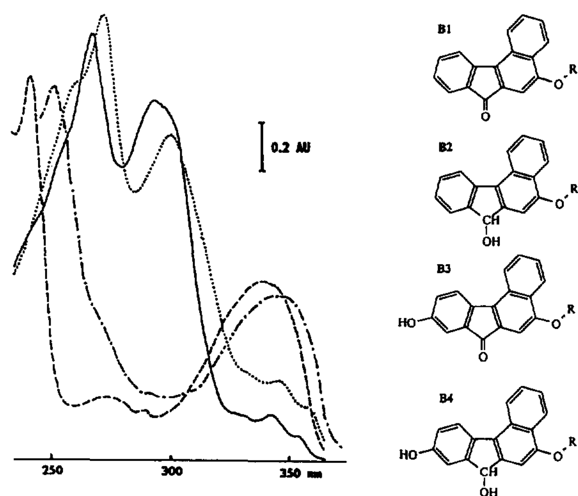

Fig. 2. UV spectra of benzo[*c*]fluorenes in the HPLC mobile phase used. Concentration of each compound was $5 \cdot 10^{-5}$ *M* (full line — 7-oxo-7*H*-benzo[*c*]fluorenes, **B1**; dashed line — 7-hydroxy-7*H*-benzo[*c*]fluorenes, **B2**; dotted line — 9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorenes, **B3**; dashed and dotted line — 7,9-dihydroxy-7*H*-benzo[*c*]fluorenes, **B4**.

absorbing 7-oxo-7*H*-benzo[*c*]fluorene chromophore, which has a characteristic UV spectrum with absorption maxima at 271 and 294 nm. Spectra of individual metabolites reflect the localization of changes in the structure due to biotransformation of benfluron molecule. In the case of N-oxidation and N-demethylation, respectively oxidative deamination, the side chain is altered, but distribution of electrons in the chromophore is not influenced. All of the 7-oxo-7*H*-benzo[*c*]fluorenes (benfluron **10**), N-oxide of benfluron **4**, mono- (**9**), bis-N-demethylated benfluron **9a** and the product of its oxidative deamination **9b** have the same spectra (see Fig. 2 and Fig. 4a).

The carbonyl reduction leading to the secondary alcohol influences the chromophore and 7-hydroxy-7*H*-benzo[*c*]fluorenes (see Fig. 1, compounds **2**, **6** and **8**) have another type of spectrum (absorption maxima at 249–250 and 340 nm, see Fig. 2 and Fig. 4b).

The cytochrome P-450-dependent oxidation of the 7-oxo-7*H*-benzo[*c*]fluorene chromophore *via* arenoxide led to the third type of chromophore change. A new auxochrome in 9-hydroxy-7-oxo-7*H*-benzo[*c*]-fluorenes (see Fig. 1 and Fig. 6, compounds **5**, **7** and **7a**) shifts the absorption maxima to 274 and 298 nm, respectively. Hence, it is easy to discriminate between the 7-oxo-7*H*-benzo[*c*]fluorene and 9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene spectra (*cf.* Fig. 2, Fig. 4a and Fig. 4c).

Spectra of the fourth group of metabolites, the 7,9-dihydroxy-7*H*-benzo[*c*]fluorenes, exhibit an analogous bathochromic shift (spectral maxima of 251 and 345 nm, see Fig. 2, compounds **1**, **3** and **3a**) in comparison with the 7-hydroxy-7*H*-benzo[*c*]-fluorenes (maxima 249–250 and 340 nm).

Metabolic products with the same chromophore in the molecule, which were indistinguishable by their UV spectra, differed in their HPLC retention times.

The data captured with a high-speed scanning UV detector were transformed by PC1000 software into a spectrochromatogram, a three-dimensional relationship in which the absorbance *A* (a dependent variable) is the function of two independent variables [retention time t_R (min) and wavelength λ (nm)]. A spectrochromatogram of a real mixture of benfluron and its metabolites is shown in Fig. 3.

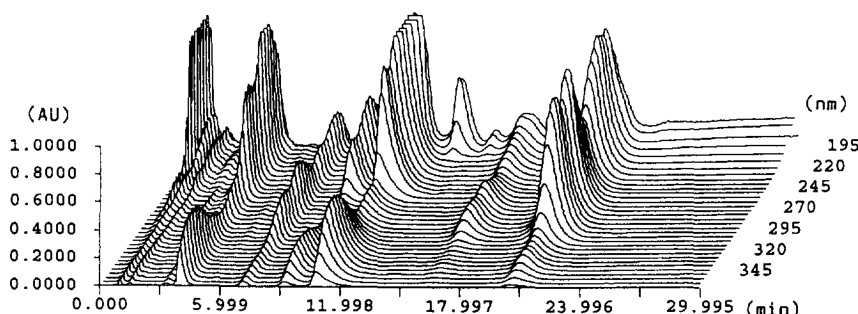

Fig. 3. Spectrochromatogram of a real mixture of benfluron and its metabolites (extract from non-induced hepatocytes).

The dual wavelength chromatogram [$A = f(t_R)$ at 295 and 340 nms] has been used usually for quantitative analysis of the mixtures.

Isochronical slices of the spectrochromatogram in retention times of separated compounds [UV spectra, $A=f(\lambda)$] are displayed in Fig. 4a–c. The underlined numbers at the top of each figure are the retention times (min) of the compound, whose spectrum is demonstrated.

Problems resulting from low concentrations of some metabolites were solved using three approaches: (A) fresh hepatocytes and isolated perfused rat liver have been used to produce a sufficient amount of metabolites for analyses, (B) standards of metabolites of "first filial generation" were used as parent compounds to get greater yields of metabolites of "second and higher filial generations" and (C) hepatocytes were isolated from rat liver in which the level of a particular P-450 isoenzyme participating in biotransformation was elevated by specific induction of its biosynthesis ("induced" rat livers).

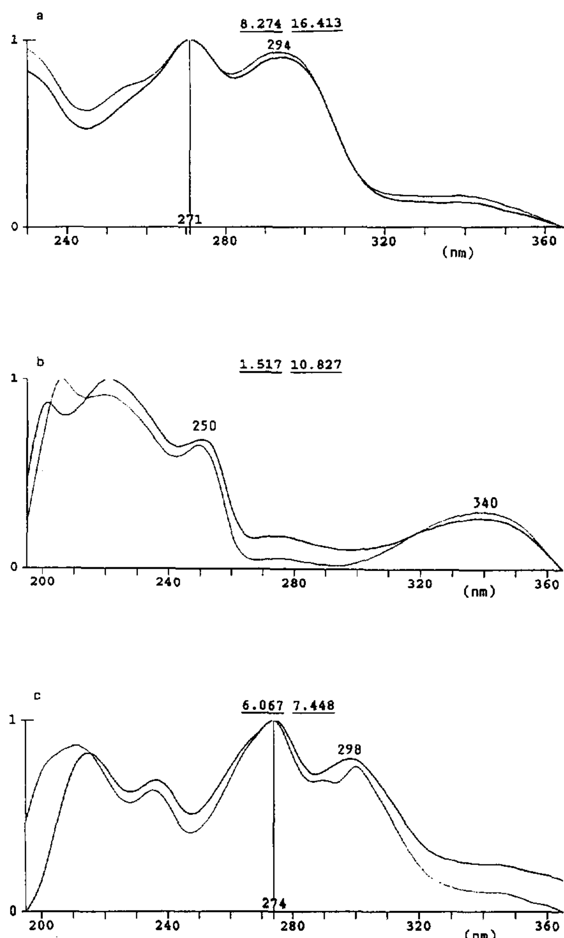

Fig. 4. Isochronical slices (UV spectra) of the spectrochromatograms in retention times of separated compounds. Fig. 4a shows a spectrum of twice N-demethylated benfluron (t_R=8.3 min) and mono N-demethylated benfluron (t_R=16.4 min); Fig. 4b shows a spectrum of reduced benfluron (t_R=10.8 min) and a conjugate with the same type of chromophore (t_R=1.5 min); Fig. 4c presents a comparison of the spectra of N-demethylated 9-hydroxy-benfluron (t_R=6.1 min) and 9-hydroxy-benfluron (t_R=7.4 min).

3.1. Use of in vitro techniques (rat hepatocytes and perfused rat liver)

The advantage of these experiments is that they are realized in a closed system without the loss of metabolites due to their distribution in the whole body and their elimination. Also, the formation of metabolites can be modulated by inhibitors or inductors of biotransformation. The analysis is often more simple as many compounds extracted from urine and faeces are not present here. The use of isolated perfused rat liver appeared to be advantageous because of the possibility of applying large amounts of the parent compound (the liver worked as a bioreactor); the products were then isolated mainly from the bile. A typical chromatogram of the bile (diluted by mobile phase) is displayed in Fig. 5. The bile was obtained from isolated rat liver perfused with a medium containing 1 mg of benfluron. Among the metabolites of the Phase I of benfluron biotransformation, i.e. the 9-hydroxy-benfluron (**7**) and its N-demethylated form (**7a**), two other prominent peaks with short retention times (1.22 a 1.88 min) were found, and both exhibited a 9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene type of spectrum. It was thought that these compounds are 9-hydroxy-benfluron conjugates. A preparative TLC followed by FAB–MS of separated compounds helped in the identification of one compound (retention time t_R= 1.88 min). The molecular mass of 495 000, corresponding to N-demethylated 9-hydroxy-benfluron-O-glucuronide (-O-glucosiduronate), has been shown in both positive and negative FAB spectra. This suggested structure will be studied further by spectroscopical and biochemical methods. The total molar

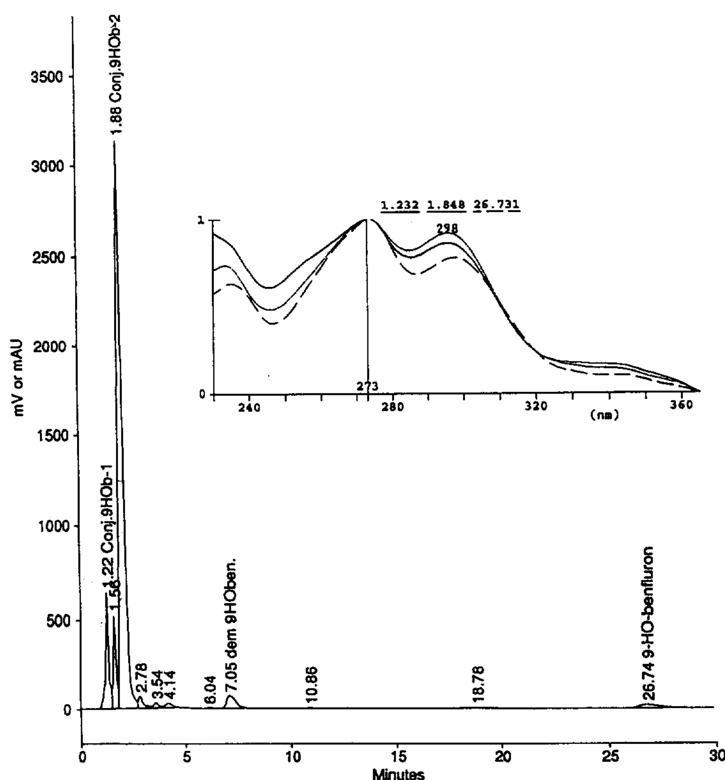

Fig. 5. Chromatogram (at 295 nm) of diluted rat bile showing the presence of the conjugates (t_R = 1.22, 1.88 min), N-demethylated 9-hydroxy-benfluron (t_R = 7.05 min) and 9-hydroxy-benfluron (t_R = 26.74 min) in mobile phase B. Inset, normalized spectra of two conjugates (full lines) compared with that of 9-hydroxy-benfluron (dashed line), proving the identity of the 9-hydroxy-benfluron moiety of the conjugates.

amount of this metabolite in the collected bile was 21–30% of administered benfluron.

3.2. Use of standards of metabolites as substrates of the biotransformation reaction and the method of alternative xenobiochemical pathways

In order to reveal and/or confirm the sequence for the formation of individual metabolites of benfluron (see Fig. 1), the four major metabolites of this compound (metabolites **4**, **7**, **8** and **9**; metabolites of the "first filial generation") were administered as substrates to isolated rat hepatocytes. The products of biotransformation of these metabolites (i.e. metabolites of the "second filial generation") were identified on the basis of their UV spectra and retention times; some new metabolites of benfluron were found this way. Also, some alternative pathways were found using this approach.

Reduced benfluron (compound **8**) undergoes reoxidation back to benfluron **10** (this is an example of a process which could not be easily found with the use of the parent compound, benfluron). The N-oxide of the reduced benfluron is formed (compound **2**, cf. the next paragraph); this compound (which is a typical metabolite of the "second filial generation") can be formed from benfluron in two ways: by reduction and then by N-oxidation (**10**→**8**→**2**) or by N-oxidation first followed by reduction of carbonyl (**10**→**4**→**2**). Also, a polar conjugate (t_R = 1.5 min) of the reduced benfluron with an unchanged aromatic chromophore has been detected (see Fig. 4b).

When benfluron-N-oxide (**4**) was used as a substrate, similar to the case found with reduced benfluron, a backward reaction to the parent benfluron

has been found. Also, the reduction of this compound has been found confirming the way (**10→4→2**) described in the preceding paragraph.

Biotransformation of 9-hydroxy-benfluron (**7**) yielded higher amounts of two newly found metabolites, N-demethylated 9-hydroxy-benfluron (see Fig. 4c and Fig. 6), 5-(2-methylaminoethoxy)-9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene, (**7a**) and N-demethylated reduced 9-hydroxy-benfluron 5-(2-methylaminoethoxy)-7,9-dihydroxy-7*H*-benzo[*c*]-fluorene, (**3a**). Also, two polar compounds with 9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorene and 7,9-dihydroxy-7*H*-benzo[*c*]fluorene chromophores with shorter retention times were found; their identification (both are probably conjugated to polar eobiotics) is in progress.

N-Demethylated benfluron (**9**) as the parent compound gave not only higher yields of reduced N-demethylated compound (**6**), as expected, but also the formation of two new compounds was confirmed. The first one is the primary amine, i.e. twice N-demethylated benfluron [t_R=8.3 min, 5-(2-aminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene, (**9a**)] and the second is 9-hydroxylated N-demethyl-benfluron (see Figs. 4c and 6, compound **7a**, alternative formation pathway *via* **10→9→7a**).

3.3. Use of isolated hepatocytes with elevated levels of cytochromes P-450

The last way to obtain higher amounts of benfluron metabolites was by the use of isolated fresh hepatocytes, in which the levels of the main enzymes of biotransformation, i.e. cytochrome P-450s, have been elevated (induced) by administration of the inducers to the experimental animals. To get higher levels of cytochrome P-450 1A isoforms, β-naphthoflavone was used; to induce the 2B family of isoenzymes, phenobarbital was used and finally the induction of the 2E1 isoform was achieved by administration of ethanol. The formation of P-450-dependent metabolites, i.e. of 9-hydroxy-benfluron (**7**, Fig. 1) and N-demethylated benfluron (**9**, Fig. 1) was significantly elevated when hepatocytes with either 1A, 2B or 2E1 induced P-450 isoforms were used. Concomitantly, the amounts of the metabolites of the "second, third,... filial generations" obtained were higher. The product of oxidative deamination of primary amine **9a**, 7-oxo-7*H*-benzo[*c*]-5-fluorenoxy-acetic acid (**9b**) with t_R=2.5 min, has been found in low concentrations in experiments with elevated levels of cytochrome P-450s (see Fig. 6).

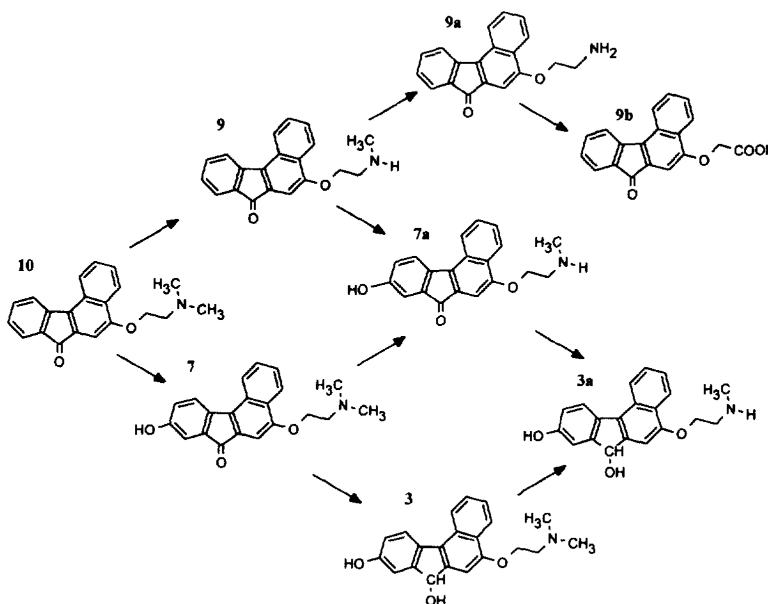

Fig. 6. Newly found metabolites of phase I of benfluron biotransformation.

The HPLC method with UV detection and (on-line) spectral analysis has been shown to be a method of choice provided that the compounds analyzed exhibit characteristic absorption spectra. The approaches described in this paper helped to identify new metabolites of benfluron and new pathways of benfluron biotransformation and contributed to the characterizion of the overall turnover of the parent drug (as the newly found compounds, mainly the conjugates, significantly increase the yield of metabolites formed). These facts are another illustration of the usefulness of these relatively cheap but reliable and convenient methods of analysis of biological samples.

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References

[1] I. Vančurová, M. Šimonová, J. Beneš and J. Křepelka, Českoslov Farm., 31 (1982) 308.
[2] J. Křepelka, I. Vančurová, J. Holubek, M. Mělka and K. Řežábek, Coll. Czech. Chem. Commun., 47 (1982) 1856.
[3] M. Mělka and J. Křepelka, Drugs of the Future, 12 (1987) 745.
[4] M. Miko, J. Křepelka and M. Mělka, Biochem. Pharmacol., 42 (1991) S214.
[5] M. Miko, J. Křepelka and M. Mělka, Anti-Cancer Drugs, 2 (1991) 289.
[6] M. Miko, J. Křepelka and M. Nobilis, Anti-Cancer Drugs, 3 (1992) 63.
[7] S. Jantová and K. Horáková, Cell Biochem. Funct., 11 (1993) 131.
[8] Z. Roubal, L. Poláková, J. Grimová, B. Kakáč, M. Peterková, V. Rejholec, P. Sedlmayer and I. Janků, Českoslov Farm., 34 (1985) 311.
[9] V. Francová, S. Šmolík, M. Schlehrová, K. Ráz, A. Selecká, Z. Franc, M. Frühaufová-Aušková, K. Řežábek, I. Vančurová and J. Křepelka, Neoplasma, 32 (1985) 529.
[10] E. Kvasničková, M. Nobilis and I.M. Hais, J. Chromatogr., 295 (1984) 201.
[11] I. Koruna, M. Ryska, L. Poláková, O. Matoušová, I. Vančurová, Z. Roubal and J. Křepelka, Českoslov Farm., 35 (1986) 451.
[12] E. Kvasničková, M. Nobilis, A. Šroler, E. Báčová and I.M. Hais, J. Chromatogr., 387 (1987) 559.
[13] M. Nobilis, E. Kvasničková, A. Šroler and I.M. Hais, Drug Metab. Interact., 9 (1991) 225.
[14] M. Nobilis, I. Vančurová, I.M. Hais, E. Kvasničková and J. Křepelka, Českoslov. Farm., 35 (1986) 68.
[15] A. Lyčka, J. Jirman, M. Nobilis, E. Kvasničková and I.M. Hais, Magn. Reson. Chem., 25 (1987) 1054.
[16] M. Nobilis, I. Vančurová, J. Křepelka, M. Mělka, A. Lyčka, J. Jirman, E. Kvasničková, I.M. Hais and M. Miko, Czechoslov. Patent. No. 267519, Federal Bureau for Inventions (PV 02914-88), Prague (in Czech), 1990.
[17] M. Nobilis and I.M. Hais, J. Chromatogr., 434 (1988) 363.
[18] I.M. Hais, M. Nobilis and E. Kvasničková, J. Chromatogr., 500 (1990) 643.
[19] P. Moldéus, J. Hogberg and S. Orrenius, Methods Enzymol., 52 (1978) 60.
[20] J. Květina and A. Guaitani, Pharmacology, 2 (1969) 65.