ELIMINATION OF BENFLURON AND ITS METABOLITES IN THE FAECES AND URINE OF RATS

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SUMMARY

The study of the biotransformation of the potential cytostatic benfluron has been continued. The elimination of benfluron and of nine of its metabolites whose structure had been established, mainly on the basis of the comparison of their IR, MS and NMR spectra with those of standards, was studied. After oral administration of 500 $mg.kg^{-1}$ to rats, the amounts of these substances in the faeces and urine were followed up by high-performance liquid chromatography for five days. Striking qualitative and quantitative differences were observed in the elimination of benfluron and its metabolites by both routes.

KEY WORDS

5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride, benfluron, cytostatics, elimination of metabolites

INTRODUCTION

Benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene hydrochloride; Fig. 1 - compound 10] was prepared in the Research Institute of Pharmacy and Biochemistry in Prague /1/. In preclinical analysis it exhibited antineoplastic properties /2/ and so the first stage of clinical testing was started.

Roubal *et al.* /3/ studied the pharmacokinetics of benfluron in experimental animals. On the basis of thin-layer chromatographic (TLC) densitometric analysis of serum extracts, the main pharmacokinetic parameters following oral (200 $mg.kg^{-1}$ in the mouse, rat and rabbit, 100 $mg.kg^{-1}$ in the dog) and intravenous administration (30 $mg.kg^{-1}$ in the rat) were established.

The absorption, distribution and elimination of ^{3}H-benfluron following oral (200 $mg.kg^{-1}$) and intravenous administration (30 $mg.kg^{-1}$) to rats was studied by Francová *et al.* /4/. After oral administration, long persistence of the label in the stomach and its wall was noted: 10% of the radioactivity administered was still found there 48 hours later. Radioactivity culminated in the small intestine between the first and 9th hour and in the large bowel about 48 hours after administration. 80% of radioactivity was eliminated in the faeces within the first week. After intravenous administration a rapid shift of the labelled compound from the blood into the organs (heart, lungs, kidney, brain and liver) was noted. Biliary elimination was considerable (54% within 12 h). 70% of the radioactivity from intravenously administered labelled compound leaves the body in the faeces, 24% in the urine.

Data on the metabolism of benfluron in animals and on the chemical structure of the products were obtained by biotransformation studies *in vitro* /5-8/ and *in vivo* /9, 10/.

The production of metabolites on the subcellular level was studied using the microsomal fraction of liver cells incubated with benfluron and the coenzymes NADPH or NADH /5, 6/. The structure of the products isolated from the incubation mixtures by preparative TLC was established by infrared and mass spectrometry. Reference compounds were prepared by chemical modification of benfluron /11, 12/ or by total synthesis /13/. Analogous studies were carried out at the cellular level using isolated hepatocytes.

Benfluron and its metabolites were estimated in the extracts from the incubates by means of HPLC /7, 8/. The inclusion of the pho-

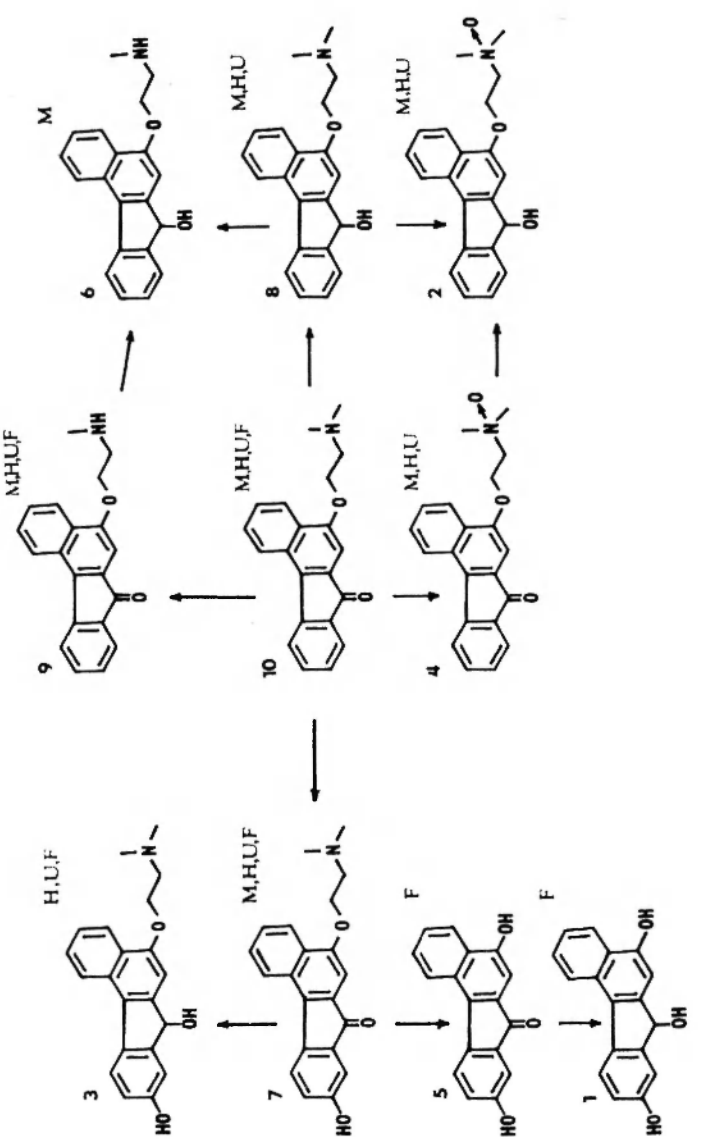

Fig. 1: A survey of the biotransformation of benfluron and of the occurrence of the benzo[c]fluorene compounds (**M** - microsomes, **H** - hepatocytes, **U** - urine, **F** - faeces). The arrows refer to hypothetical pathways.

todiode array detector in the chromatographic set-up made it possible to characterize the metabolites according to their UV-spectra /8/ (Fig. 2).

The metabolites of benfluron which were found in the extracts from the *in vitro* incubates are surveyed in Figure 1. Benfluron (**10**) is oxidized in the side chain to its N-oxide (**4**) and N-demethylated product (**9**). The reduction of the ketones **4**, **9** and **10** results in the respective secondary alcohols, 7-hydroxy-derivatives **2**, **6** and **8**. In addition, the phenolic metabolite of benfluron (**7**) was found which, after the isolation of a substantial amount from faeces, was identified by Fourier-Transform NMR as 9-hydroxy-benfluron /10/.

The same metabolites were produced by the incubation with isolated hepatocytes where, in addition, 5-(2-dimethylaminoethoxy)-7,9-dihydroxy-7*H*-benzo[*c*]fluorene (**3**) was revealed.

Koruna *et al.* /9/ studied the metabolites of benfluron found in the urine of the rat, rabbit, mini-pig and man by means of mass spectrometry. He confirmed the presence of most of the metabolites just mentioned. In the case of phenolic derivatives, the position of the hydroxyl has not been specified. Several minor metabolites not produced *in vitro* or occurring below the detection threshold of TLC and HPLC were revealed /9/.

The aim of the present paper is the identification of the main metabolites of benfluron in the urine and faeces of rats and monitoring of their elimination from the organism.

MATERIALS AND METHODS

Laboratory animals

Three male rats (*Rattus norvegicus* var. *alba*, Wistar type) from the Trebeš breeding station of the Faculty of Pharmacy were employed. They weighed 250 g (rat *I*), 280 g (rat *II*) and 400 g (rat *III*). They were allowed access to water and food pellets *ad libitum* before and during the experiment.

Materials

Benzo[c]fluorene derivatives (see Figure 1). A glossary of their names and code numbers follows:

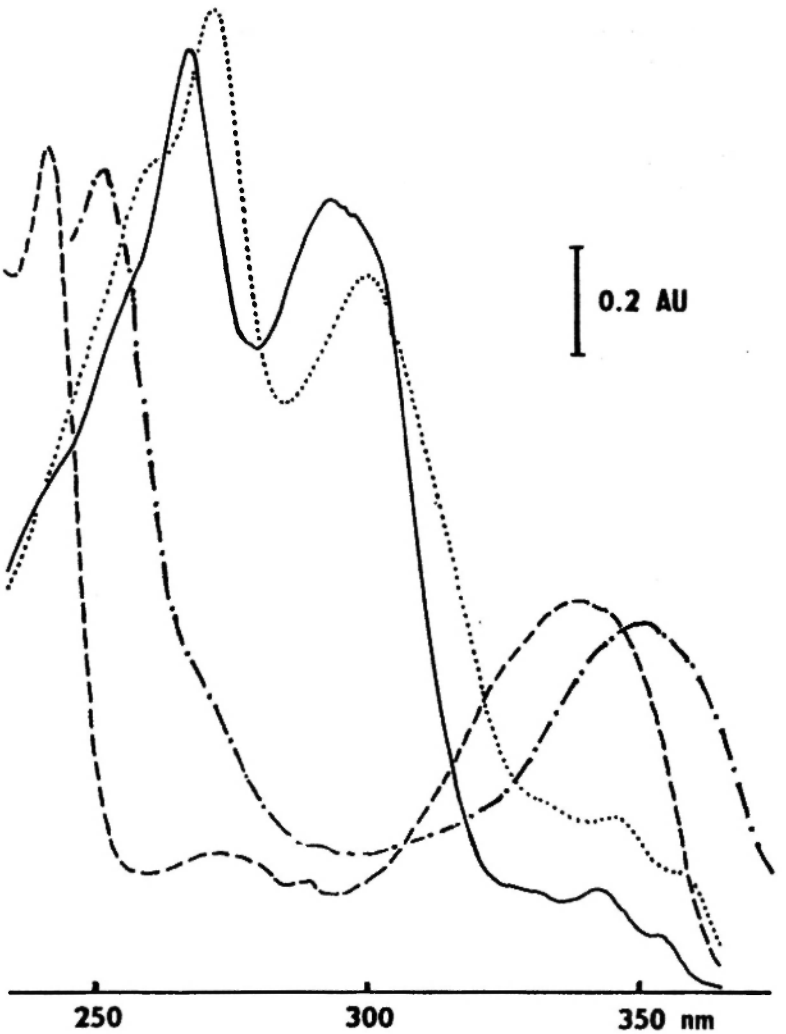

Fig. 2: UV spectra of benzo[c]fluorenes in the HPLC mobile phase. Concentration 5.10^{-5} mol.l^{-1}. ——— 7-oxo-7*H*-benzo[c]fluorenes, ---------- 7-hydroxy-7*H*-benzo[c]fluorenes, ... 9-hydroxy-7-oxo-7*H*-benzo[c]fluorenes, . - . - . - . - . - 7,9-dihydroxy-7*H*-benzo[c]fluorenes.

5,7,9-trihydroxy-7*H*-benzo[*c*]fluorene (**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-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-dimethylaminoethoxy)-7-hydroxy-7*H*-benzo[*c*]fluorene (**8**)
5-(2-methylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene (**9**)
5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo[*c*]fluorene (benfluron **10**, 353.9 g.mol^{-1}).

Code numbers of the individual compounds correspond to their elution sequence from the column.

Reference samples **4** and **10** were obtained from the Research Institute of Pharmacy and Biochemistry in Prague; the remaining compounds were prepared in our laboratory by chemical synthesis (**6-9**) /11, 13/ or by isolation from the faeces **5,7** /10/. Metabolites **1** and **3** were prepared by the Meerwein -Ponndorf-Verley reduction of compounds **5** and **7**, which leaves their phenolic hydroxyl intact. The reduction with a 2- to 3-fold molar excess of aluminium isopropoxide in anhydrous 2-propanol yielded almost 90% of the substances **1** or **3**, respectively. Both were isolated from the reaction mixture and purified by preparative TLC. They were recrystallized after conversion to hydrochlorides.

Mobile phase components. Acetonitrile and nonylamine (analytical reagent grade and purum, Fluka Chemie AG, Buchs), methanol, 2-propanol (anal. reag. grade, redistilled), phosphoric acid (anal. reag. grade, all Lachema, Brno, Czechoslovakia).

Chromatography (see Figures 3 and 4)

Chromatographic set-up consisted of a high pressure pump HPP 4001 (Laboratory Instruments, Prague), syringe loading sample injector with 10 μl loop - model 7125 (Rheodyne, USA), CGC columns 150 x 3.3 mm with Separon SGX C 18 7 μm (Laboratory Instruments, Prague), a PU 4021 multi-channel photodiode-array UV-Vis detec-

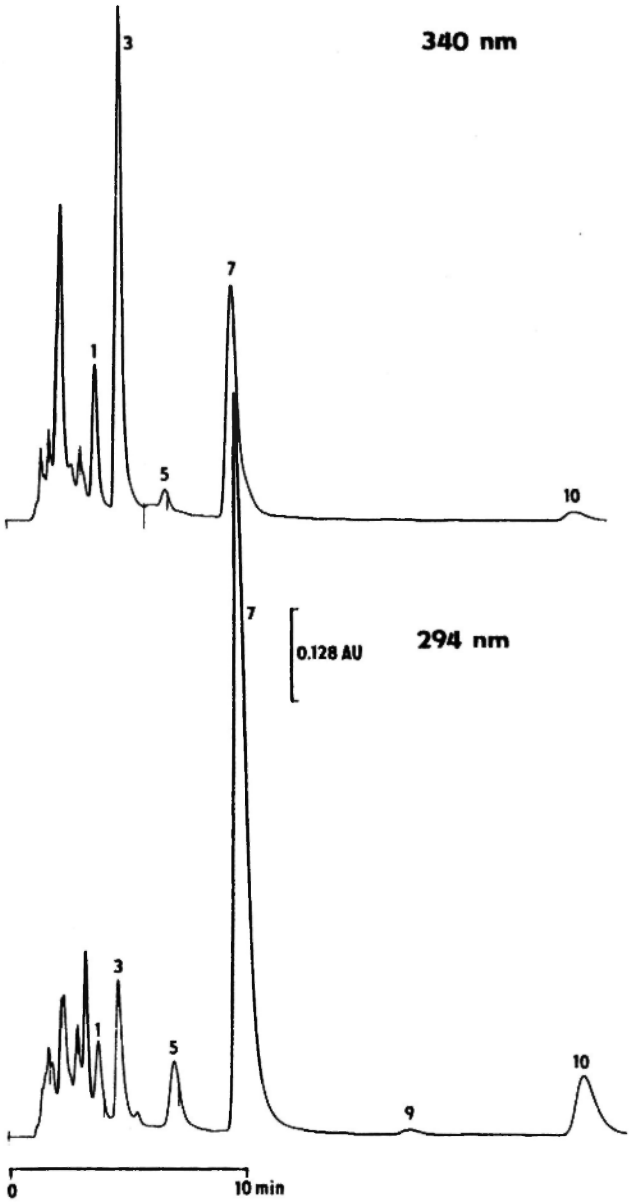

Fig. 3: Chromatogram of the faecal extract (rat *III*, 48-72 h after administration of benfluron). The dry residue was dissolved in 10 ml of mobile phase and 10 μl was used for HPLC. $F = 0.5$ ml.min^{-1}.

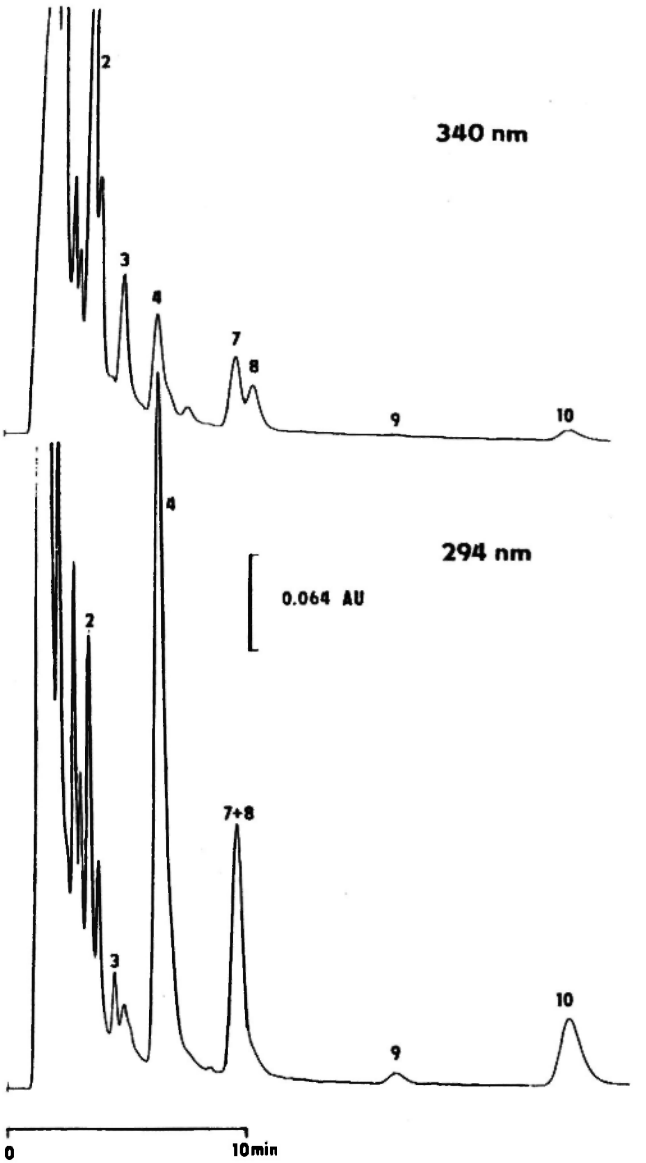

Fig. 4: Chromatogram of the urine extract (rat *III*, 48-72 h after administration of benfluron). The dry residue was dissolved in 1 ml of mobile phase and 10 μl was used for HPLC. $F = 0.5$ ml.min^{-1}.

tor (Pye Unicam, Cambridge, UK) and two PU 4810 computing integrators (Pye Unicam, Cambridge, UK).

Mobile phase consisted of nonylamine buffer, acetonitrile and 2-propanol (2:2:1, v/v/v). The buffer was made by mixing 2 ml nonylamine with 990 ml water, adjusting to pH 7.4 with a solution of phosphoric acid (2 $mol.l^{-1}$) and making up to 1000 ml with water.

Spectrophotometer

UV-Vis-spectrophotometer SP 8-200 (Pye Unicam, UK) was used for the study of the spectra and the calculation of the molar absorption coefficients.

Computation

Calculations were carried out using an IBM-compatible AT computer (Falcon Technol., Kent, WA, U.S.A.). To speed up the calculations and their visual and graphics presentation, software was written in GWBASIC and is available from the authors on request.

Biotransformation and extraction of benzo[*c*]fluorenes

Saturated aqueous solution of benfluron (500 $mg.kg^{-1}$) was administered by stomach tube to the rats in light ether anaesthesia. The animals were placed individually in metabolic cages. Urine and faeces were collected at 24-hour intervals for 5 days. The samples were processed immediately.

The urine was alkalized with 7 ml of 15% aqueous ammonia to pH 9-10 and repeatedly (3 times) extracted with 10 ml ethyl acetate.

Faeces were triturated and extracted repeatedly (3 times) with small volumes (10-25 ml according to the amount of faeces eliminated during 24 hours) of ethyl acetate containing 5% of triethylamine. The extract was filtered off from the suspension.

Ethyl acetate extracts were evaporated *in vacuo* (max 40°C) to dryness. The residues were dissolved in a known volume (usually 1 ml for urine extract and 10 ml for faeces extract) of the mobile phase to be used in HPLC.

HPLC analysis of benzo[*c*]fluorenes

The procedure and the evaluation of chromatographic analyses based on the molar absorption coefficients (Table 1) has been described previously /7, 8/.

RESULTS

In the course of the present study (Figures 3 and 4), two further benzo[*c*]fluorene derivatives were discovered which had not been detected in the *in vitro* experiments. Metabolite **5** is an O-dealkylation product of 9-hydroxy-benfluron. Metabolite **1** is the reduction product of metabolite **5**. Molar absorption coefficients of metabolites **1**, **3** and **5** for 294 and 340 nm were established to supplement the table of these coefficients published earlier /7/ (see Table 1). Figures 5 and 6 show the integrated excretions (faeces and urine, respectively) of the benflurone metabolites in terms of percentage of administered dose. Means and standard deviations are given for all time intervals. Substances demonstrated in both faeces and urine are shown by unbroken lines; substances shown in only one of the two excretions are shown by broken lines. Sums of the identified benzofluorenes in the respective excretions are presented in Figure 7.

DISCUSSION

The spectra of benzo[*c*]fluorenes (Figure 2) show that we are dealing with four spectrally differing benzo[*c*]fluorene classes. This can be exploited in the identification of the HPLC peaks using the photodiode array detector.

Substance **6** (reduced N-demethylated benfluron), which had been identified *in vitro* /5/, could not be detected in the urine or faeces.

The peaks of the phenolic benfluron derivative **7** and of the 7-dihydro-benfluron **8** overlap in the urine chromatogram (see Figure 4). A method of dealing mathematically with this situation has been described /7, 8/. Interestingly, 7-dihydro-benfluron **8**, which is a major product found in the body and in the *in vitro* incubates, was not detectable in the faeces and thus did not interfere with the estimation of **7**. This was confirmed by recording the spectra at

TABLE 1

The values of molar absorption coefficients of benzo[*c*]fluorenes

	ε_i^λ [$m^2 . mol^{-1}$]	
Compounds	**294 nm**	**340 nm**
7-Oxo-7*H*-benzo[*c*]fluorenes (**4, 9, 10**)	$2.94 . 10^3$	$0.38 . 10^3$
7-hydroxy-7*H*-benzo[*c*]fluorenes (**2, 6, 8**)	$0.37 . 10^3$	$1.47 . 10^3$
9-hydroxy-7-oxo-7*H*-benzo[*c*]fluorenes (**5, 7**)	$2.44 . 10^3$	$0.64 . 10^3$
7,9-dihydroxy-7*H*-benzo[*c*]fluorenes (**1, 3**)	$0.518 . 10^3$	$1.22 . 10^3$

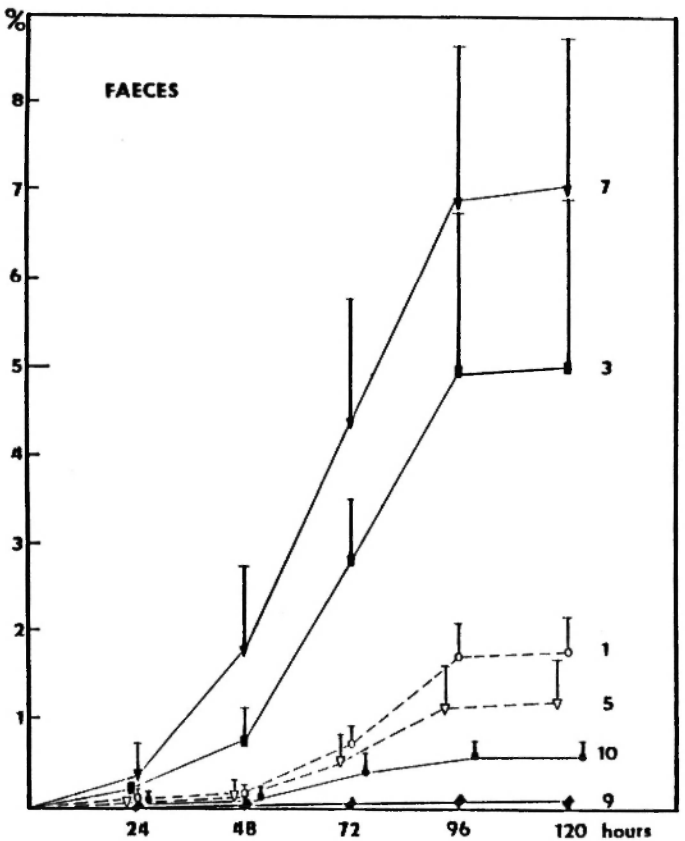

Fig. 5: Integrated elimination in the faeces of benzo[*c*]fluorenes after the intragastric administration of 500 $mg.kg^{-1}$ of benfluron. Mean values and standard deviations in mole % (n = 3).

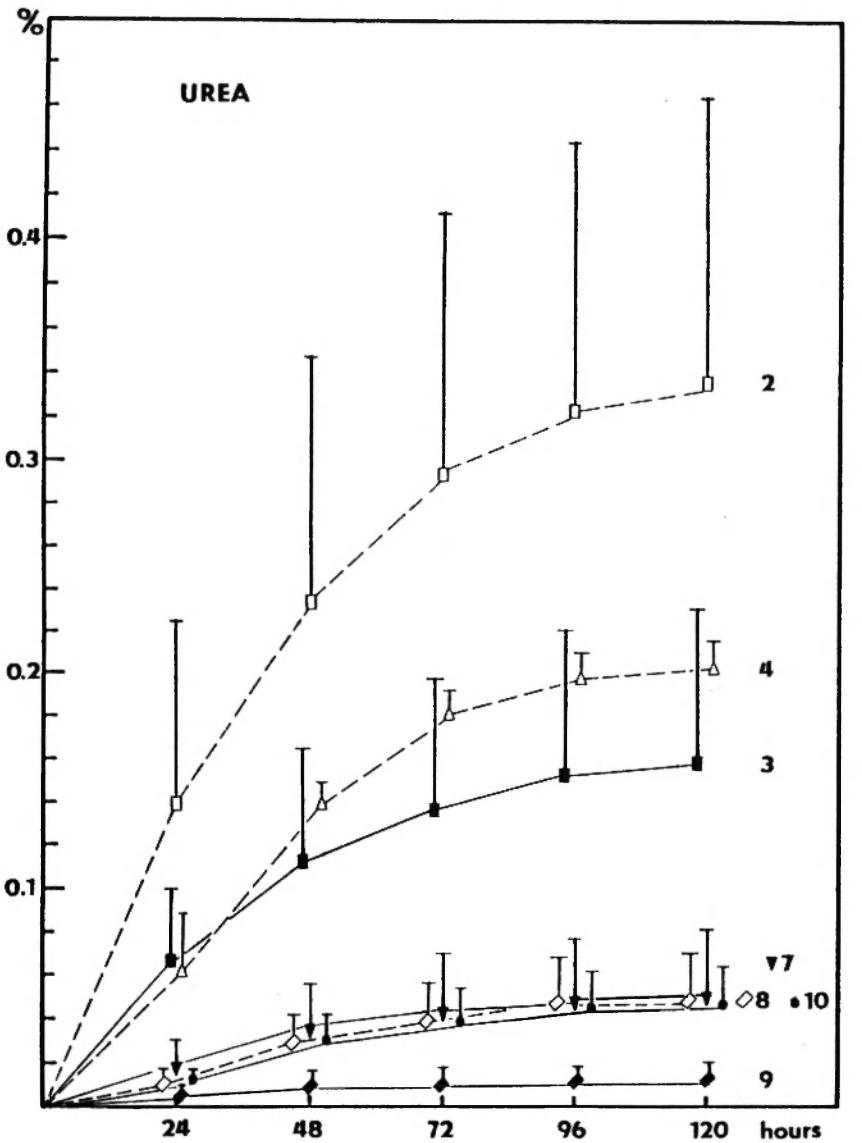

Fig. 6: Integrated elimination in the urine of benzo[c]fluorenes after the intragastric administration of 500 mg.kg^{-1} of benfluron. Mean values and standard deviations in mole % (n = 3).

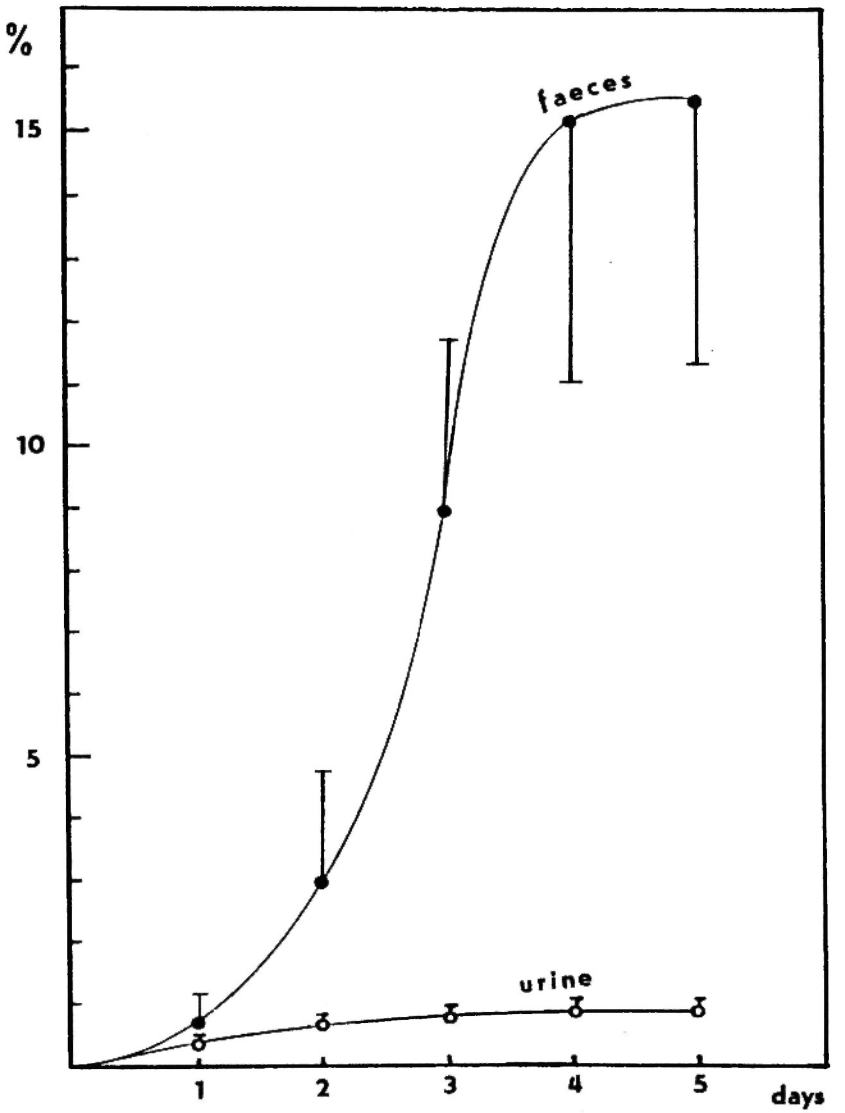

Fig. 7: Integrated elimination of the sum of identified benzo[*c*]fluorenes after the intragastric administration of 500 mg.kg^{-1} of benfluron. Mean values and standard deviations in mole % (n = 3).

different time points during elution of the respective concentration peak.

The retention times of the N-oxide of benfluron **4** and of the phenolic metabolite 5 are also close to one another, yet **5** only occurs in the faecal extracts, where **4** is absent. In urinary extracts **4**, but not **5**, was found.

In the faeces, the phenolic metabolites **7**, **3**, **1** and **5** are prevalent. The N-demethylated benfluron **9** was present in low amounts. There are at least three arguments favouring the production of **5** and **1** from **7** and **3** by intestinal bacteria: They were not found in the *in vitro* incubates with microsomes or hepatocytes. Their excretion in the faeces increases after an apparent initial lag, suggesting that they need prolonged exposure to the intestinal environment. They were not found in preliminary experiments with antibiotics which kill intestinal flora (unpublished results). It cannot be determined to what extent the animal organism (biliary excretion) and the bacterial flora participate in the excretion of other metabolites, especially the phenols **7** and **3**.

In the urine we found the products of N-oxidation (the N-oxide **4** and its 7-dihydro form **2**), of aromatic hydroxylation (9-hydroxy-benfluron **7** and 7-dihydro form **3**), N-demethylated benfluron **9** (low amount) and 7-dihydro-benfluron **8**. It was not possible to detect **1** and **5** in the urine. They might not have been absorbed from the gut, or, if absorbed, they may have undergone conjugation. Admittedly we did not look for conjugates.

Peaks (cf. Figures 3 and 4) which did not correspond to the nine substances for which we had reference samples, by their retention times or their spectra, were not considered.

As shown by the slopes of the lines, maximum levels in the faeces are reached on the 3rd or 4th day (Fig. 5) after the administration of benfluron. According to Fig. 6, urinary elimination of benzo[*c*]fluorenes reaches its maximum on the 2nd day (in case of some metabolites, on the first day).

Quantitative differences in the total amounts of metabolites eliminated in the urine and in the faeces are very striking (Fig. 7). The conclusion of Francová *et al.* /4/, namely that elimination is mainly through the gastrointestinal tract, has been confirmed. From the amount of benfluron administered, 10-18% is excreted in the faeces in the form of the drug and of its five identified metabolites. Through the uropoietic system, on the other hand, only 0.7-0.9% of the amount

of benfluron administered is excreted in the form of the drug and of its six identified metabolites. The fate of the rest remains unknown. In a separate experiment we studied the elimination of the benzo[*c*]fluorenes up to the 11th day following administration of benfluron and we could not detect any benfluron or its known metabolites after the 5th day.

Francová *et al.* /4/ demonstrated considerable affinity of benzo[*c*]fluorenes to some organs. One of the possible explanations of the difference between the dose administered and the sum of benfluron plus its known metabolites eliminated would be the gradual release from tissues and subsequent elimination at levels which are beyond the sensitivity of our techniques. Considerable elimination of benfluron and its biotransformation products in the bile was noted by Francová *et al.* /4/; the enterohepatic cycle would prolong the retention of benzo[*c*]fluorenes in the organism.

On the basis of a more sensitive mass-spectrometric method, Koruna *et al.* /9/ reported some 22 metabolites of benfluron. Although some of them were present only in small amounts, benfluron metabolites which remained unidentified in our study may have contributed to the difference from the overall metabolites /4/. Strongly polar metabolites may show low recovery in the extraction technique used. Some further degradation products of benzo[*c*]fluorenes may escape detection at the wavelengths 294 and 340 nm employed.

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