Study of the Biotransformation of Benfluron Using the Isolated Perfused Rat Liver

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Introduction
Benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7H-benzo[g][1,4]diazepine hydrochloride] is a potential antineoplastic agent exhibiting interesting pharmacodynamic properties in experiments carried out in vitro (animal and human cells) and in vivo (2,9,10,11). Biotransformation of human cells and in vitro (2,9,10,11). Biotransformation of benfluron was also studied with the use of experimental in vivo and in vitro methods. A number of metabolites generated during Phase I biotransformation have been detected (see Fig. 1) (4.13). A high-performance liquid chromatographic method was developed to determine benfluron and its metabolites in extracts from biological samples (7). However, previous studies on benfluron biotransformation revealed significant differences in recovery measurements, i.e. a difference in the total amount of benfluron given to the rat and the sum of the nine metabolites found in laces and in urine over eleven days after oral administration of the parent compound. Oral administration of benfluron has been shown to lead to very poor absorption due to strong affinity of this compound to the rat stomach wall (1.14). The second possibility of how to explain the disproportion of recovery in the previous experiment is that 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 formed during Phase II biotransformation).

Key words: further metabolites of Phase I and Phase II biotransformation of the potential cytostatic agent benfluron with special regard to the conjugation processes. Its pharmacokinetic profile during the perfusion was also estimated. The rat liver was isolated from the body and perfused in vitro using a recirculating perfusion system. Benfluron was added to the reservoir as a bolus in doses of 200, 100, 30 mg/kg of body weight and 1 mg/perfusate volume and also as a continual infusion in a dose of 0.1 mg/min in separate series of experiments. The following metabolites formed during Phase I biotransformation were found in the perfusion fluid as well as in the bile: benfluron N-oxide, 9-hydroxy benfluron, demethylated 9-hydroxy benfluron, demethylated benfluron, and reduced benfluron. The major Phase II metabolite found in the bile samples was the glucuronide of 9-hydroxy benfluron. The pharmacokinetic profile of benfluron in IPRL indicated its main disposition and metabolic pathway, i.e. its rapid extraction from perfusate by the liver (t1/2α = 3.76 min). 9-Hydroxybenfluron followed O-glucuronidation and excretion to the bile. It was revealed that 12.5 % of the total dose of the parent compound was excreted to the bile in the form of conjugates during the first hour of perfusion, 32 % during 1.5 hour, and 70 % during 2 hours after the administration of benfluron. The conjugates with glucuronic acid represented 96-98 % of all metabolites found in the bile.

Material and Methods

Chemicals
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 to prepare Krebs-Henseleit bicarbonate buffer. (HPLC grade, Merck), nonylamine (purum, Fluka), chloroform, methanol, 2-propanol, phosphoric acid (85%), aqueous ammonia (26%), triethylamine,
Fig. 1: Chemical structures of benfluron (compound No. 10) and its metabolites with their hypothetical pathways.

Fig. 2: Diagram of a recirculating liver perfusion apparatus:
1. thermometer;
2. constant head device;
3. electromanometer;
4. three-way stopcock;
5. liver platform with organ;
6. vial to collect bile;
7. flow meter;
8. thermostatically controlled reservoir;
9. thermostat;
10. filter;
11. peristaltic pump;
12. pneumoxide;
13. oxygenator;
14. manometer;
15. thermostatic control unit;
16. constant temperature cabinet.

Laboratory animals
Male rats Wistar Han II (Rattus norvegius var. alba, conventional breeding facility of the Research Institute for Pharmacy and Biochemistry, Konářovice nad Labem, Czech Republic; 250-300 g) were used. They were fasted overnight and were allowed free access to water before the experiment. The experiments were approved by the local ethics committee.

Liver Perfusion
The rat liver was perfused in vitro using a modified surgical and perfusion technique described previously (8). Animals were anaesthetized with pentobarbital (60 mg/kg) before surgery. Freshly prepared and filtered albumin- and erythrocyte-free Krebs-Henseleit bicarbonate buffer (pH 7.4), supplemented with glucose (0.1 %), polynvinylpyrolidone (3.5 %) as the plasma expander, and heparin (6.7 IU/ml) and bubbled with humidified mixture of 95 % CO₂ and 5 % O₂, was delivered to the portal vein catheter. A flow rate of about 4 ml/g liver/min was maintained. The temperature of the perfusion cabinet and perfusion medium was thermostatically controlled at 37±0.5°C. Perfusion was conducted using the recirculating mode. Cannulation of the common bile duct permitted collection of bile, the flow rate of which was determined gravimetrically. An initial stabilization period of 30 min was allowed before adding benfluron to the perfusion medium.

The perfusate flow rate, bile production and organ appearance were determined during the perfusion, organ weight and organ histopathology were determined after the perfusion. The perfusate flow rate, bile production and organ appearance were determined during the perfusion, organ weight and organ histopathology were determined after the perfusion.

Sample preparation
The samples of perfusion liquid were alkalized 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 collected bile was only diluted with the mobile phase used in HPLC or in methanol for preparative TLC.

Chromatography
A Thermo Separation Products chromatograph setup was used. An HPLC column LiChrocart 125 x 4 mm with a precolumn LiChrospher 100 RP-18 (Merck) were used. The samples were assayed using a Spectra Focus high speed scanning UV detector. Detection was performed in dual wavelength mode (295 and 340 nm) or in high-speed scanning mode (range 195-365 nm).

A preparative TLC was used for the isolation of the newly found metabolites and their conjugates in bile. For more details see the literature (12).

Calculation
A Table Curve 2D software (SPSS Inc., version 4) was used to calculate pharmacokinetic data (t₁/₂α= half-time associated with the rapid elimination phase, t₁/₂β= half-time associated with the slow elimination phase) from mean perfusate and bile concentrations of benfluron and its metabolites. Bile excretion rate of metabolite was expressed as mean ± standard deviation.

Results
All data presented in this chapter were obtained after administration of 1 mg of benfluron into the IPRL system (see discussion). Using the IPRL method, the following metabolites of benfluron formed during Phase I biotransformation were found in the perfusion liquid as well as in the bile:...
and ethyl acetate (all of analytical grade, Lachema) were used for sample preparation, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Nonylamine buffer for the mobile phase and five benzo[c]fluorenes standard mixtures (compounds 4, 7, 8, 9 and 10, synthesis: M. Nobilis) were used for HPLC assay.

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In the first series of experiments, IPRL preparations were perfused with different bolus doses of benfluron: 200, 100 and 30 mg/kg (n = 2 for each dose) and 1 mg/perfusate volume (n = 4). These experiments were performed to define the metabolic profile of benfluron and to assess its various metabolites in the perfusate and bile. Perfusion and bile samples were collected at 15-min intervals after the addition of benfluron. Additional two IPRL experiments were conducted in which the livers were perfused with constant infusion rate of benfluron (0.1 mg/min) to produce a sufficient quantity of conjugates for their further identification. Bile samples were collected at 10-min intervals during 120 min of perfusion.

Sample preparation
The samples of perfusion liquid were alkalized 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 collected bile was only diluted with the mobile phase used in HPLC or in methanol for preparative TLC.

Chromatography
A Thermo Separation Products chromatograph setup was used. An HPLC column LiChroCART 125 x 4 mm with a precolumn LiChrospher 100 RP-18 (Merck) were used. The samples were assayed using a Spectra Focus high-speed scanning UV detector. Detection was performed in dual wavelength mode (295 and 340 nm) or in high-speed scanning mode (range 195-365 nm). A preparative TLC was used for the isolation of the newly found metabolites and their conjugates in bile. For more details see the literature (12).

Liquid Chromatography-Mass Spectrometry
A Beckman System Gold setup (pump 125S, diode-array UV detector 168) and a Finnigan MAT setup (LCQ ion trap mass spectrometer coupled with a liquid chromatograph by an electrospray interface) were used for metabolites identification (LC/MS). It allowed to follow molecular masses of the compounds in the sample as well as to perform fragmentation of selected ions.

Calculation
A Table Curve 2D software (SPSS Inc., version 4) was used to calculate pharmacokinetic data ($t_{1/2\alpha}$ half-time associated with the rapid elimination phase, $t_{1/2\beta}$ half-time associated with the slow elimination phase) from mean perfusate and bile concentrations of benfluron and its metabolites. Bile excretion rate of metabolite was expressed as mean ± standard deviation.

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All data presented in this chapter were obtained after administration of 1 mg of benfluron into the IPRL system (see discussion). Using the IPRL method, the following metabolites of benfluron formed during Phase I biotransformation were found in the perfusion liquid as well as in the bile:

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1. thermometer; 2. constant head device; 3. electromanometer; 4. three-way stopcock; 5. liver platform with organ; 6. vial to collect bile; 7. flow meter; 8. thermostatically controlled reservoir; 9. thermostat; 10. filter; 11. peristaltic pump; 12. pneumoxide; 13. oxygenator; 14. manometer; 15. thermostatic control unit; 16. constant temperature cabinet.
Evaluation of the pharmacokinetic parameters revealed the halftime of the rapid distribution phase of benfluron ($t_{1/2A}$) = 1.76 min. The halftime of the elimination phase of benfluron was determined to be $t_{1/2B}$ = 63.30 min. The course of elimination of benfluron from the perfusion medium in dependence on time is shown on Fig. 3. Graphic representation of excretion of the principal metabolite (glucuronide 9-hydroxy benfluron) into bile in dependence on time is shown in Fig. 4. The halftime of achieving its steady-state phase of excretion was estimated to be 10.40 min.

### Discussion

The use of the IPRL method made it possible to find metabolites of Phase I and Phase II biotransformation of benfluron. The presented results clearly show that benfluron undergoes massive biotransformation in the liver compartment, above all by hydroxylation in position 9 and subsequent conjugation with glucuronic acid.

It was not possible to implement the original intention of finding minority metabolites by saturating biotransformation pathways by using large doses of benfluron. The use of high benfluron concentrations in the IPRL system resulted in marked changes in the examined parameters of functional capacity of the isolated liver, i.e. a decrease in perfuse flow, a decrease in oxygen consumption, and failure of biliary excretion. For this reason the doses were gradually decreased from 200 mg/kg to 1 mg/volume of perfusion medium. This dose did not result in the described changes and neither did continual infusion at a rate of 0.1 mg/min which was employed to produce sufficient amounts of metabolites intended for their identification by the LC/MS method. Interpretation of the changes in the functional capacity of the liver preparation after administration of large doses of benfluron can be based on a recent paper by Kopecký F. and Kopecká B. (3), which solves the physico-chemical properties of benfluron in aqueous medium in dependence on the ionic strength of the solution. Benfluron hydrochloride is a substance relatively well soluble in aqueous media. However, it has been found that in the presence of potassium chloride (and other electrolytes) in approximate osmotic concentrations of molecules, which could correspond to other conjugates (but their full identification has not been completed yet).

Conjugates excreted into bile in the course of 2 hours of perfusion represent 70 % (Table 1) of the administered dose of the parent drug and 96-98 % of all metabolites found. It gives evidence for a large share of conjugation mechanisms in biotransformation of benfluron by the liver and suggests possible enterohepatic kinetics of benfluron in vivo conditions. The construction of a probable scheme of benfluron biotransformation is shown in Fig. 1.

#### References

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### Table 1: Percentile representations of the conjugates from the administered dose in bile in vitro in dependence on time.

| Time of perfusion after benfluron administration [h] | Conjugates [cumulative % from administered dose] |
|-----------------------------------------------------|--------------------------------------------------|
| 1                                                   | 12±5                                             |
| 1.5                                                 | 32±11                                            |
| 2                                                   | 70±19                                            |

Recalculation of ion concentrations of employed Krebs-Henseleit solution and the achieved concentrations of benfluron in perfusion medium ($c_{benfluron}$ = 1.1·10⁻⁵ mol/l) has revealed that the used concentration of benfluron in a dose of 1 mg/volume of perfusion medium lies just below the limit of the concentration region of the formation of the above-mentioned multimers.

An analysis of absorption spectra obtained by high-speed UV scanning detection revealed the products of Phase I biotransformation, i.e. demethylated 9-hydroxy-benfluron and twice demethylated 9-hydroxy-benfluron, including other previously described metabolites (see Results). In detailed analysis of elution zones in the region of short retention times, the spectra characteristic of benzo[α]fluorene structures were found. For this reason the HPLC method was modified, which resulted in prolongation of elution times of metabolites and made possible to separate the found structures. Their isolation and identification using the LC/MS method have revealed that it is the product of conjugation of 9-hydroxy-benfluron with glucuronic acid. In the regions of short retention times (HPLC), the method of mass spectrometry has revealed other masses of molecules, which could correspond to other conjugates (but their full identification has not been completed yet).

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**Fig. 3.** The elimination kinetics of benfluron from the perfusate after administration of 1 mg of benfluron into the IPRL system.

**Fig. 4.** The kinetics of biliary excretion of the principal metabolite of benfluron (conjugate of 9-hydroxy-benfluron) after administration of 1 mg of benfluron into the IPRL system.
Evaluation of the pharmacokinetic parameters revealed the half-time of the rapid distribution phase of benfluron ($t_{1/2\alpha}$) = 3.76 min. The half-time of the elimination phase of benfluron was determined to be $t_{1/2\beta}$ = 63.30 min. The course of elimination of benfluron from the perfusion medium in dependence on time is shown in Fig. 3. Graphic representation of excretion of the principal metabolite (glucuronide of 9-hydroxy benfluron) into bile in dependence on time is shown in Fig. 4. The half-life of achieving its steady state phase of excretion was estimated to be 10.40 min.

It revealed, in a short retention time of HPLC analyses, the main metabolite of benfluron, identified by LC/MS as the product of conjugation of 9-hydroxy benfluron with glucuronic acid formed during the Phase II biotransformation, i.e. O-glucuronide of 9-hydroxy benfluron.

### Recalculation of ion concentrations of employed Krebs-Henseleit solution and the achieved concentrations of benfluron in perfusion medium

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During the last November we recalled the 70th anniversary of opening the new public hospital in Hradec Královec, the institution that subsequently has become a basis for the University Hospital of the new local Medical Faculty. Since that anniversary there has already elapsed more than half a year and today's reminder may thus be taken for rather delayed.

Nevertheless, the purport of this article is not only to commemorate the very famous day when the long lasting desire of doctors and various men of light and learning of Hradec Královec came to its fulfilment. I would like to demonstrate also all the efforts, which had preceded this hospital creation.

The care for human health was developing during the history only very slowly. Things that we nowadays consider for quite natural ones were neither dreamed of for many centuries. Today's hospitals were preceded in the Middle Ages, as well as even for a long part of the New Ages, by so called sick houses. Their tasks did not lie in the proper treatment; they rather represented mere shelters for old and sick people. The first such sick houses were established by Church and consisted mostly from very modest institutions, as proved by the photograph of Jan Smit that documents the by a miracle preserved small baroque sick house in Rabštejn upon Střela, erected most probably by Servits in the 18th century.

Also in Hradec Královec and its close vicinity such sick houses arose and disappeared again. The existence of the first one, established most probably as soon as in the 13th century by the commend (monasterial settlement) of German Sick House Brethren, German Knights of the Cross, is documented in written from the year 1362. Another sick house was founded in the middle of the 14th century at Saint Anna's Church. By the end of 14th century the magistrate and aldermen together with the parish priest from Holy Ghost founded a big sick house with the St. Anthony Church on the riverside of Orlice. All these sick houses came to a lot of harm during the Hussite wars. That big one at Orlice river ceased to exist during the fortification works in Hradec Královec. Besides the above mentioned sick houses for old and sick people also the special houses for leprous, and later on also for plague and cholera patients were established during the Middle Ages.

As late as by the end of the 18th and beginning of 19th centuries the character of hospitals in this country, as well as in all over Central Europe, started changing into the today's form of existence. In Hradec Královec, that was in the year 1765 by the decision of Emperor Joseph the 2nd converted into the military fortified town, the situation was rather difficult. Vast suburbs had been taken down and the historical town centre on the hill above the rivers Labe and Orlice confluence were squeezed by the star-shaped walls. The whole city became a bazaar housing estate with continious existence of offices, trades, church and school institutions, and, above all, housing the strong military garrison. It was quite natural then, that at first the military hospital...