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Disposition study of a new potential antineoplastic agent dimefluron in rats using high-performance liquid chromatography with ultraviolet and mass spectrometric detection

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Abstract

The disposition of a new potential antineoplastic drug dimefluron after an oral administration to rats was investigated. Dimefluron, 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride, was administered in a single oral dose (250 mg kg^{-1} of body weight) in the form of an aqueous solution via a gastric probe. Dimefluron metabolites were being searched for in rat faeces. Synthetic standards of the expected phase I metabolites (the products of *O*- and *N*-desmethylation, *N*-oxidation and carbonyl reduction of dimefluron) were prepared and used together with dimefluron and internal standard in the development of two HPLC bioanalytical methods based on different separation principles. The first separation of dimefluron and the phase I metabolites was tested on a 250 mm × 4 mm chromatographic column with LiChrospher 60 RP-selectB 5 μm (Merck) using an isocratic mobile phase containing 0.01 M nonylamine buffer (pH 7.4) and acetonitrile in the 1:2 ratio (v/v). The second separation was performed on a 250 mm × 4 mm chromatographic column Discovery HS F5, 5 μm (Supelco) using a linear gradient mode with the mobile phase containing acetonitrile and phosphate buffer (0.05 M KH_2PO_4, pH 3). The flow rate was 1 ml min^{-1} in both cases. UV detection was performed in the dual wavelength mode, with 317 nm having been used for dimefluron and all 7*H*-benzo[*c*]fluoren-7-one metabolites, 367 nm for 7H-benzo[*c*]fluoren-7-ol metabolites. A higher homologue of dimefluron served as an internal standard. The identity of the dimefluron metabolites in biological samples was confirmed using HPLC–MS experiments.

The elimination study showed that the concentration maximum for dimefluron and its metabolites in rat faeces was reached 48 h after the administration of the parent drug. *O*-Desmethylated derivatives of dimefluron prevailed among the phase I metabolites.

Keywords: Potential antineoplastic dimefluron; Metabolites in faeces; HPLC with UV and MS detection

1. Introduction

A potential antineoplastic agent dimefluron [3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride, see Fig. 1, compound 1] was synthesized in the laboratories of Institute of Experimental Biopharmaceutics in Hradec Králové as one of the proposed structural modifications of benfluron [1].

The starting structure from the group of basic benzo[*c*]fluorene derivatives, benfluron (5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride [2–4]), exhibited an interesting spectrum of pharmacodynamic properties in experiments carried out in vitro and in vivo [5–8].

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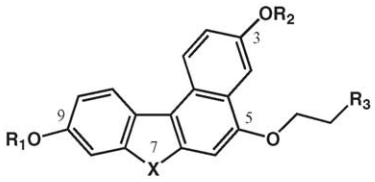

Compound	X	R_1	R_2	R_3
1	C=O	$-CH_3$	$-CH_3$	$-N(CH_3)_2$
2	C=O	-H	$-CH_3$	$-N(CH_3)_2$
3	C=O	$-CH_3$	-H	$-N(CH_3)_2$
4	C=O	-H	-H	$-N(CH_3)_2$
5	C=O	$-CH_3$	$-CH_3$	$-NH(CH_3)$
6	C=O	$-CH_3$	$-CH_3$	$-N(CH_3)_2O$
7	CH-OH	$-CH_3$	$-CH_3$	$-N(CH_3)_2$
8	C=O	$-CH_3$	$-CH_3$	$-CH_2N(CH_3)_2$

Fig. 1. Chemical structures of benzo[*c*]fluorene derivatives under study: dimefluron (DMF) (compound 1); 9-*O*-desmethyl-DMF (compound 2); 3-*O*-desmethyl-DMF (compound 3); 3,9-*O*-didesmethyl-DMF (compound 4); *N*-desmethyl-DMF (compound 5); DMF *N*-oxide (compound 6); C_7-reduced-DMF (compound 7); higher homologue of DMF (compound 8).

The disposition of benfluron in experimental animals was studied by TLC [9–12], HPLC [13–15], MS [16] and NMR [17] methods using a comparison of the metabolites with their synthesized standards [18,19]. During xenobiochemical studies, the biotransformation products of arylhydroxylation, *N*-desmethylation, *N*-oxidation, carbonyl reduction and also of conjugation with glucuronic acid were identified [14,15,20–23] and the enzymes involved in their biotransformation were characterized [24]. The elimination study of benfluron in rats showed that the principal phase I metabolite in faeces was 9-hydroxy-benfluron and that this metabolite was eliminated in the form of a conjugate with glucuronic acid [14,15,23].

Unfortunately, the preclinical tests of benfluron revealed certain negative pharmacokinetic, distribution and xenobiochemical properties, for which its further research and development was terminated. Attention was paid to some structural analogues of benfluron, including its 3,9-dimethoxy derivative, dimefluron.

Dimefluron was tested on rabbits and its potential chronic effects on the cardiac function, biochemical, haematological and other physiological parameters were compared with another intercalating antineoplastic agent, daunorubicin [25–28]. Only some aspects of dimefluron metabolism in vitro and in vivo have been studied so far [29,30].

The goal of this study was to develop chromatographic methods for the disposition studies of dimefluron, since insufficient amount of information about the pharmacokinetics and metabolism of dimefluron in experimental animals has been published to date. For this purpose, chemical reference standards of six expected metabolites of dimefluron and a higher homologue of dimefluron (internal standard) were synthesized and various bioanalytical HPLC methods based on ultraviolet diode-array and mass spectrometry detection were developed. The most promising of these methods was validated and used for the identification and determination of dimefluron metabolites in biomatrices (extracts from rat faeces).

2. Experimental

2.1. Chemicals, preparations and materials

Dimefluron, 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride ($C_{23}H_{24}ClNO_4$, MW = 413.89 g mol^{-1}, Fig. 1, compound 1); 9-*O*-desmethyldimefluron, 9-hydroxy-3-methoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride ($C_{22}H_{22}ClNO_4$, MW = 399.87 g mol^{-1}, Fig. 1, compound 2); 3-*O*-desmethyldimefluron, 3-hydroxy-9-methoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride ($C_{22}H_{22}ClNO_4$, MW = 399.87 g mol^{-1}, Fig. 1, compound 3); 3,9-*O*-didesmethyldimefluron, 3,9-dihydroxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one, base ($C_{21}H_{19}NO_4$, MW = 349.38 g mol^{-1}, Fig. 1, compound 4); *N*-desmethyldimefluron, 3,9-dimethoxy-5-(2-methylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride ($C_{22}H_{22}ClNO_4$, MW = 399.87 g mol^{-1}, Fig. 1, compound 5); dimefluron *N*-oxide ($C_{23}H_{23}NO_5$, MW = 393.43 g mol^{-1}, Fig. 1, compound 6); C_7-reduced dimefluron, 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol ($C_{23}H_{25}NO_4$, MW = 379.45 g mol^{-1}, Fig. 1, compound 7) and a higher homologue of dimefluron, 3,9-dimethoxy-5-(2-dimethylaminopropoxy)-7*H*-benzo[*c*]fluoren-7-one base ($C_{24}H_{25}NO_4$, MW = 391.46 g mol^{-1}, Fig. 1, compound 8) were synthesized in our laboratories and used as the standards in the elimination study. Hydrobromic acid (48% in water) and acetic acid (99.8%, p.a., both Lachema Brno, Czech Republic) were used for the *O*-desmethylation of dimefluron. Sodium borohydride (powder, >98%, Janssen-Chimica, Belgium) was employed in the carbonyl reduction of dimefluron. Diethyl azodicarboxylate (Fluka Chemie AG, Buchs, Switzerland) was used for *N*-desmethylation of dimefluron. Hydrogen peroxide (30% water solution, Lachema Brno, Czech Republic) was used for *N*-oxidation of dimefluron. 3,9-Dimethoxy-5-hydroxy-7*H*-benzo[*c*]fluoren-7-one ($C_{19}H_{14}O_4$, MW = 306.31 g mol^{-1}, synthesized in our laboratory) and 3-(dimethylaminopropyl chloride hydrochloride (MW = 158.07 g mol^{-1}, Sigma–Aldrich) were used for the preparation of the internal standard. Acetonitrile, methanol (both HPLC grade, Merck, Darmstadt, Germany), acetic acid (99%, analytical grade, HiChem s r.o., Prague, Czech Republic), ammonium acetate (Fractopur®, Merck, Darmstadt, Germany), ammonium hydroxide (26% aqueous solution of NH_3, all Lachema Brno, Czech Republic), ethyl acetate, formic acid (87% p.a.), hydrochloric acid (35% water solution of HCl, analytical grade), orthophosphoric acid (85% p.a.), potassium dihydrogenphosphate, sodium

hydroxide (all analytical grade, Lachema Brno, Czech Republic), nonylamine (purum; >97%), triethylamine (purum; >98%, both Fluka Chemika), ultra-high-quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga Ltd., Bucks, England) were used for the liquid–liquid extraction of biomatrices and chromatography of dimefluron derivatives. Dimethyl sulfoxide-d_6 (Armar AG, Germany) was used in NMR experiments.

Nonylamine buffer was made by mixing 1.83 ml of nonylamine with 990 ml of UHQ water, adjusting to pH 7.4 with a solution of orthophosphoric acid (2 mol l^{-1}) and making up to 1000 ml with water.

Triethylammonium acetate (ammonium acetate) buffer was prepared from a 10 mmol l^{-1} solution of triethylamine (ammonium hydroxide) in UHQ water, pH was adjusted to 7.4 by 2 mol l^{-1} acetic acid.

Phosphate buffer (50 mmol l^{-1}, pH 3) was made by mixing 6.81 g of KH_2PO_4 with 990 ml of UHQ water, adjusting to pH 3 with a solution of orthophosphoric acid (2 mol l^{-1}) and making up to 1000 ml with water.

Ammonium acetate buffer (5 mmol l^{-1}, pH 3) was prepared in an analogous way from 0.385 g CH_3COONH_4 per liter, pH was adjusted to the required value by the addition of formic acid (consumption ca 1.8 ml per liter).

Chromatographic plates (200 mm × 200 mm × 0.5 mm silica gel layers on glass plates) for the preparative thin-layer chromatographic separations of the synthesized standards of dimefluron metabolites were made on a Camag Automatic TLC Plate Coater (Muttenz, Switzerland) using a suspense of Silica gel 60HF_{254} for thin-layer chromatography (Merck, Germany) in a methanol–triethylamine mixture (1:1, v/v) poured on glass plates.

Preparative thin-layer chromatography (TLC) with a mobile phase of chloroform–methanol–triethylamine (70:10:5, v/v/v) was used for the separation of the reaction mixtures. The reaction mixture was dissolved in a minimum volume of the mobile phase, the solution was then transferred onto the start of a chromatographic plate and developed. The colour bands of silica gel, containing individual compounds were scraped off from the chromatogram. Each silica gel portion containing an individual compound was suspended in methanol and silica gel from the suspension was filtered off on a sintered glass filter with porosity under 40 μm. The filtrate (methanolic solution of an isolated compound) was evaporated to dryness. The purity of each individual compound was evaluated by HPLC with ultraviolet photodiode-array detection (HPLC–DAD), HPLC–MS and NMR experiments.

2.2. NMR analyses

A Varian Mercury-Vx BB 300 NMR spectrometer was used for the NMR analyses of the synthetic standards of dimefluron metabolites. The NMR spectra were recorded at 300 MHz for ^{1}H, and 75 MHz for ^{13}C. Chemical shifts are given as δ values in ppm, the coupling constants are given in Hz. Analytical sample (15–20 mg) was dissolved in deuterated dimethyl sulfoxide (0.7 ml) and the solution was transferred via pipette into an NMR tube (203 mm length, 5 mm diameter).

2.3. HPLC–MS analyses

High-performance liquid chromatography–mass spectrometry (HPLC–MS) analyses were performed on an Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) and a liquid chromatograph consisting of a model 616 pump with a quaternary gradient system, a model 996 photodiode array detector, a model 717+ autosampler, a thermostated column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA). Mass spectra were recorded in the range *m*/*z* 15–1000 using the positive-ion and negative-ion electrospray ionization (ESI). The pressure and flow rate of the nebulising gas were 70 psi and 12 l min^{-1}, respectively. The temperature of the drying gas was 365 °C. The mass spectrometer was tuned to give an optimum response for *m*/*z* 350. The extracts from the rat faeces or synthetic compounds were separated using a linear gradient elution: 0 min–40% A and 60% B, 20 min–80% A and 20% B, 60 min–80% A and 20% B, where A is acetonitrile and B is ammonium acetate buffer (C = 5 mmol l^{-1}, pH 3). The samples were dissolved in the mobile phase and 100 μl of the solution was injected into HPLC–MS system.

The positive-ion and negative-ion electrospray ionization mass spectra of all compounds were also measured using the direct infusion technique under the following conditions: the flow rate and pressure of the nebulising gas 4 l min^{-1} and 10 psi, respectively, temperature of the drying gas 300 °C. For direct infusion ESI experiments, the samples were dissolved in acetonitrile–water (1:1, v/v).

2.4. Syntheses of the standards of expected phase I dimefluron metabolites

2.4.1. *O*-Desmethylation of dimefluron (preparation of compounds 2–4 in Fig. 1 and in Section 2.1)

Dimefluron (1.3 g, 3.36 mmol) was dissolved in a mixture of hydrobromic acid (5 ml) and acetic acid (100 ml) and the solution heated under reflux for 1.5 h. The acids were then evaporated and the reaction mixture was separated by preparative thin-layer chromatography (see Section 2.1). The retention factors and colours of individual separated compounds were as follows: 3,9-*O*-didesmethyl-DMF (R_f = 0.35, gray green); 3-*O*-desmethyl-DMF (R_f = 0.53, red brown); 9-*O*-desmethyl-DMF (R_f = 0.61, violet); dimefluron (DMF; R_f = 0.89, red); the isolated bases of compounds 2 and 3 were converted to hydrochlorides and recrystallized.

2.4.1.1. 9-O-Desmethyldimefluron, hydrochloride of compound 2 ($C_{22}H_{22}ClNO_4$, MW = 399.87 g mol^{-1}). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.10 (s, 1H, OH), 10.01 (bs,

1H, N.HCl), 8.41 (d, 1H, J = 9.34 Hz, H1), 7.86 (d, 1H, J = 8.24 Hz, H11), 7.65 (d, 1H, J = 2.75 Hz, H4), 7.33 (dd, 1H, J = 9.34 Hz, J = 2.75 Hz, H2), 7.07 (s, 1H, H6), 6.97 (d, 1H, J = 2.47 Hz, H8), 6.86 (dd, 1H, J = 8.24 Hz, J = 2.47 Hz, H10), 4.579 (t, 2H, J = 4.67 Hz, OCH_2), 3.96 (s, 3H, OCH_3), 3.66 (t, 2H, NCH_2), 2.89 (s, 6H, NCH_3)

^{13}C NMR (75 MHz, DMSO-d_6) δ 193.7, 159.3, 158.1, 152.5, 137.3, 136.3, 134.6, 130.8, 128.9, 127.2, 124.1, 123.6, 120.5, 119.7, 111.9, 103.1, 100.2, 63.2, 55.9, 55.4, 42.9.

Positive-ion ESI mass spectra of 9-*O*-desmethyldimefluron base, $C_{22}H_{21}NO_4$, MW = 363; MS: *m/z* 402, $[M + K]^+$, 5%; *m/z* 386, $[M + Na]^+$, 2%; *m/z* 364, $[M + H]^+$, 100%. MS^2 of 364: *m/z* 319, $[M + H - CH_3NHCH_3]^+$, 100%. Negative-ion ESI mass spectra: MS: *m/z* 362, $[M - H]^-$, 100%; *m/z* 290, $[M - H\text{-}(CH_3)_2NCH_2CH_2]^{-\bullet}$, 36%. MS^2 of 362: *m/z* 317, $[M - H\text{-}CH_3NHCH_3]^-$, 2%; *m/z* 290, $[M - H\text{-}(CH_3)_2NCH_2CH_2]^{-\bullet}$, 100%; *m/z* 275, $[C_{17}H_7O_4]^-$, 15%.

2.4.1.2. 3-O-Desmethyldimefluron, hydrochloride of compound 3 ($C_{22}H_{22}ClNO_4$, MW = 399.87 g mol^{-1}). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.48 (bs, 1H, OH), 8.39 (d, 1H, J = 9.07 Hz, H1), 7.94 (d, 1H, J = 8.25 Hz, H11), 7.58 (d, 1H, J = 2.20 Hz, H4), 7.31 (dd, 1H, J = 9.06 Hz, J = 1.97 Hz, H2), 7.07 (d, 1H, J = 2.20 Hz, H8), 7.02 (s, 1H, H6), 6.99 (dd, 1H, J = 8.24 Hz, J = 2.20 Hz, H10), 4.63–4.54 (m, 2H, OCH_2), 3.82 (s, 3H, OCH_3), 3.70–3.60 (m, 2H, NCH_2), 2.92 (s, 6H, NCH_3).

^{13}C NMR (75 MHz, DMSO-d_6) δ 193.3, 159.7, 158.1, 152.4, 137.2, 136.5, 136.2, 131.3, 128.3, 127.4, 123.8, 122.9, 120.9, 118.1, 110.5, 105.9, 99.6, 63.1, 55.9, 55.6, 43.1.

Positive-ion ESI mass spectra of 3-*O*-desmethyldimefluron base, $C_{22}H_{21}NO_4$, MW = 363; MS: *m/z* 402, $[M + K]^+$, 6%; *m/z* 386, $[M + Na]^+$, 3%; *m/z* 364, $[M + H]^+$, 100%. MS^2 of 364: *m/z* 319, $[M + H - CH_3NHCH_3]^+$, 100%. Negative-ion ESI mass spectra: MS: *m/z* 362, $[M - H]^-$, 100%. MS^2 of 362: *m/z* 317, $[M - H\text{-}CH_3NHCH_3]^-$, 3%; *m/z* 290, $[M - H\text{-}(CH_3)_2NCH_2CH_2]^{-\bullet}$, 100%; *m/z* 275, $[C_{17}H_7O_4]^-$, 10%.

2.4.1.3. 3,9-O-Didesmethyldimefluron, base of compound 4 ($C_{21}H_{19}NO_4$, MW = 349.38 g mol^{-1}). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.35 (d, 1H, J = 9.21 Hz, H1), 7.83 (d, 1H, J = 8.10 Hz, H11), 7.50 (d, 1H, J = 2.75 Hz, H4), 7.23 (dd, 1H, J = 9.21 Hz, J = 2.75 Hz, H2), 6.96 (s, 1H, H6), 6.92 (d, 1H, J = 2.47 Hz, H8), 6.83 (dd, 1H, J = 8.10 Hz, J = 2.47 Hz, H10), 4.29 (t, 2H, J = 5.21 Hz, OCH_2), 2.89 (t, 2H, J = 5.22 Hz, NCH_2), 2.37 (s, 6H, NCH_3).

^{13}C NMR (75 MHz, DMSO-d_6) δ 193.8, 157.9, 157.9, 152.9, 137.1, 136.6, 134.9, 131.5, 128.3, 127.4, 123.9, 122.9, 120.7, 119.4, 111.7, 105.6, 99.3, 66.2, 57.6, 45.4.

Positive-ion ESI mass spectra of 3,9-*O*-desmethyldimefluron base, $C_{21}H_{19}NO_4$, MW = 349; MS: *m/z* 350, $[M + H]^+$, 100%. MS^2 of 350: *m/z* 305, $[M + H - CH_3NHCH_3]^+$, 100%. Negative-ion ESI mass spectra:MS: *m/z* 697, $[2M - H]^-$, 4%; *m/z* 348, $[M - H]^-$, 100%. MS^2 of 348: *m/z* 303, $[M - H\text{-}CH_3NHCH_3]^-$, 3%; *m/z* 276, $[M - H\text{-}(CH_3)_2NCH_2CH_2]^{-\bullet}$, 100%.

2.4.2. N-Desmethylation of dimefluron (preparation of compounds 5 in Fig. 1 and Section 2.1)

Dimefluron base (1.5 g, 4.7 mmol) was dissolved in methanol (40 ml), diethyl azodicarboxylate (1.5 g, 8.6 mmol) was added and the reaction mixture was stirred at ambient temperature for 1 h. The temperature was then shortly elevated to the boiling point of methanol. The reaction mixture was acidified by 100 ml of 1 M HCl, heated and maintained at reflux for half of an hour. Methanol was evaporated and pH of the reaction mixture was adjusted to value of 10 with 1 M NaOH. The reaction mixture was extracted into ethyl acetate, the organic layer filtered, dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. *N*-Desmethyldimefluron was isolated from the reaction mixture using preparative TLC (see Section 2.1) and then converted into the corresponding hydrochloride.

2.4.2.1. N-desmethyldimefluron, hydrochloride of compound 5 ($C_{22}H_{22}ClNO_4$, MW = 399.87 g mol^{-1}). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.25 (bs, 2H, NH.HCl), 8.49 (d, 1H, J = 9.34 Hz, H1), 7.99 (d, 1H, J = 8.51 Hz, H11), 7.77 (d, 1H, J = 1.95 Hz, H4), 7.42–7.32 (m, 1H, H2), 7.15–7.06 (m, 2H, H6, H8), 7.06–6.98 (m, 1H, H10), 4.57–4.45 (m, 2H, OCH_2), 3.99 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.55–3.42 (m, 2H, NCH_2), 2.68 (bs, 3H, NCH_3).

^{13}C NMR (75 MHz, DMSO-d_6) δ 193.3, 159.8, 159.4, 153.0, 136.8, 136.3, 136.2, 130.9, 129.2, 127.2, 123.9, 123.8, 120.6, 118.4, 110.6, 103.7, 100.0, 64.3, 56.0, 55.9, 47.4, 33.0.

Positive-ion ESI mass spectra of *N*-desmethyldimefluron base, $C_{22}H_{21}NO_4$, MW = 363; MS: *m/z* 727, $[2M + H]^+$, 2%; *m/z* 364, $[M + H]^+$, 100%; *m/z* 333, $[M + H - CH_3NH_2]^+$, 5%. MS^2 of 364: *m/z* 333, $[M + H - CH_3NH_2]^+$, 100%; *m/z* 321, $[M + H - CH_3N{=}CH_2]^+$, 5%; *m/z* 307, $[M + H - CH_3N{=}CHCH_3]^+$, 17%.

2.4.3. Reduction of the carbonyl function into a secondary alcoholic group (preparation of compound 7 in Fig. 1)

The base of dimefluron (1 g, 2.65 mmol) was dissolved in methanol (40 ml), sodium borohydride (0.3 g, 7.94 mmol) was added to the solution and the reaction mixture was stirred under ambient temperature for 1 h and then heated at reflux for half of an hour. The solvent was removed under reduced pressure, the residue diluted by 5% aqueous NaOH (20 ml), and the product extracted into ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4. After solvent removal, the base of 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol was obtained.

2.4.3.1. C_7-reduced dimefluron, base of compound 7 ($C_{23}H_{25}NO_4$, MW = 379.45 g mol^{-1}). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.51 (d, 1H, J = 9.34, H1), 8.04 (d, 1H,

J = 8.51 Hz, H11), 7.59 (d, 1H, J = 2.75 Hz, H4), 7.30 (dd, 1H, J = 9.34 Hz, J = 2.75 Hz, H2), 7.22 (s, 3H, H6), 7.20 (d, 1H, J = 2.61 Hz, H8), 6.94 (dd, 1H, J = 8.51 Hz, J = 2.61 Hz, H10), 5.87 (bs, 1H, OH), 5.39 (s, 1H, CH), 4.34–4.23 (m, 1H, OCH_2), 3.88 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 2.82 (t, 2H, J = 5.27 Hz, NCH_2), 2.30 (s, 6H, NCH_3).

^{13}C NMR (75 MHz, DMSO-d_6) δ 158.1, 156.7, 152.8, 149.9, 143.6, 133.3, 126.9, 126.6, 125.7, 124.2, 122.1, 119.1, 113.5, 111.2, 103.6, 102.0, 74.1, 67.1, 57.9, 55.5, 55.2, 45.9.

Positive-ion ESI mass spectra of C_7-reduced dimefluron, $C_{23}H_{25}NO_4$, MW = 379; MS: m/z 380, $[M+H]^+$, 100%; m/z 362, $[M+H-H_2O]^+$, 4%. MS^2 of 380: m/z 362, $[M+H-H_2O]^+$, 100%; m/z 319, $[M+H-H_2O-CH_3N-CH_2]^+$, 47%; m/z 305, $[M+H-H_2O-CH_3N{=}CHCH_3]^+$, 55%; m/z 289, $[M+H-H_2O-CH_3N{=}CH_2-CH_2O]^+$, 29%. Negative-ion ESI mass spectra: MS: m/z 378, $[M-H]^-$, 100%. MS^2 of 378: m/z 363, $[M-H-CH_3]^{-\bullet}$, 30%; m/z 350, $[M-H-CO]^-$, 93%; m/z 335, $[M-H-CH_3N{=}CH_2]^-$, 5%; m/z 318, $[M-H-CO-CH_3OH]^-$, 10%; m/z 306, $[M-H-(CH_3)_2NCH_2CH_2]^{-\bullet}$, 100%; m/z 291, $[M-H-(CH_3)_2NCH{=}CHOH]^-$, 15%; m/z 277, $[M-H-CO-(CH_3)_2NCH_2CH_3]^-$, 21%; m/z 260, $[M-H-CO-(CH_3)_2NCH_2CH_2O]^{-\bullet}$, 5%; m/z 247, $[M-H-CO-(CH_3)_2NCH_2CH_3-CH_2O]^-$, 16%.

2.4.4. N-Oxidation of dimefluron (preparation of compounds 6 in Fig. 1 and Section 2.1)

Dimefluron hydrochloride (0.2 g, 0.48 mmol) was dissolved in ethanol (20 ml) and 7 ml of concentrated ammonium hydroxide and 7 ml of hydrogen peroxide were added to the solution. The reaction mixture was stirred at 40 °C for 3 h. Ethanol was then distilled off and the product was extracted into chloroform and the solution dried over anhydrous Na_2SO_4. After solvent removal, the crude *N*-oxide of 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one was purified by preparative TLC (chloroform–methanol–concentrated ammonium hydroxide, 70:30:5, v/v/v).

2.4.4.1. Dimefluron N-oxide, compound 6 ($C_{23}H_{23}NO_5$, $MW = 393.43\ g\ mol^{-1}$).

^{1}H NMR (DMSO-d_6) δ 8.43 (d, 1H, J = 9.21 Hz, H1), 7.96 (d, 1H, J = 8.24 Hz, H11), 7.51 (d, 1H, J = 2.61 Hz, H4), 7.33 (dd, 1H, J = 9.21 Hz, J = 2.61 Hz, H2), 7.11 (s, 1H, H6), 7.10 (d, 1H, J = 2.61 Hz, H8), 7.01 (dd, 1H, J = 8.25 Hz, J = 2.61 Hz, H10), 4.83–7.70 (m, 2H, NCH_2), 3.90 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.75–3.63 (m, 2H, CH_2), 3.15 (s, 6H, NCH_3).

^{13}C NMR (DMSO-d_6) δ 193.3, 159.7, 159.3, 153.0, 136.4, 136.3, 130.9, 129.4, 127.3, 123.8, 123.8, 120.7, 118.4, 110.6, 102.3, 99.9, 72.8, 68.6, 63.3, 60.1, 55.9, 55.5.

Positive-ion ESI mass spectra of dimefluron *N*-oxide, $C_{23}H_{23}NO_5$, MW = 393; MS: m/z 809, $[2M+Na]^+$, 36%; m/z 787, $[2M+H]^+$, 43%; m/z 432, $[M+K]^+$, 15%; m/z 416, $[M+Na]^+$, 100%; m/z 394, $[M+H]^+$, 42%. MS^2 of 394: m/z 377, $[M+H-OH]^{+\bullet}$, 2%; m/z 333, $[M+H-(CH_3)_2NOH]^+$, 5%; m/z 305, $[M+H-(CH_3)_2NOCH_2CH_3]^+$, 100%. Negative-ion ESI mass spectra: MS: m/z 820, $[2M+Cl]^-$, 57%; m/z 428, $[M+Cl]^-$, 100%.

2.4.5. Higher homologue of dimefluron (preparation of compound 8 in Fig. 1 and Section 2.1)

Sodium (0.5 g, 21.74 mmol) was dissolved in 50 ml of 2-methyl-2-propanol (*tert*-butyl alcohol) and 3,9-dimethoxy-5-hydroxy-7*H*-benzo[*c*]fluoren-7-one (1 g, 3.26 mmol) in *tert*-butyl alcohol was added to the solution with stirring. When the reaction mixture turned blue, a suspension of 3-dimethylaminopropylchloride hydrochloride (1.5 g, 9.5 mmol) in a small amount of *tert*-butyl alcohol was added in several portions. The reaction mixture was stirred and heated at reflux under unaqueous conditions for half of an hour. When the colour turned red, the reaction was complete. *tert*-Butyl alcohol was distilled off, the reaction mixture diluted by 5% aqueous NaOH, the product extracted into ethyl acetate and the extract dried over anhydrous Na_2SO_4. After solvent removal, the crude base of 3,9-dimethoxy-5-(2-dimethylaminopropoxy)-7*H*-benzo[*c*]fluoren-7-one was purified by preparative TLC (see Section 2.1).

2.4.5.1. Homo-dimefluron, base of compound 8 ($C_{24}H_{25}NO_4$, $MW = 391.46\ g\ mol^{-1}$).

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.34 (d, 1H, J = 9.21 Hz, H1), 7.87 (d, 1H, J = 8.24 Hz, H11), 7.45 (d, 1H, J = 2.74 Hz, H4), 7.28 (dd, 1H, J = 9.21 Hz, J = 2.74 Hz, H2), 7.04 (d, 1H, J = 2.48 Hz, H8), 6.96 (dd, 1H, J = 8.24 Hz, J = 2.47 Hz, H10), 6.93 (s, 1H, H6), 4.19 (t, 2H, J = 6.32 Hz, OCH_2), 3.89 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 2.48 (t overlapped, 2H, J = 6.33 Hz, NCH_2), 2.20 (s, 6H, NCH_3), 2.05–1.92 (m, 2H, CH_2).

^{13}C NMR (75 MHz, DMSO-d_6) δ 193.4, 159.6, 159.2, 153.6, 136.4, 136.3, 136.0, 130.9, 129.4, 127.2, 123.7, 123.7, 120.5, 118.2, 110.5, 102.2, 99.6, 66.7, 56.0, 55.4, 45.3, 35.5, 26.8.

Positive-ion ESI mass spectra: MS: m/z 392, $[M+H]^+$, 100%; m/z 347, $[M+H-CH_3NHCH_3]^+$, 3%. MS^2 of 392: m/z 347, $[M+H-CH_3NHCH_3]^+$, 100%; m/z 319, $[M+H-(CH_3)_2NCH_2CH_3]^+$, 25%.

In addition, the NMR, APCI–MS and ESI–MS spectra of dimefluron were measured for the sake of comparison:

^{1}H NMR (300 MHz, $CDCl_3$) δ 2.92 (6H, s CH_3N), 3.67 (2H, bt, J = 4.5 Hz, CH_2N), 3.81 (3H, s CH_3O), 3.94 (3H, s CH_3O), 4.56 (2H, bt, J = 4.5 Hz, CH_2O), 6.97 (1H, dm overlapped, J = 8.2 Hz, Ar), 6.95–7.10 (2H, m, Ar), 7.30 (1H, dm, J = 9.2 Hz, Ar), 7.55–7.65 (1H, m, Ar), 7.88 (1H, dm, J = 8.2 Hz, Ar), 8.35 (1H, dm, J = 9.2 Hz, Ar).

^{13}C NMR (75 MHz, $CDCl_3$) δ 193.5, 159.9, 159.5, 152.8, 137.0, 136.4, 136.3, 130.9, 129.3, 127.3, 124.0, 123.8, 120.7, 118.4, 110.7, 103.2, 100.3, 63.2, 55.99, 55.95, 55.5, 43.0.

Positive-ion ESI mass spectra of dimefluron base, $C_{23}H_{23}NO_4$, MW = 377; MS: m/z 400, $[M+Na]^+$, 4%; m/z 378, $[M+H]^+$, 100%; m/z 333, $[M+H-CH_3NHCH_3]^+$, 3%. MS^2 of 378: m/z 333, $[M+H-CH_3NHCH_3]^+$, 100%.

2.5. Rats and biological material

Dimefluron was administered in a single oral dose of 250 mg kg^{-1} of body weight to three male rats (*Rattus norvegicus* var. *alba*, Wistar type, weighing 270–290 g, from the Konárovice breeding station) in the form of a saturated aqueous solution via a gastric probe. These experiments were approved by the institutional ethics committee. The rats were placed individually into cages in which they had free access to water and food pellets before and during the experiment. Faeces of individual rats were collected each 24 h (in the intervals 0–24, 24–48, 48–72, 72–96, 96–120, 120–144, 144–168 h after the administration). The collected faeces were processed immediately.

2.6. Extraction of faeces and sample preparation

Each portion of faeces was triturated and extracted repeatedly (three times) with small volumes of ethyl acetate containing 5% of triethylamine (10–25 ml of mixture according to the amount of faeces eliminated during 24 h). For the homogenization of faeces, a vigorous stirring of faeces with the above-mentioned extraction mixture was used (magnetic stirrer, 40 °C for a few minutes). The extraction medium containing benzo[*c*]fluorene derivatives was filtered off from the dispersed biomatrix and the filtrate dried over anhydrous Na_2SO_4. The ethyl acetate extract was then evaporated in a glass tube (stream of nitrogen, max. 40 °C) to dryness. The dry extract in the glass tube was dissolved in 2 ml of methanol. Fifty microliters of this methanolic mixture were spiked by 50 μl of 10^{-3} M aqueous solution of homodimefluron (I.S.), diluted with 1900 μl of the mobile phase, vigorously mixed and centrifuged (10 000 × *g* for 10 min). One hundred microliters of the supernatant were injected onto the chromatographic column.

2.7. HPLC with ultraviolet photodiode-array detection

Routine chromatographic analyses were performed using a Thermo Electron (formerly Thermo Finnigan) chromatograph (San Jose, CA, USA). The chromatographic system was composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100-μl sample loop, UV6000 LP photodiode array detector with Light Pipe Technology, FL3000 fluorescence detector, SN4000 system controller and a data station (Intel-Pentium 4 CPU 1.6 GHz, RAM 256 MB, HDD 40 GB) with the ChromQuest 4 analytical software (Thermo Electron Inc., San Jose, CA, USA) working under the Windows 2000 operating system (Microsoft Corporation).

The efficiency of two chromatographic systems was evaluated in the separation of dimefluron, its phase I metabolites and internal standard in the extracts from the faeces.

System A consisted of a LiChroCART® 250-4 mm chromatographic column with LiChrospher 60 RP-selectB, 5 μm (Merck) and a mobile phase containing 0.01 M nonylamine buffer (pH 7.4) and acetonitrile in the 1:2 ratio (v/v), and the column was rinsed in the isocratic mode.

System B consisted of a Discovery 250-4 mm chromatographic column containing a HS-F5, 5 μm stationary phase (Supelco) and a mobile phase containing 0.05 M KH_2PO_4 buffer pH 3 and acetonitrile. The chromatographic analysis started with the mobile phase composition acetonitrile–0.05 M KH_2PO_4 buffer pH 3 (25:75, v/v). A linear gradient, increasing the proportion of acetonitrile in the mobile phase gradually to 60% over 20 min was applied, and an isocratic mobile phase composition of acetonitrile–0.05 M KH_2PO_4 buffer pH 3 (60:40, v/v) was used in the time interval of 20–28 min in the analysis. After equilibration (time interval of 28–33 min) preceding the initial chromatographic conditions, the next sample could be analysed.

The flow rate was 1 ml min^{-1} in both chromatographic systems. UV detection was performed in the dual wavelength mode, with 317 nm having been used for dimefluron and all 7*H*-benzo[*c*]fluoren-7-one metabolites, 367 nm for all 7*H*-benzo[*c*]fluoren-7-ol metabolites (see Fig. 2). In recording the UV spectra, a photodiode-array mode (in the range of 195–380 nm with a 1 nm distance) was used. The identity of the dimefluron metabolites in biological samples was confirmed using HPLC–MS experiments (see Section 2.3).

2.8. Calibration

Standard 10^{-3} M stock solutions (41.4 mg of dimefluron, 40.0 mg of 9-*O*-desmethyl-DMF, 40.0 mg of 3-*O*-desmethyl-DMF, 34.9 mg of 3,9-*O*-didesmethyl-DMF (base), 40.0 mg of *N*-desmethyl-DMF, 39.3 mg of DMF *N*-oxide, 37.9 mg of C_7-reduced-DMF (base) and 39.1 mg of homoDMF (base), each in 100 ml of the respective UHQ-water solution were prepared. The compounds used in the form of bases were dissolved in UHQ-water containing an equimolar amount of hydrochloric acid. Lower concentrations (10^{-4} M, 10^{-5} M) of each compound were obtained by the dilution with UHQ water. A calibration series of dimefluron + its metabolites/homodimefluron (IS) mixtures with the concentrations of 0.05, 0.25, 0.5, 2.5, 10, 17.5, 25 and 35 nmol/ml of each analyte, and with the same homodimefluron concentration (25 nmol/ml) was made. Six individual samples were prepared at each calibration level. The same concentrations were used to make a calibration curve with a drug-free faeces extract spiked with dimefluron, its metabolites and homodimefluron (using appropriate concentrations in order to keep the volumes at a minimum). This calibration series was measured using the UV detector under the conditions mentioned in Section 2.7. The extraction procedures were the same as described in Section 2.6.

2.9. Testing and statistical evaluation of the analytical procedure

As mentioned in Section 2.8, eight-level calibration series with six analyses at each concentration level was measured.

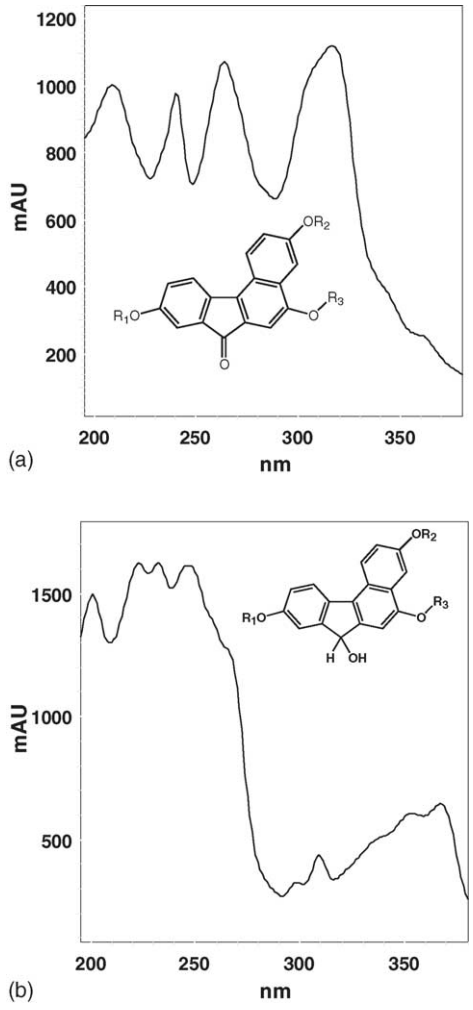

Fig. 2. (a) UV spectra of 3,9-dimethoxy-7*H*-benzo[*c*]fluoren-7-one chromophore (dimefluron and all 7-one metabolites, maxima: 209, 240, 264 and 317 nm). (b) UV spectra of 3,9-dimethoxy-7*H*-benzo[*c*]fluoren-7-ol chromophore (C_7-reduced dimefluron and all 7-ol metabolites, 201, 223, 232, 247, 299, 309, 354, 367 nm).

On-line statistical processing of the calibration analyses by the least-squares method was performed automatically using the ChromQuest software. The linearity of the calibration curve from the aqueous solutions of dimefluron, its metabolites and homodimefluron (IS) and from the drug-free faeces extracts spiked with the above-mentioned analytes was tested and evaluated [$y = kx + q$, where x is the concentration ratio of dimefluron (or one of its metabolites, respectively) to homodimefluron (IS) and y is the corresponding peak–area ratio of dimefluron (one of its metabolites)/homodimefluron (I.S.)] and the correlation coefficient (r) was expressed. The accuracy was determined as a relative error (%) found on the standard curve. The precision of the method, expressed as the relative standard deviation (percentage of coefficients of variation; R.S.D. = 100 S.D./mean), was also assessed. Both statistical parameters were calculated for each concentration level. The range of the applicability of the HPLC method was enclosed within the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). The lower limit of quantification (LLOQ) was determined as the lowest concentration on the standard calibration curve which was measured with a precision of 20% and accuracy of 80 or 120%. Upper limit of quantification (ULOQ) was equal to the highest concentration in the eight-level faeces extract calibration. The recovery of dimefluron and its six metabolites was also calculated [31,32].

3. Results and discussion

3.1. Syntheses of the standards and their NMR spectra

For the development of new HPLC methods and for the preparation of calibration samples, the standards of the expected dimefluron metabolites and a suitable internal standard were required. Consequently, simple synthetic and degradation methods starting from dimefluron and its precursor (3,9-dimethoxy-5-hydroxy-7*H*-benzo[*c*]fluoren-7-one) were employed. Given the functional groups present in dimefluron and our previous experience with the biotransformation of benfluron, the following pathways in phase I dimefluron metabolism were expected: *O*-desmethylation, *N*-desmethylation, *N*-oxidation and carbonyl reduction. The products of these metabolic reactions could be identified in various biomatrices after the administration of dimefluron, hence their standards had to be synthesized.

O-Desmethylation, *N*-desmethylation, *N*-oxidation and carbonyl reduction were performed by the above chemical modifications of dimefluron (see Sections 2.4.1–2.4.4). Under the conditions described in Section 2.4.1, three *O*-desmethylated products (compounds 2–4 in Fig. 1) arose from the parent compound 1 and were separated by preparative TLC (see Section 2.1). The other reaction led to relatively pure final products, but for analytical purposes, all these compounds (5–7 in Fig. 1) were purified by preparative TLC as well. Some of the basic products were converted into their salts (hydrochlorides) for a better crystallization.

Before the development of the chromatographic conditions for the HPLC bioanalyses of dimefluron and its phase I metabolites, a suitable internal standard had to be identified. In some of our previous papers [33–35], good experience with the structural homologues of the drugs under study was made. Consequently, a higher homologue of dimefluron (homodimefluron, compound 8 in Fig. 1), possessing an additional methylene group in the side chain of molecule was prepared by the Williamson synthesis from the dimefluron precursor (3,9-dimethoxy-5-hydroxy-7*H*-benzo[*c*]fluoren-7-one) and 3-dimethylaminopropyl chloride.

The structure and purity of individual compounds 2–8 (see Fig. 1) was confirmed by NMR, HPLC–DAD and HPLC–MS experiments.

3.2. Mass spectra of the dimefluron derivatives

The individual compounds in real samples were unambiguously identified using the following HPLC–MS approach. First, the first-order and MS^n spectra of all standards were measured and interpreted both in the positive-ion and negative-ion electrospray ionization mode (see Section 2.3). A mixture of all standards was then analysed by HPLC–MS and HPLC–MS–MS to verify that the mass spectra are identical to those obtained in the direct infusion experiments. The following step was the analysis of real samples of three rat faeces. No significant differences were found among individual samples, and the results are therefore discussed together. All synthetic standards (see Section 2.4) were clearly identified in the rat faeces extracts, but the concentration of compound 4 (3,9-*O*-didesmethyl-DMF) was very low.

The comparison of observed ions with the structures showed the characteristic fragmentation behaviour for individual functional groups, which enabled us the identification of other metabolites, the standards of which were not synthesized, based on the following rules. The molecular weights (MW) were determined according to $[M+H]^+$ ions in the positive-ion and $[M-H]^-$ ions in the negative-ion ESI mass spectra, which was further confirmed by the less abundant $[M+Na]^+$ and $[M+K]^+$ ions in some cases. The compounds without a hydroxy group (compounds 1, 5, 8 and 13 in Table 1) did not provide any signal in the negative-ion mode except for the *N*-oxide (compound 6), where the adduct ions $[M+Cl]^-$ and $[2M+Cl]^-$ were found, but with very low sensitivity. The presence of the dimethylamino group was indicated by the neutral loss of CH_3NHCH_3 ($\Delta m/z$ 45) or $CH_3N{=}CH_2$ ($\Delta m/z$ 43), while the presence of the methylamino group corresponded to the loss of CH_3NH_2 ($\Delta m/z$ 31) together with a minor loss of $CH_2N{=}CH_2CH_3$ ($\Delta m/z$ 57). The presence of *N*-oxide was characterized by the neutral losses of $CH_3N(OH)CH_3$ ($\Delta m/z$ 61) and $(CH_3)_2NOCH_2CH_3$ ($\Delta m/z$ 89), by a slightly increased relative abundance of molecular adducts with alkali metal ions and dimeric ions $[2M+H]^+$ as well as by an increased fragmentation in the first-order mass spectra. When the 7-carbonyl group was reduced to the secondary hydroxy group, the loss of water ($\Delta m/z$ 18) was the base peak in the MS/MS spectrum unlike other compounds containing 7-carbonyl function. The other typical neutral losses were observed after the primary loss of water. Compounds with the primary hydroxy group in positions 3- or 9- could be easily distinguished from the 7-hydroxy derivatives, because the loss of water in the first case occurred after the loss of alkylamines and was not a predominate peak in the spectrum.

All information valuable for the characterization of phase I metabolites is summarised in Table 1 together with the identification of new metabolites (compounds 9–15), the standards of which were not prepared. The MWs, fragmentation behaviour and retention order of compounds 9–15 are in agreement with the proposed structures depicted in Table 1. In addition to phase I metabolites, there is certain evidence of phase II metabolites with lower retention times as well, but their concentrations in our extracts were too low and the chromatographic resolution must also be improved for their positive identification.

3.3. Sample preparation procedure

As mentioned in our previous study [14], the principal route of elimination of phase I benfluron metabolites was the gastrointestinal tract of rats. With a view to the similarity of benfluron and dimefluron, it was expected that prevailing amounts of the phase I dimefluron metabolites would

Table 1
List of compounds identified in the extracts of rat faeces by HPLC–MS and HPLC–MS–MS

Compound no.[a]	t_R (min)[b]	MW	Hydroxyl group (position)[c]	Alkylamino group (substituent R_3 in Fig. 1)
1	50.5	377	–	$(CH_3)_2N-$
2	28.3	363	Yes (9)	$(CH_3)_2N-$
3	32.5	363	Yes (3)	$(CH_3)_2N-$
4	16.3	349	Yes (3, 9)	$(CH_3)_2N-$
5	45.2	363	–	CH_3NH-
6	47.0	393	–	$(CH_3)_2NO-$
7	35.4	379	Yes (7)	$(CH_3)_2N-$
8	55.8	391	–	$(CH_3)_2NCH_2-$
9	33.8	397	Yes (3 or 9)	$(CH_3)_2N-$
10	29.4	349	Yes (3)	CH_3NH-
11	24.6	349	Yes (9)	CH_3NH-
12	28.3	393	Yes (unknown)	$(CH_3)_2N-$
13	24.6	379	–	CH_3NH-
14	20.1	365	Yes (3, 7)	$(CH_3)_2N-$
15	18.7	365	Yes (7, 9)	$(CH_3)_2N-$

[a] 9: 3-*O*-desmethyl-chlordimefluron or 9-*O*-desmethyl-chlordimefluron; 10: 3-*O*-desmethyl-*N*-desmethyldimefluron; 11: 9-*O*-desmethyl-*N*-desmethyldimefluron; 12: hydroxydimefluron; 13: *N*-desmethyl-C_7-reduced –7-methoxydimefluron; 14: 3-*O*-desmethyl-C_7-reduced dimefluron; 15: 9-*O*-desmethyl-C_7-reduced dimefluron. See Fig. 1 for structures of 1–8 and Fig. 6 for structures of 10–11 and 13–15.

[b] Retention times in HPLC–MS system (see Section 2.3).

[c] Compounds without hydroxy groups do not provide signal in the negative-ion ESI mode.

be found in the faeces as well. For this reason the primary search for phase I dimefluron metabolites was oriented towards this solid, relatively worse extractable biomatrix. Ethyl acetate with triethylamine was found to be a good extraction medium for phase I dimefluron metabolites of various polarity. A small amount of triethylamine (proton scavenger) in this medium served to convert the possibly present salts of dimefluron metabolites in the excrements into their better extractable bases.

Ethyl acetate and triethylamine were evaporated from the extracts and the dry extract was dissolved in methanol, which is compatible with the mobile phase used in the HPLC determination. The methanolic solution was spiked with the internal standard. In accord with our assumptions, the faeces contained a large number and amounts of colour phase I dimefluron metabolites and had to be repeatedly diluted before the HPLC analysis.

3.4. Chromatography

The development of chromatographic conditions, which would be suitable for the separation of dimefluron and its phase I metabolites, started by testing chromatographic system used in the separation of benfluron derivatives [13–15]. Benfluron and its metabolites were separated on various reversed phase columns C_{18}, using a mobile phase containing nonylamine buffer (pH 7.4), acetonitrile and 2-propanol (2:2:1, v/v/v). These conditions were sufficient for the resolution of all benfluron derivatives during 30 min. The employment of these chromatographic conditions for dimefluron and its metabolites failed due to an unsatisfactory resolution of isomeric 3-*O*-desmethyl-DMF and 9-*O*-desmethyl-DMF. In addition, most of the peaks in the chromatogram were asymmetric which was result of the interaction of the basic analytes with the residual silanols on the surface of non-endcapped octadecylsilyl silica gel. Hence, new chromatographic conditions for dimefluron derivatives were being searched for.

In the *chromatographic system A* (see Section 2.7), a chromatographic column LiChrospher RP-selectB (Merck) with the chemically bound octylsilane (C_8) on the silica gel surface was tested. The starting silica material was optimized to suppress the undesirable interactions with the basic analytes. The mobile phase used for the separation of benfluron derivatives was simplified by the elimination of 2-propanol, and its composition was optimized to achieve a satisfactory resolution of all dimefluron derivatives under study (see compounds in Fig. 1). Fig. 3 shows a chromatogram of dimefluron derivatives in the mobile phase containing 0.01 M nonylamine buffer (pH 7.4) and acetonitrile in the 1:2 ratio (v/v). The chromatographic analysis of compounds 1–7 lasted 45 min with the resolution of individual compounds being satisfactory, but the peaks were asymmetric to a high degree. The most fundamental limitation of this chromatographic system was a very long retention time (more then 120 min) of the internal standard (homodimefluron, compound 8 in Fig. 1) under the above-mentioned conditions and such a chromatographic system was unsuitable for serial analyses.

In the *chromatographic system B* (see Section 2.7), a chromatographic column containing an HS-F5, 5 μm stationary phase (pentafluorophenylpropyldimethylsilyl silica gel) was used. The composition of an acidic mobile phase based on a mixture of acetonitrile and a phosphate buffer of pH 3 was being changed using a linear gradient during the first 20 min of the chromatography and then maintained in the isocratic mode until the completion of the analysis. As apparent from Fig. 4a, the whole analysis lasted 28 min, all eight dimefluron derivatives including the internal standard (compounds 1–8 in Fig. 1) were well separated and, importantly, the shape of all peaks exhibited a much higher degree of symmetry than those in the chromatographic system A. Hence, the

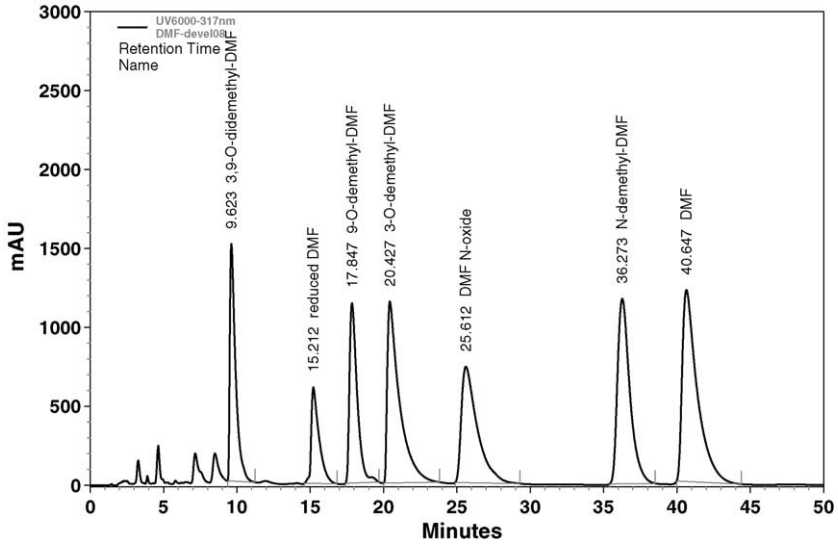

Fig. 3. Chromatographic separation of dimefluron and its metabolites on a RP-selectB (octylsilyl silica gel) column using an isocratic mobile phase containing nonylamine buffer (pH 7.4)–acetonitrile in the 1:2 ratio (v/v).

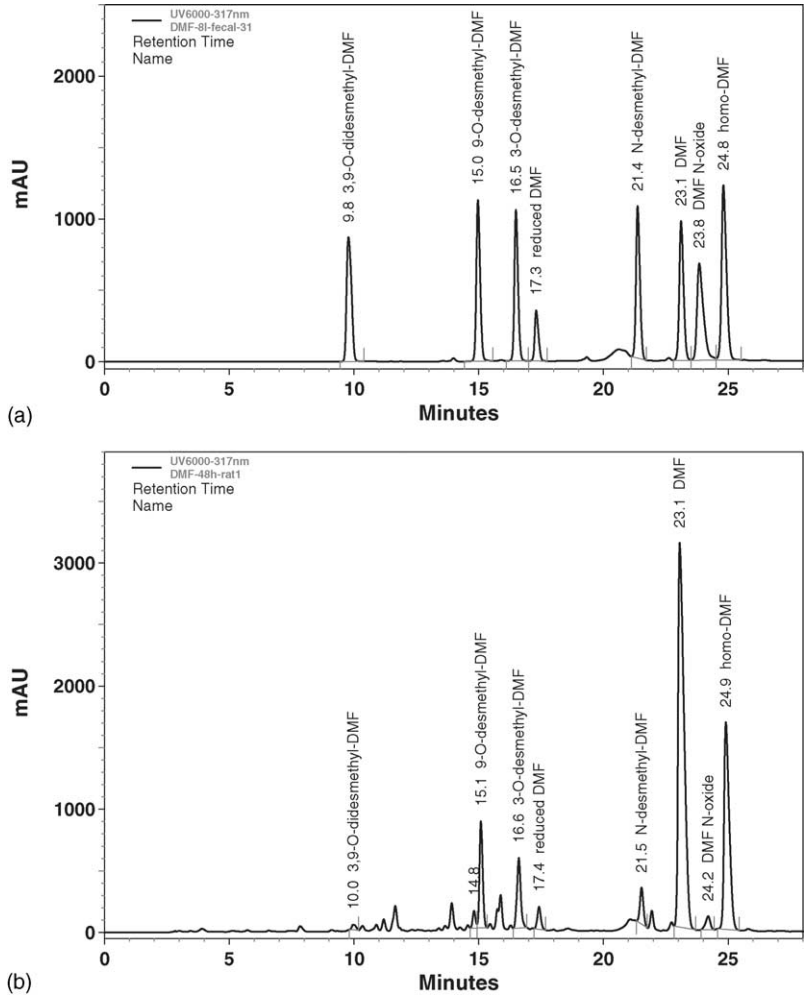

Fig. 4. (a) Chromatographic separation of dimefluron and its metabolites on a Discovery HS-F5 (pentafluorophenylpropyldimethylsilyl silica gel) column using a gradient mobile phase containing phosphate buffer (pH 3)–acetonitrile (see Section 2.7, system B). Extract from drug-free rat faeces spiked with the individual metabolites, dimefluron and homodimefluron (I.S.). (b) Chromatographic separation under the same conditions (see Section 2.7, system B). Extract from the rat faeces collected in the interval 24–48 h after the oral administration of dimefluron; the sample was spiked with homodimefluron (I.S.).

chromatographic system B was validated and chosen for routine analyses.

Photodiode-array UV–vis detection (DAD) under the conditions described in Section 2.7 was employed not only for the determination of dimefluron and those metabolites, the synthetic standards of which were available, but also in the search for and preliminary identification of all other benzo[*c*]fluorene derivatives discovered in the extracts. Spectral analysis of the acquired 3D-chromatograms (spectrochromatograms) enabled us to identify two types of benzo[*c*]fluorene compounds (7*H*-benzo[*c*]fluoren-7-one and 7*H*-benzo[*c*]fluoren-7-ol) according to their characteristic UV-spectra displayed in Fig. 2. In this way, some new minor metabolites, having the 7*H*-benzo[*c*]fluoren-7-one and 7*H*-benzo[*c*]fluoren-7-ol chromophore were discovered in the spectrochromatograms. Their final structure was elucidated by HPLC–DAD–MS experiments.

The possibility of the fluorescence detection employment was also tested. It was observed that only 7*H*-benzo[*c*]fluoren-7-ol derivatives exhibited fluorescence at the excitation maxima of 245 and 361 nm and the emission maxima were found at the wavelengths of 427 and 736 nm.

The developed bioanalytical method for the determination of dimefluron derivatives involving a liquid–solid extraction of faeces and based on the ultraviolet photodiode-array detection was validated. The validation parameters are summarised in Table 2. The calibration curves were found to be linear in the range of 0.05–35 nmol/ml. The value of LLOQ varied in dependence on a particular compound (0.05–2.5 nmol/ml). The recovery for different metabolites ranged between 75 and 89%.

3.5. Elimination of dimefluron and its metabolites via gastrointestinal tract of rats

The developed and validated bioanalytical method was used for the study of dimefluron disposition in rats. The aim of this study was to elucidate the structures of individual phase

Table 2
Validation results of the bioanalytical LSE–HPLC–DAD method for dimefluron and its metabolites

Compound (no. in Fig. 1 and Table 1)	Regression equation	r	Precision (%)	Accuracy (%)	LLOQ (nmol/ml)	ULOQ (nmol/ml)
3,9-*O*-Didesmethyl-DMF	$y = 0.9517x + 0.0155$	0.9988	1.15–7.18	97.94–106.41	0.5	35
9-*O*-Desmethyl-DMF	$y = 0.9502x + 0.0122$	0.9997	1.68–5.80	99.49–114.31	0.5	35
3-*O*-Desmethyl-DMF	$y = 0.9211x + 0.0103$	0.9998	0.79–3.53	99.24–101.98	2.5	35
Reduced DMF	$y = 0.2954x + 0.003$	0.9999	0.55–4.62	99.57–108.81	2.5	35
N-Desmethyl-DMF	$y = 0.9128x + 0.019$	0.9978	3.94–14.53	98.44–110.79	0.5	35
DMF	$y = 0.9729x + 0.0133$	0.9996	1.02–5.27	98.33–114.17	0.5	35
DMF *N*-oxide	$y = 0.9833x + 0.0528$	0.9997	0.49–4.62	89.79–114.43	0.05	35

I dimefluron metabolites and to evaluate their elimination from the organism from the viewpoint of time dependence and total balance dependence (administered molar amount of dimefluron versus the sum of eliminated molar amounts of dimefluron and its metabolites).

9-*O*-Desmethyldimefluron and 3-*O*-desmethyldimefluron were identified as the principal phase I metabolites (see Fig. 4b). In an analogous elimination study of benfluron [14], 9-hydroxybenfluron was found to be the principal phase I metabolite of benfluron in faeces. With a view to the fact that the biotransformation precursors of phenolic metabolites (9-hydroxybenfluron in this case) are nucleofilic arenoxides (epoxides), which may be very toxic for the organism given their possible influence on nucleic acids and proteins, the dimefluron phase I metabolism leading to *O*-desmethylation products seems less hazardous for the organism than the aryl-hydroxylation of benfluron.

The other expected or newly found phase I dimefluron metabolites (products of *N*-desmethylation, *N*-oxidation and carbonyl reduction) were eliminated in minor amounts, as shown in Fig. 5 and Table 1.

The elimination of dimefluron and its phase I metabolites culminated in the time interval of 24–48 h following the administration of the parent compound (see Fig. 5) and the

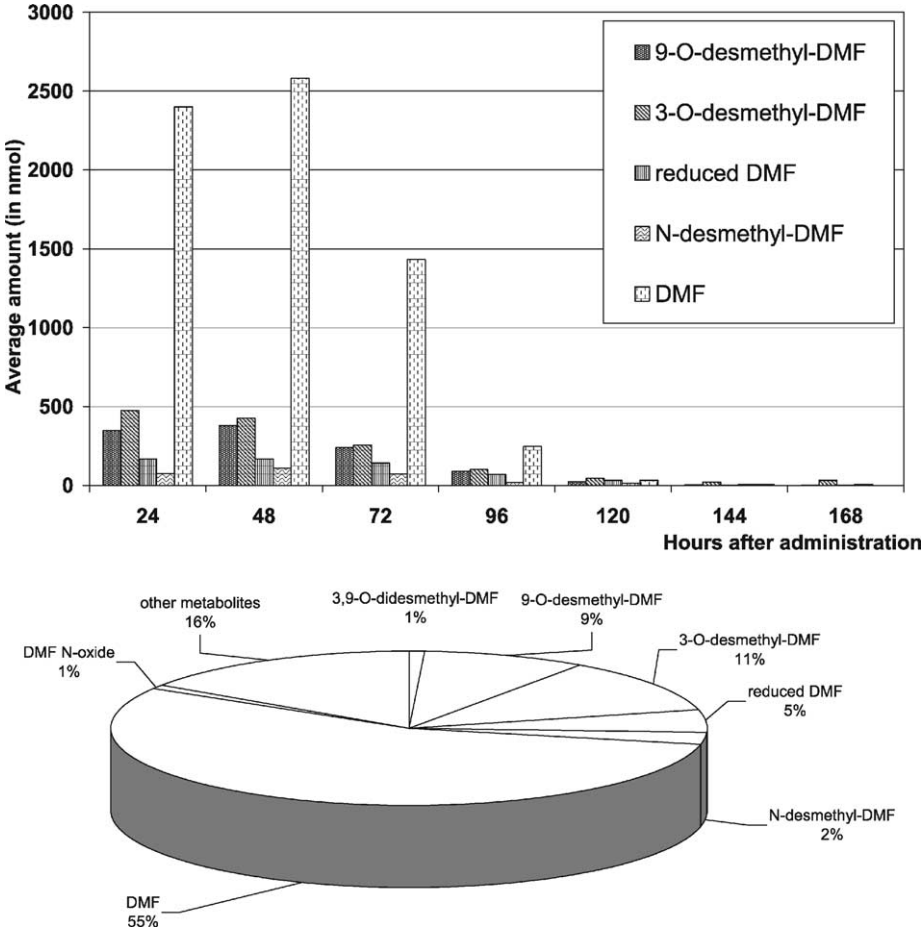

Fig. 5. (a) Average molar amount of dimefluron and its four principal phase I metabolites in the extracts from rat faeces. (b) Percentage of the total elimination of dimefluron and its phase I metabolites in the extracts from rat faeces.

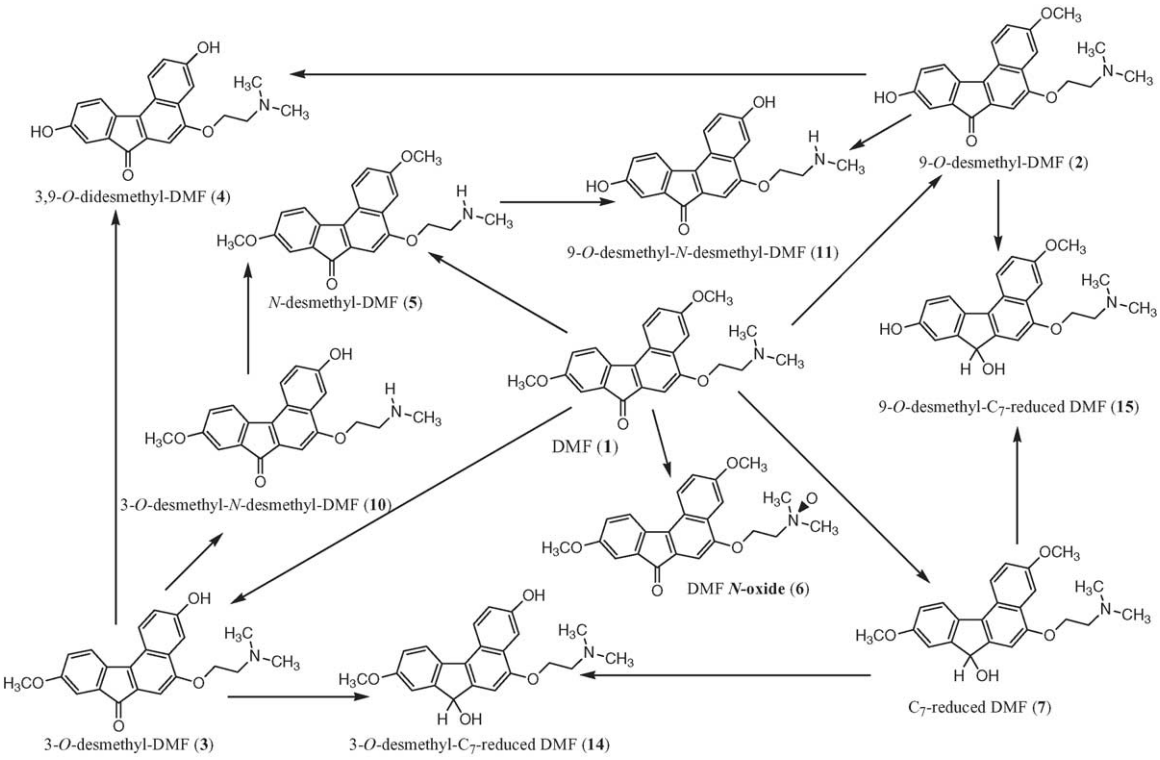

Fig. 6. Presumptive survey of the phase I dimefluron biotransformation (the numbering of individual compounds is the same as in Fig. 1 and Table 1).

major amount of unconjugated benzo[*c*]fluorene derivatives (89.4%, average from three rats) left the organism over the first three days. Presumptive survey of the phase I dimefluron biotransformation based on the results of HPLC–DAD–MS analyses is given in Fig. 6. In addition to the metabolites, whose synthetic standards were available (see compound 1–8 in Table 1), some new benzo[*c*]fluorene structures were discovered in the extracts from rat faeces: the products of a consecutive *O*- and *N*-desmethylation (compounds 10 and 11 in Table 1) and products of *O*-desmethylation and C_7-carbonyl reduction (compounds 14 and 15 in Table 1). Also small amounts of hydroxydimefluron (compound 12, position of hydroxyl unknown) were identified. The finding of compounds 9 (3-*O*-desmethyl-chlordimefluron or 9-*O*-desmethyl-chlordimefluron) and 13 (*N*-desmethyl-C_7-reduced-7-methoxydimefluron) is according to the actual xenobiochemical knowledge hardly explicable.

The balance study showed that only 7.3% of dimefluron and its phase I metabolites (of the administered molar amount of dimefluron = 100%) left the rat organism via the gastrointestinal tract during the next 7 days after the administration. This percentage corresponds to a similar total amount of benfluron and its phase I metabolites found in our former experiments [14]. The remaining amount could have been eliminated via another route (by uropoietic system) or in the form of phase II metabolites (conjugates), which were not investigated in this study because they are not extractable into ethyl acetate.

4. Conclusions

Two chromatographic systems differing in the stationary phases used, in the pH value of the mobile phase and in the mode of elution (isocratic versus gradient) were tested in the development of a new bioanalytical method involving the liquid–solid extraction of animal faeces and subsequent high-performance liquid chromatographic determination of dimefluron and its phase I metabolites based on the ultraviolet and mass spectrometric detections. Better results were achieved using a pentafluorophenylpropylsilyl silica gel column rinsed with the mobile phase of acetonitrile–phosphate buffer pH 3 in a gradient mode. After the validation of the bioanalytical method, this chromatographic system was applied to the disposition study of dimefluron in rats. The elimination of dimefluron and its phase I metabolites in the faeces was studied after an intragastric administration of dimefluron to three male rats. Maximum concentrations of dimefluron derivatives in the excrements were achieved in the time interval of 24–48 h after the administration. 9-*O*-Desmethyldimefluron and 3-*O*-desmethyldimefluron were the major metabolites found in the rat faeces, while the metabolic products of *N*-desmethylation, *N*-oxidation and carbonyl reduction were found in lower concentrations. According to the balance study, only 7.3% of the administered molar amount of dimefluron were eliminated in the form of an unchanged parent compound and its phase I metabolites by the gastrointestinal tract, the fate of the remaining amount of

benzo[*c*]fluorene derivatives will be elucidated in our further studies.

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