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SEPARATION AND DETERMINATION OF IMIPRAMINE AND ITS METABOLITES FROM BIOLOGICAL SAMPLES BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive method for the determination of imipramine and seven of its major metabolites in biological samples is described. The extraction procedures used are based on the partition properties which were determined for each metabolite. The extracted drug and its metabolites are in some cases transformed into derivatives. They are finally separated and determined by gas chromatography. The sensitivity reached is 0.01–0.05 μg, except for 2-hydroxy-desmethylimipramine (0.2–0.3 μg). Examples of analyses using tissues, bile, and liver perfusion medium are given.

Several authors[1–10] have described the determination of imipramine and some of its metabolites in biological materials. Thin layer chromatography lends itself to the detection of all known imipramine metabolites, though the quantitative evaluation methods[11,12] are of limited accuracy. Thin layer chromatography followed by elution and U.V.-spectrophotometry proved not sensitive enough for studies of imipramine metabolism. Gas–liquid chromatography (GLC), due to its high sensitivity, proved to be the method of choice for obtaining accurate values in metabolic experiments. Gas chromatography has been used for the detection of imipramine and desipramine as early as 1961 by GILLETTE *et al.*[13]. More recently gas chromatography has been used for the determination of the antidepressant dibenzepine and six metabolites by LEHNER *et al.*[14] and for phenothiazines by MCMARTIN AND STREET[15].

In addition to GLC procedures this paper also deals with the determination of the partition coefficients of imipramine and its metabolites between organic solvents and aqueous buffers. Based on these values suitable extraction procedures have been developed for tissues, bile, and perfusion medium. Simple methods for the synthesis of derivatives to be used for the final gas chromatography are also described.

MATERIALS AND METHODS

Substances and abbreviations

Imipramine(IP), desmethylimipramine (DMI), desdimethylimipramine (DDMI), 2-hydroxy-imipramine (2-OH-IP), 2-hydroxy-desmethylimipramine (2-OH-DMI),

imipramine-N-oxide (IPNO), iminodibenzyl (IDB), 2-hydroxyiminodibenzyl (2-OH-IDB) were kindly given by Geigy Ltd., Basle, and dibenzepine (DBZ) by Dr. A. Wander Ltd., Berne. All reagents used were analytical grade purity.

Partition coefficients between aqueous and organic phases

Isotonic phosphate buffer[16], pH 7.4, and *n*-hexane or chloroform, respectively, were used. Previous to partition of imipramine or its metabolites, the phases were saturated with respect to each other by shaking for 5 min. The substance was then dissolved in the aqueous phase (5 ml) and shaken with the organic phase (5 ml) in a centrifuge tube (20 ml) for 30 min at 20°. The initial concentration in the aqueous phase and the final concentrations after equilibration were determined with a Unicam U.V.-spectrophotometer SP 800.

The variation of the partition coefficients as a function of pH was determined by using 1,2-dichloroethane, diethyl ether (peroxide-free) and *n*-heptane as organic phases, phosphate buffers (pH 5.0–7.0), borax buffers[17] (pH 7.0–8.8) and glycine buffers[18] (pH 9.0–12.4) as aqueous phases.

Extraction procedures

Imipramine and its metabolites are extracted from tissue homogenates by solvent extraction. IPNO is separated from the mixture since it undergoes pyrolysis to IDB and other products in the gas chromatography column. The isolated IPNO is reduced to IP and determined as such. The remaining metabolites are further separated into non-phenolic and phenolic bases. Dibenzepine (DBZ) is added as an internal standard.

Rat tissues containing imipramine and its metabolites were homogenized in isotonic phosphate buffer, pH 7.4, 1:3, v/v at 0–5° in a Potter-Elvehjem glass homogenizer. Test extractions were carried out with imipramine, imipramine metabolites, and dibenzepine added to the homogenate. For the initial step of extraction the homogenate was brought to pH 10.0 by adding 1 *N* NaOH and concentrated ammonia.

5 ml portions of homogenates of liver, lung, kidney, and fat tissues (pH 10.0) were shaken three times with equal volumes of diethyl ether (5 ml) for 15 min, then centrifuged and the organic phases reextracted with carbonate–bicarbonate buffer[19], pH 10.0. Imipramine-N-oxide was extracted from the aqueous phases by shaking four times with equal volumes of 1,2-dichloroethane. The dichloroethane extracts were dried with anhydrous Na_2SO_4 and evaporated with a stream of nitrogen (99.9 %).

After separation of IPNO the phenolic bases (2-OH-IP, 2-OH-DMI, 2-OH-IDB) and the non-phenolic bases (IP, DMI, DDMI, IDB, DBZ) were separated by the following procedure. The combined ether extracts were shaken three times for 15 min with 3 ml portions of 1 *N* HCl. The acid extract was made alkaline to pH 10.0 with NaOH and ammonia and shaken three times with 3 ml portions of *n*-heptane–isoamyl alcohol (99:1, v/v). The combined organic phases containing the non-phenolic bases were dried with Na_2SO_4 and evaporated with a nitrogen stream.

The phenolic bases contained in the aqueous phase (pH 10) were extracted with three 3 ml portions of ether and reextracted with three 3 ml portions of 1 *N* KOH. The aqueous phase was titrated to pH 10 by adding conc. HCl and then ammonia. DBZ was also added at this stage to serve as an internal standard for the determination of the phenolic bases. The latter were finally extracted from the alkaline phase

with three 3 ml portions of ether. The combined ether phases were dried and evaporated as indicated above.

Brain homogenates, which never contained phenolic metabolites, were shaken for 15 minutes at pH 10–11 four times with equal volumes of *n*-heptane–isoamyl alcohol (99:1,v/v). The combined organic phase was reextracted four times with 4 ml portions of 0.1 *N* HCl. The acid extracts were then adjusted to pH 10 and extracted with four 5 ml portions of 1,2-dichloroethane. The combined organic phases containing the non-phenolic bases were dried and evaporated.

The low blanks with bile and perfusion medium* allowed another simplified extraction method: 2 ml perfusion medium or 0.5 ml bile were titrated to pH 10.0 with NaOH and ammonia and extracted with three 3 ml portions of ether for 15 min. The organic phases were washed with carbonate–bicarbonate buffer pH 10.0 and the aqueous and organic phases processed as described for homogenates, separation into phenolic and non-phenolic groups, however, was not necessary.

The number of extractions (*n*) required theoretically for an optimum extraction yield of the major imipramine metabolites has been calculated from the distribution coefficients at pH 10 as determined experimentally.

Gas–liquid chromatography

Apparatus. Gas chromatograph Perkin Elmer 801, equipped with a flame ionisation detector (H_2 35 ml/min, air 340 ml/min) and recorder (Honeywell 1 mV, chart speed 16.5 mm/min). The peak areas were evaluated with an electronic integrator (Perkin Elmer D 24). The pyrex glass column (length 2 m, diameter 2.5 mm) was packed with SE-30 (a methyl polysiloxane) on Anakrom ABS. Nitrogen (30 ml/min) was used as a carrier gas. The temperatures of the injector, column and detector were kept at 320, 240 and 260°, respectively. The substances were dissolved in CS_2–$CHCl_3$ (7:3, v/v) and volumes of 0.5–1 μl were injected with a Hamilton syringe.

Sample preparation. The isolated metabolites, with the exception of IPNO, were acetylated in a glass stoppered tube at room temperature by the following procedure: 0.5 ml acetic anhydride and 0.05 ml of dry pyridine were added to the extracts. After 3 h the reaction mixture was evaporated with a stream of nitrogen.

IPNO was reduced to IP with titanium(III) chloride in the following manner: 1 ml of a solution prepared by mixing 5 ml $TiCl_3$ (Merck), 5 ml H_2O and 1 ml conc. HCl was added to 1 ml of the aqueous IPNO solution and heated to 50° for 10 min. After cooling DBZ was added and the pH of the solution was adjusted to 10 by adding 5 *N* NaOH and concentrated ammonia. IP and DBZ were extracted by shaking with two 4 ml portions of *n*-heptane–isoamyl alcohol (99:1, v/v) for 15 min. The organic phase was dried with Na_2SO_4 and evaporated with a stream of nitrogen. This extract was used directly for gas chromatography since the tertiary amines IP and DBZ cannot be acetylated.

Quantitative evaluation. DBZ was used as internal standard. The peaks were evaluated depending on their shape: (1) by the peak height method; (2) by measuring the peak area by the approximation method of CONDAL-BOSCH[20] (height times width in 15 and 85 % of height); (3) by exact integration of the peak area with the electronic

* Medium for perfusion of isolated rat liver: 40 vol. washed bovine erythrocyte suspension, 60 vol. Ringer phosphate–bicarbonate solution containing 1.5 g/100 ml crystallized bovine albumin, 0.4 g/100 ml glucose and 7.5 mg/100 ml aureomycin.

integrator. The absolute amounts were calculated by means of the following formula:

$$m_i = \frac{A_i \cdot Ff^i_{DBZ} \cdot m_{DBZ}}{A_{DBZ}} \ (\mu g)$$

where

m_i = Amount of substance in μg
A_i = Measured value for peak i
Ff^i_{DBZ} = Correction factor for substance i based on DBZ

$$= \frac{m_i \cdot \text{measured value peak}_{DBZ}}{m_{DBZ} \cdot \text{measured value peak}_i}$$

m_{DBZ} = Amount of internal standard DBZ in μg
A_{DBZ} = Measured value for peak$_{DBZ}$

Retention time t_R, correction factors, and linearity of the flame ionisation detector. The constancy of the retention times of IP, its metabolites, and DBZ was tested under the optimal conditions outlined above. The amounts injected were in the range of 0.05–0.5 μg. The correction factors were based on the internal standard (DBZ) and determined by injecting standard mixtures of IP, IDB, DBZ and the acetylated imipramine metabolites in various concentrations. Each solution was chromatographed three or four times. The correction factors were plotted against the amount of substance. Linearity of the flame ionisation detector is expressed by a straight line parallel to the abscissa.

RESULTS AND DISCUSSION

Partition coefficients: extraction of imipramine and its metabolites from biological material

Table I gives the partition coefficients of imipramine and its metabolites between an aqueous phase, pH 7.4, and *n*-hexane or chloroform, respectively. The substances are listed in the order of increasing polarity.

TABLE I

PERCENTAGE OF IMIPRAMINE AND ITS METABOLITES IN THE ORGANIC PHASE AFTER PARTITION WITH ISOTONIC PHOSPHATE BUFFER pH 7.4

Substance	*n-Hexane*	*Chloroform*
IP	99.4 ± 1.4*	99.9 ± 0.8*
IDB	99.2 ± 1.2	99.6 ± 1.1
DDMI	70.5 ± 2.3	99.1 ± 1.8
DMI	65.1 ± 2.1	98.5 ± 1.6
2-OH-IDB	44.8 ± 2.0	98.0 ± 1.2
2-OH-IP	25.2 ± 2.0	97.4 ± 1.5
IPNO	10.0 ± 2.2	97.2 ± 1.5
2-OH-DMI	6.3 ± 2.0	55.2 ± 2.1

* Standard deviations from 8 experiments.

The partition coefficients indicate the distribution of the imipramine metabolites over a wide range of polarity. The order of the metabolites is identical in both systems used, 2-OH-DMI being the most polar one. The glucuronides of the phenolic metabolites have not been included in our series, however preliminary partition experiments indicate that their polarity is still considerably higher than that of 2-OH-DMI. Fig. 1 shows that an optimal yield of 2-OH-DMI can be achieved by extraction with diethyl ether at pH 10.0 $\pm$ 0.2. Under the same conditions the partition of IPNO, independent of pH, is 71.9 $\pm$ 1.8 % in 1,2-dichloroethane and 7.8 $\pm$ 2.0 % in ether, thus permitting the separation of IPNO.

The minimum number, n, of extractions required for practically quantitative (> 98 %) isolation of the substances (ratio of phase volumes 1:1) is 2 for ether and n-heptane and 2–3 for 1,2-dichloroethane (Table II). The calculated α values given in Table II were confirmed by test extractions from biological materials.

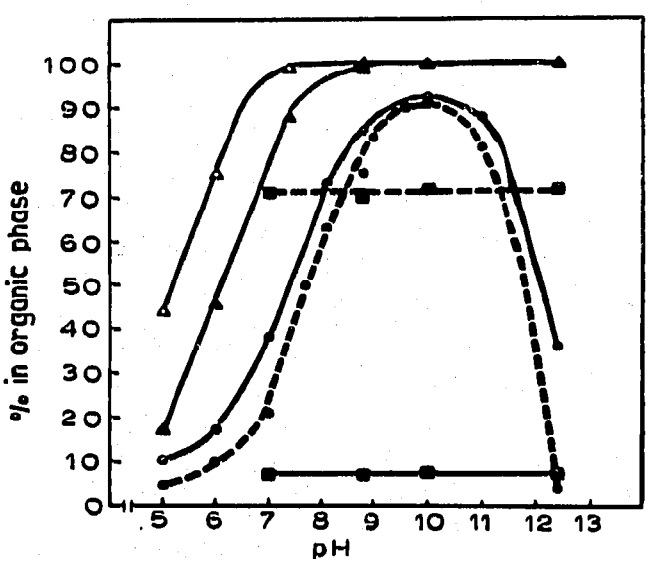

Fig. 1. Partition of IP (△); DMI (▲); 2-OH-DMI (●); and IPNO (■) between aqueous buffer solutions and diethyl ether(————) or 1,2-dichloroethane (– – – –) respectively, as a function of pH value.

Gas–liquid chromatography

The acetylation of DMI, DDMI, 2-OH-IP, 2-OH-DMI and 2-OH-IDB was carried out at room temperature with reaction times from 15 min to 3 h. A maximum yield of 98 % was reached after 2 h. The tertiary amines, IP, IDB and DBZ, are not altered by the procedure.

Anakrom ABS coated with 1.5 % SE-30 shows optimum separation properties (240–260° isothermal). Carbon disulfide proved to be a most suitable solvent for the substances used. The response of the flame ionisation detector to CS_2 is minimal; the peak area of this solvent is 1/100 that of methanol, thus its peak does not interfere with the first peak (IDB) of the analyzed materials. Chloroform has to be added to the solvent since the solubility of the phenolic metabolites in CS_2 is limited. The optimum ratio CS_2–$CHCl_3$ is 7:3,v/v. The solvent properties are hardly altered by the addition of $CHCl_3$ in the ratio mentioned.

TABLE II

MEASURED AND CALCULATED VALUES AS CRITERIA FOR THE EXTRACTION OF IMIPRAMINE AND ITS METABOLITES

Substance	*Diethyl ether*				*1,2-Dichloroethane*				*n-Heptane*			
	% in organic phase	K_c	n^*	α^*	*% in organic phase*	K_c	n^*	α^*	*% in organic phase*	K_c	n^*	α^*
IPNO	7.8 ± 2.0	0.08	2	0.85	71.9 ± 1.8	2.6	3	0.02				
2-OH-DMI	92.9 ± 1.8	13.00	2	0.005	91.1 ± 1.4	10.2	2	0.008	13.8 ± 2.2	0.2	4	0.59
IP	100**				100**				100**			
DMI	98.2 ± 1.5	54.40	2	0.001	98.0 ± 2.0	49.0	2	0.001	98.4 ± 1.4	61.5	2	0.001
DBZ	100**				100**				95.1 ± 2.0	19.4	2	0.002

* $\alpha = \left(\frac{v_1}{v_1 + K_c \cdot v_2}\right)^n$ where v_1 = volume of aqueous phase (5 ml)
v_2 = volume of organic phase (5 ml)
n = number of extractions
K_c = partition coefficient
α = proportion of substance in aqueous phase (pH 10) after n extractions.

** No substance detectable in aqueous phase after one extraction.

The retention times, t_R of imipramine, its metabolites and DBZ are constant in the test range of 0.05–5.0 μg (see Table III). This table also lists the correction factors as based on DBZ. The sensitivity of the flame ionisation detector for all tested substances is linear in the 0.2–3.0 μg range (Fig. 2). Due to the constant capacity of the detector it must be assumed that linearity also exists when samples of less than 0.2 μg are injected.

A standard chromatogram of IP, its metabolites and DBZ extracted from buffer solutions and acetylated is given in Fig. 3. The chromatograms of extracts from

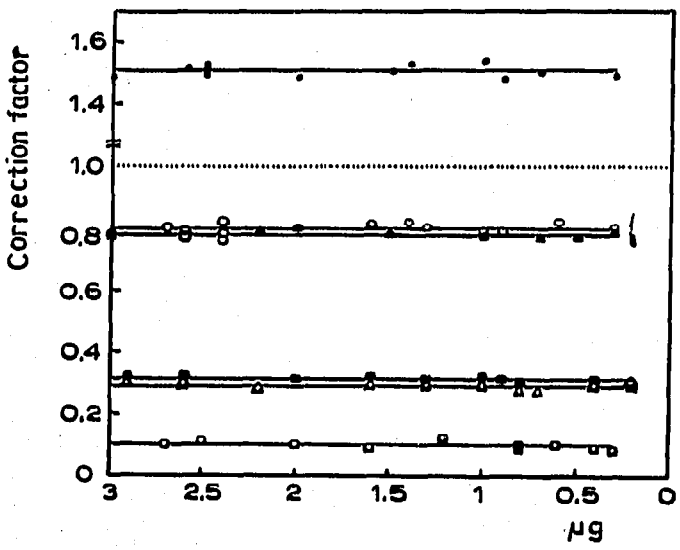

Fig. 2. Correction factors based on DBZ = 1 of IDB (□); IP (△); 2-OH-IDB-Ac (■); 2-OH-IP-Ac (●); DDMI-Ac (▲); and DMI-Ac (O). Linearity of flame ionisation detector.

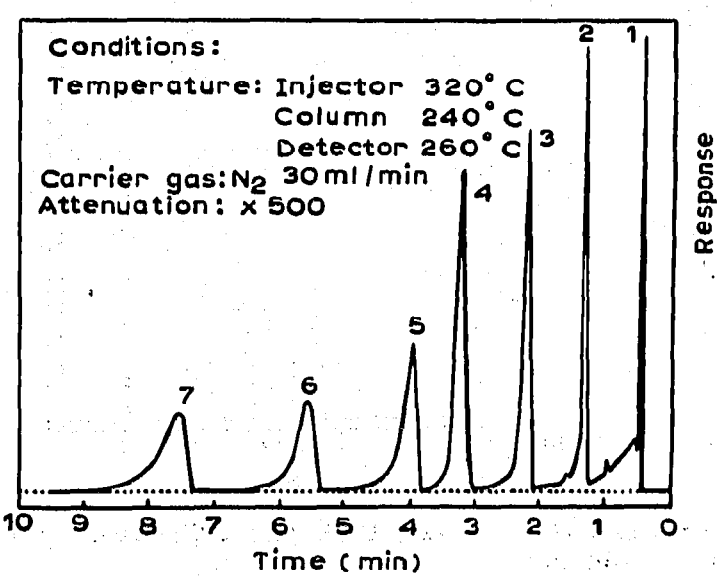

Fig. 3. Standard gas chromatogram. Solvent (1); IDB (2); IP (3); 2-OH-IDB-Ac (4); DBZ (5); 2-OH-IP-Ac (6); and DMI-Ac (7). Peak areas correspond to the amount of substance (1.5–2.5 μg).

TABLE III

GAS CHROMATOGRAPHIC CHARACTERISTICS OF IMIPRAMINE AND ITS METABOLITES

Substance i	*Retention time (min) (column temp. 240°)*	*Correction factor* $Ff_{DBZ}^{i} \pm SD$	*Evaluation method*
IDB	1.30–1.33 (18)*	0.10 ± 0.008 (18)*	peak height
IP	2.18–2.24 (20)	0.29 ± 0.01 (18)	peak height
2-OH-IDB-Ac	3.15–3.21 (18)	0.31 ± 0.01 (23)	peak height
Dibenzepine	3.87–3.93 (20)	1.00	peak height and electronic integration
2-OH-IP-Ac	5.45–5.57 (16)	1.51 ± 0.02 (18)	peak height
DDMI-Ac	6.84–6.90 (16)	0.78 ± 0.02 (14)	electronic integration
DMI-Ac	7.27–7.42 (16)	0.80 ± 0.02 (22)	electronic integration

* Number of experiments in parentheses.

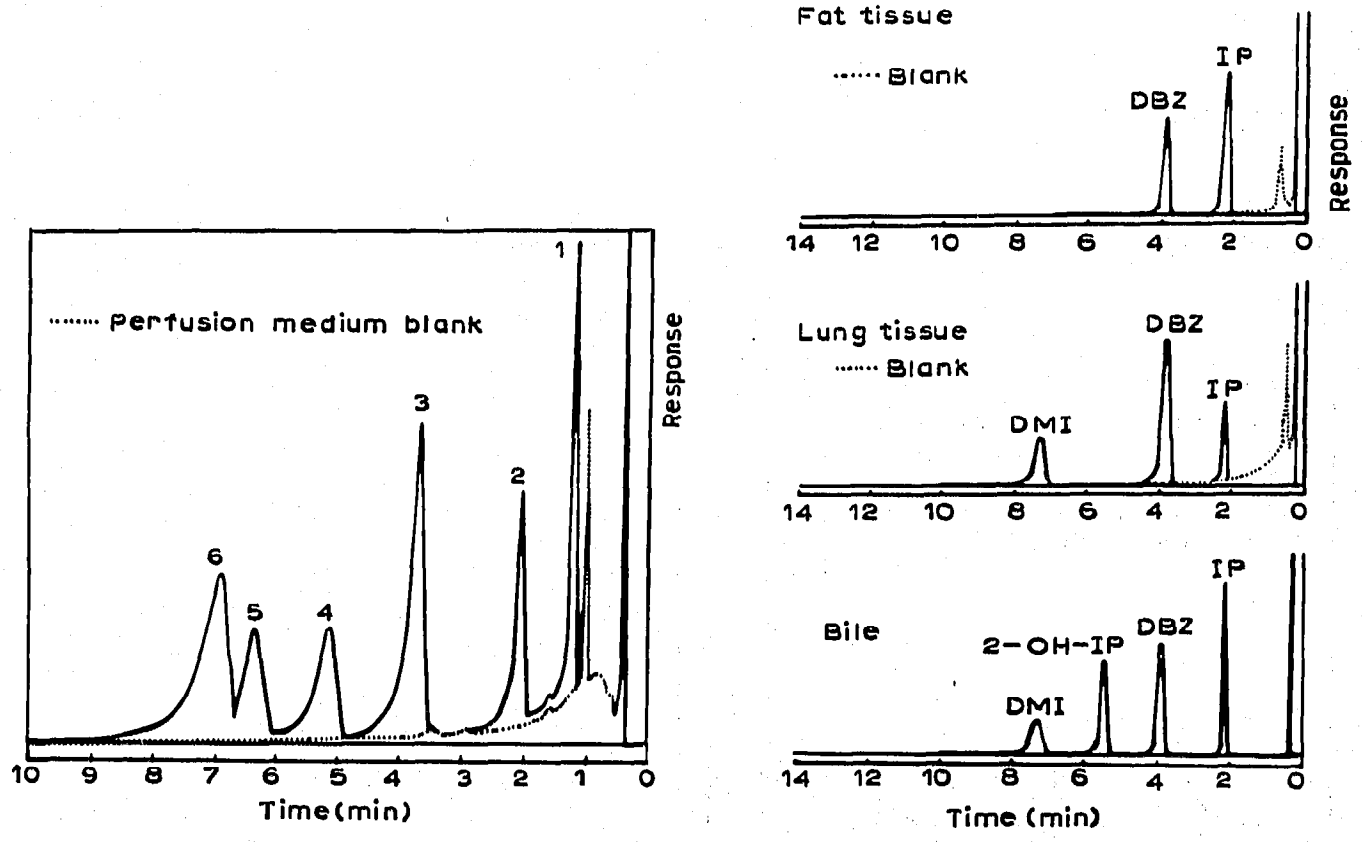

Fig. 4. Gas chromatogram of perfusion medium. IDB (1); IP (2); DBZ (3); 2-OH-IP-Ac (4); DDMI-Ac (5); and DMI-Ac (6); Same conditions as Fig. 3.

Fig. 5. Gas chromatograms of tissues from rats treated with imipramine, and of bile from a liver perfusion experiment.

biological materials containing no IP or IP-metabolites showed no peaks with retention times identical to those of the mentioned substances. Extracts from liver, kidney and brain showed unidentified peaks in the t_R range of 0–2 min, partially interfering with the IDB peak only. The separation of the peaks of DMI-Ac and DDMI-Ac is incomplete, however graphical evaluation has proved to be satisfactory. Chromatograms of extracts of perfusion medium are shown in Fig. 4; chromatograms from current metabolic experiments in Fig. 5.

The sensitivity of the gas chromatography determination is $<$ 0.01 μg for IDB; 0.01 μg for IP and 2-OH-IDB-Ac; 0.02 μg for DBZ and 2-OH-IP-Ac; 0.05 μg for DDMI-Ac and DMI-Ac. In order to reach these sensitivity values the extracts have to show a high degree of purity. The sensitivity of 2-OH-DMI-Ac is only 0.2–0.3 μg. Since there is considerable tailing of 2-OH-DMI-Ac, an irreversible adsorption of a part of this substance has to be assumed. The retention times of 2-OH-DMI-Ac at column temperatures of 240 and 260° are 11.5 $\pm$ 0.4 and 9.4 $\pm$ 0.2 min, respectively (t_R of DBZ at 260° = 2.15 $\pm$ 0.03 min).

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