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Shift of the high-performance liquid chromatographic retention times of metabolites in relation to the original drug on an RP8 column with acidic mobile phase

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Abstract

The effect of the structural change in the metabolization of drugs on the HPLC retention time with an RP8 column with an acetonitrile–phosphate buffer (pH 2.3) as the mobile phase was investigated at model compound pairs of 29 functionalization reactions. A more or less typical region for $T_M = \log(k'_M/k'_D)$ was found for each of these reactions (with k'_M and k'_D being the capacity factors of the metabolite and the drug, respectively), which can be explained by an increase or a decrease of the hydrophilic properties caused by the structural change. This effect is superimposed by an essential influence of the unchanged part of the molecule and in some cases by special intramolecular interactions like the hydrogen bond. Despite the more complicated structure of real drugs the results obtained at the model compound pairs were confirmed for most of the 55 metabolite/drug pairs. The practical use of the T_M values as a support to distinguish between different metabolites in the HPLC-DAD analysis of intoxications is demonstrated with cases of poisoning with diphenhydramine, propafenone and methaqualone.

Keywords: Structure–retention relationship; Metabolization

1. Introduction

In the last decade high-performance liquid chromatography with a photodiode array detector (HPLC–DAD) has become one of the methods most frequently used in systematic toxicological analysis [1–9], by which in 'general unknown' intoxication cases the presence or absence of a large number of illegal or therapeutic drugs, pesticides or other toxic compounds must be elucidated in a relatively short time. By computerised comparison of all peaks in the chromatogram of an extract from blood, urine or tissue with a library of UV spectra and retention parameters of a large number of toxic compounds, the constituents can be identified. A subsequent semiquantitative determination of the concentration is possible via the peak areas also stored in such libraries.

In order to exclude an acute intoxication it is necessary to identify as far as possible each peak in the chromatograms of such sample extracts. Very often these chromatograms are complicated by peaks of several drug metabolites. Since as a rule these metabolites are not at hand in the investigating laboratory, one generally tries to use other criteria for the identification. In most cases the similarity of

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the UV spectra between the metabolites and the original drug is used, but also the regular appearance of a certain peak in all intoxications with the same drug points to a metabolite.

On the other hand, the metabolization should give also rise to a characteristic shift of the retention time, which should be another helpful criterion used to assign a metabolite peak to one of the different known metabolization reactions of a drug, e.g. aromatic or aliphatic hydroxylation, desamination, decarboxylation etc.

The relationship between chemical structure and retention parameters (capacity factors k') in reversed-phase liquid chromatography was thoroughly investigated by many authors in the last 20 years, and excellent reviews were published relating to this topic, in the context of retention mechanism and the possibility of a prediction of k', by Golushko et al. [10,11], Dorsey et al. [12,13], Kaliszan [14,15], Lochmüller et al. [16], Tchapla et al. [17], Schoenmakers et al. [18,19] and Smith et al. [20,21]. This work is based on correlations with a range of physical and structural properties like size, planarity, dipole moment or octanol–water partition coefficients (log P). A good correlation was found between the effect of substituents on k' and the Hansch π and Rekker f functional group constants, which are used in the more general quantitative structure–activity relationships (QSAR) for the calculation of log P from the molecular structure [22]. Furthermore, for the prediction of k' from the structural formula computer expert systems were developed [11], in which log k' is calculated as a summation of fragment increments of the partial molar volume and of the interaction energy with the mobile phase.

It follows from these results described in the literature that the specific structural change of a metabolization reaction should lead to a typical shift of k', which should to a certain degree be independent of the structure of the molecular part unchanged by the reaction. Investigations with the purpose of a retention prediction of metabolites were already carried out by Valko et al. [23], who calculated average values for $\Delta\log k'$ of, e.g. C-hydroxylation, N-demethylation or decarboxylation.

In order to study the relationship between the type of the metabolization reaction and the shift of the capacity factor k' a series of model compound pairs was investigated for the most important phase I metabolizations by HPLC at an RP8 column with a phosphate buffer/acetonitril mobile phase. In each model compound pair one substance could theoretically have been formed from the other by metabolization. The RP8 column and the acidic mobile phase were chosen since these are conditions established in the systematic toxicological analysis by HPLC–DAD. For confirmation of the results obtained with the model compounds, a series of real drug/metabolite pairs was also included in these investigations.

2. Experimental

2.1. Substances

The substances chosen as model compounds were purchased from Merck, Aldrich or Riedel de Haen and were used without further purification. The drugs and metabolites were either purchased from Sigma or were generously donated at our disposal by a large number of manufacturers. Acetonitril (UVASOL, transmission >80% at 195 nm) was purchased from Merck.

2.2. HPLC techniques and conditions

The HPLC measurements were carried out using a Shimadzu HPLC device consisting of a pump LC-6A, an autosampler SIL-9A, a photodiode array detector SPD-M6A, a computer 386 DX33 and a printer HP Laser Jet 4. The SPD-M6A is operated by the software CLASS.

The HPLC column was a Lichrosorb RP8, 5 μm, 250×4.0 mm (Merck). The isocratic mobile phase consisted of acetonitrile–phosphate buffer pH 2.3 (37:63, v/v) and was degassed by a stream of helium. All measurements were carried out at room temperature (20–25°C). For the determination, a time t_0 of an unretained peak urea was chosen. Since histamine hydrochloride had exactly the same time but a much higher absorbance, this compound was used for the regular control of t_0. Furthermore, in order to control the experimental conditions, 5-(4-methylphenyl)-5-phenylhydantoine (MPPH) was used as a reference substance with a medium retention time in each series of measurements.

3. Results and discussion

3.1. Investigations of model substance pairs

For the investigation of the effect of the metabolization reaction on k' relatively simple aromatic or heteroaromatic model compounds were chosen. The aromatic group is necessary as a prerequisite of the UV detection. Since also an effect of the unchanged part of the molecule on the shift of k' is expected, between 2 and 9 pairs were involved for each functionalization reaction. Only at the aromatic hydroxylation was the effect of the unchanged part of the molecule investigated more thoroughly at 32 compound pairs. As a criterion, T_M for the quantitative characterisation of the metabolization effect $\log(k'_M/k'_D)$ is used. This is in agreement with the description of the functional group contributions to the retention [20].

$$\text{Drug } (k'_D) \rightarrow \text{Metabolite } (k'_M) \qquad (1)$$

$$T_M = \log k'_M - \log k'_D = \log (k'_M/k'_D)$$

The k' values of the model compounds as well as the T_M values of the compound pairs are given in Table 1 for 29 different functionalization reactions. For each reaction the pairs are listed in the order of increasing T_M. For the majority of the reactions a characteristic, but more or less broad, T_M region (see also Fig. 1) is found according to a typical change of the lipophilic or hydrophilic properties of the reaction centre. In Fig. 1 this region is shown for 18 of the reactions including the data obtained with real drug/metabolite pairs from Table 3. The reactions (10), (14), (18) and (21) with a varying group R', R'' or X as well as the reactions (13) and (28), which are represented by only two very similar compound pairs, are omitted in Fig. 1. This is also the case with reactions (3), (4) and (27), which are not very common in drug metabolism.

A structural interpretation of the change of k' is possible on the basis of the molecular mechanisms of retention in reversed-phase liquid chromatography [12,17]. $T_M<0$ is found in all cases, in which the reaction leads to an increase of the hydrophilic properties of the molecule and, therefore, to a stronger interaction with the aqueous mobile phase, like aliphatic or aromatic hydroxylation, oxidation of alkyl groups to carboxylic groups, removal of *N*- or *O*-alkyl groups from amines, amides or ethers, hydrolysis of esters or reduction of nitro compounds to amines. In alkyl-substituted aromatics the introduction of a hydroxy group into the alkyl group decreases k' more than into the aromatic ring, as is obvious from the formation of the cresols and phenylmethanol from toluene ($T_M = 0.538$ to -0.500 and -0.760, respectively) or of *p*-hydroxyethylbenzene and the phenylethanols from ethylbenzene ($T_M = -0.553$ and 0.813 to -0.822, respectively). $T_M<0$ is also found in the reductive removal of Cl, Br or I from halogenated aromatic compounds and in the hydrolysis of the thiocarbonyl group to the carbonyl group.

$T_M>0$ is observed always, if a hydrophilic substituent is removed or is transformed into a less hydrophilic group, since then the metabolite is less stabilized than the parent drug by the interaction with the mobile phase. This is the case in the removal of the amino group (cf. reactions 15 and 18), which is particularly hydrophilic in the acidic mobile phase because of the protonation to ammonium compounds which are more strongly stabilised by the mobile phase. This effect decreases with increasing size of *N*-alkyl substituents as shown in the series benzylamine ($T_M = 0.854$), *N*-methylbenzylamine ($T_M = 0.745$), *N*-ethylbenzylamine ($T_M = 0.606$) and *N*-*i*-propylbenzylamine ($T_M = 0.529$), since the loss of the hydrophilic ammonium group is increasingly compensated by the loss of the hydrophobic alkyl group. Also the hydrolysis of carboxylic amides into carboxylic acids leads to an increase of the retention time although the *N*-atom is not protonated in this case. Another example of $T_M>0$ is the decarboxylation of carboxylic acids.

For five of the reactions given in Table 1 the results can be compared with the $\Delta\log k'$ values ($\equiv T_M$) described by Valko et al. [23], who investigated 11 drug/metabolite pairs at three different RP18 columns in a methanol/phosphate buffer pH 7.8 mobile phase. These authors found for the aliphatic hydroxylation $\Delta\log k' = -0.180$ to -0.498 (our results, reaction 5: $T_M = -0.05$ to -0.82), for the aromatic hydroxylation -0.37 to -0.77 (our results, reaction 6: -0.19 to -0.55), for the *N*-demethylation of amines -0.183 to -0.290 (our

Table 1
Capacity factors k' of conceivable drug/metabolite model substance pairs for the simulation of the effect of the metabolization reactions on reverse phase retention

Model substance pair 'Drug/Metabolite'	k'_D	k'_M	T_M
R-CH_2-OH→R-COOH $T_M = -0.07$ to 0.17 (1)			
2-Phenylethanol/Phenylacetic acid	1.68	1.43	−0.069
3-Hydroxymethylpyridine/Nicotinic acid	0.24	0.21	−0.053
O-Phenylethylenglycol/Phenoxyacetic acid	1.38	1.24	−0.048
Phenylmethanol/Benzoic acid	1.35	1.63	0.081
4-Methoxyphenylmethanol/4-Methoxybenzoic acid	1.10	1.61	0.166
2-Aminophenylmethanol/2-Aminobenzoic acid[a]	0.296	1.14	0.586
R-CHOH-R′→R-CO-R′ $T_M = 0.16$ to 0.32 (2)			
1-Phenylethanol/Acetophenone	1.71	2.49	0.163
4-Methoxyphenylmethanol/4-Methoxybenzaldehyde	1.10	1.83	0.222
1-Phenylpropanol/Propiophenone	2.42	4.37	0.257
Phenylmethanol/Benzaldehyde	1.35	2.70	0.301
Diphenylmethanol/Benzophenone	4.85	10.21	0.323
R-CHO→R-COOH $T_M = -0.22$ to −0.18 (3)			
Benzaldehyde/Benzoic acid	2.70	1.63	−0.219
4-Dimethylaminobenzaldehyde/4-Dimethylaminobenzoic acid	2.96	1.80	−0.215
4-Chlorobenzaldehyde/4-Chlorobenzoic acid	4.05	2.58	−0.195
4-Cyanobenzaldehyde/4-Cyanobenzoic acid	1.99	1.32	−0.179
R-CHOH-COOH→R-COOH+CO_2 $T_M = 0.16$ to 0.48 (4)			
α-Hydroxy-β-phenyl-propionic acid/Phenylacetic acid	0.993	1.43	0.159
α-Hydroxyphenylacetic acid/Benzoic acid	0.543	1.63	0.477
Alk-H→Alk-OH $T_M = -0.82$ to −0.05 (5)			
Ethylbenzene/1-Phenylethanol	11.13	1.71	−0.813
Ethylbenzene/2-Phenylethanol	11.13	1.68	−0.822
Toluene/Phenylmethanol	7.77	1.35	−0.760
Phenylacetic acid/2-Hydroxphenylacetic acid	1.43	0.543	−0.421
2-Aminotoluene/2-Aminobenzylalcohol	0.366	0.296	−0.092
N-Ethylaniline/2-Anilinoethanol	0.308	0.254	−0.084
Ar-H→Ar-OH $T_M = -0.55$ to −0.19 (6)			
Ethylbenzene/4-Ethylphenol	11.13	3.11	−0.553
Naphthalene/β-Naphthol	11.46	3.22	−0.551
Toluene/*m*-Hydroxytoluene	7.77	2.25	−0.538
Toluene/*p*-Hydroxytoluene	7.77	2.25	−0.538
Naphthalene/α-Naphthol	11.46	3.45	−0.522
Indol/4-Hydroxyindol	3.76	1.14	−0.520
Benzene/Phenol	4.56	1.43	−0.504
Toluene/*o*-Hydroxytoluene	7.77	2.46	−0.500
Indol/5-Hydroxyindol	3.77	1.23	−0.485
Benzoic acid/*p*-Hydroxybenzoic acid	1.63	0.551	−0.471
Phenol/*p*-Hydroquinone	1.43	0.497	−0.458
Aniline/4-Aminophenol	0.216	0.077	−0.448
Benzaldehyde/4-Hydroxybenzaldehyde	2.70	0.986	−0.437
Biphenyle/4-Phenylphenol	17.26	6.37	−0.433
Anisol/Guajacol	3.97	1.53	−0.415
Benzaldehyde/3-Hydroxybenzaldehyde	2.70	1.17	−0.365
Phenylacetic acid/4-Hydroxyphenyl acetic acid	1.43	0.612	−0.369
Nitrobenzene/*p*-Nitrophenol	3.65	1.73	−0.324
α-Naphthol/1,6-Dihydroxynaphthalene	3.45	1.61	−0.332
β-Naphthol/1,6-Dihydroxynaphthalene	3.22	1.61	−0.303
L-Phenylalaninmethyl ether/L-Tyrosineethyl ether	0.400	0.200	−0.301

Table 1. Continued

Model substance pair 'Drug/Metabolite'	k'_D	k'_M	T_M
N-Phenylethanolamine/*p*-Hydroxy-*N*-phenylethanolamine	0.254	0.131	−0.288
N-methylaniline/4-*N*-methylaminophenol	0.312	0.166	−0.274
α-Naphthol/1,5-Dihydroxynaphthalene	3.45	1.86	−0.267
β-Naphthol/1,5-Dihydroxynaphthalene	3.22	1.86	−0.238
Acetophenone/*m*-Hydroxyacetophenone	2.49	1.45	−0.236
Quinoline/8-Hydroxyquinoline	0.454	0.266	−0.232
Nitrobenzene/*m*-Nitrophenol	3.65	2.36	−0.189
Nitrobenzene/*o*-Nitrophenol[a]	3.65	2.84	−0.109
Benzaldehyde/Salicylaldehyde[a]	2.70	2.43	−0.045
Benzoic acid/Salicylic acid[a]	1.63	1.71	0.021
Quinoline/2-Hydroxyquinoline[a]	0.454	1.24	0.438
Ar-CH_3→Ar-COOH T_M = −0.79 to −0.61 (7)			
p-Xylene/*p*-Methylbenzoic acid	13.18	2.16	−0.786
Toluene/Benzoic acid	7.77	1.63	−0.679
p-Methylphenol/*p*-Hydroxybenzoic acid	2.25	0.551	−0.611
o-Methylphenol/Salicylic acid[a]	2.84	1.71	−0.221
2-Methylpyridine/2-Pyridinecarboxylic acid[a]	0.189	0.123	−0.187
p-Aminotoluene/*p*-Aminobenzoic acid[a]	0.246	0.570	0.365
o-Aminotoluene/*o*-Aminobenzoic acid[a]	0.366	1.14	0.493
RR'N-CH_3→RR'N-H T_M = −0.33 to −0.11 (8)			
4-(*N*-Methylamino)-phenol/4-Aminophenol	0.166	0.077	−0.334
p-Dimethylaminobenzophenone/*p*-Aminobenzophenone[b]	13.51	3.91	−0.268
N-Methylacridone/Acridone	6.02	3.22	−0.272
N-Methylphenothiazine/Phenothiazine	29.06	18.46	−0.197
N-Methylaniline/Aniline	0.312	0.216	−0.160
N,*N*-Dimethylaniline/N-Methylaniline	0.420	0.312	−0.129
N,*N*-Dimethylbenzylamine/N-Methylbenzylamine	0.320	0.243	−0.120
N-Methyl-*p*-anisidine/*p*-Anisidine	0.347	0.270	−0.109
N-Methylbenzylamine/Benzylamine	0.243	0.189	−0.109
RR'N-C_2H_5→RR'N-H T_M = −0.25 to −0.15 (9)			
N-Ethylbenzylamine/Benzylamine	0.335	0.189	−0.249
N,*N*-Diethylaniline/*N*-Ethylaniline	0.462	0.308	−0.176
N-Ethylaniline/Aniline	0.308	0.216	−0.154
RR'N-R"→RR'N-H T_M = −1.40 to −0.37 (depending on R") (10)			
N-Phenylbenzylamine/Aniline R" = CH_2-C_6H_5	5.66	0.216	−1.40
N-Phenylglycine/Aniline R" = CH_2-COOH	0.955	0.216	−0.646
N-*i*-Propylaniline/Aniline R" = *i*-C_3H_7	0.616	0.216	−0.455
N-*i*-Propyl-benzylamine/Benzylamine R" = *i*-C_3H_7	0.439	0.189	−0.366
R-CO-NR'-R"→R-CO-NR'-H T_M = −0.30 to −0.12 (depending on R") (11)			
N,*N*-Diethylbenzamide/Benzamide[b] R" = C_2H_5	3.09	0.766	−0.301
N-Methylbenzamide/Benzamide R" = CH_3	1.21	0.766	−0.198
N,*N*-Dimethylbenzamide/*N*-Methylbenzamide R" = CH_3	1.60	1.21	−0.121
R-O-CH_3→R-O-H T_M = −0.63 to −0.25 (12)			
1-Methoxynaphthalene/1-Hydroxynaphthalene	14.61	3.45	−0.627
2-Methoxynaphthalene/2-Hydroxynaphthalene	13.03	3.22	−0.607
p-Aminoanisol/*p*-Aminophenol	0.270	0.077	−0.545
Anisol/Phenol	3.97	1.43	−0.444
p-Hydroquinonedimethylether/*p*-Hydroquinone	1.38	0.497	−0.443
p-Methoxyphenylacetic acid/*p*-Hydroxyphenylacetic acid	1.37	0.612	−0.349
Veratrol/Guajacol	2.68	1.53	−0.245
R-O-C_2H_5→R-O-H T_M = −0.60 (13)			
4-Ethoxyacetanilide/4-Hydroxyacetanilide	1.84	0.466	−0.597
4-Ethoxyaniline/4-Hydroxyaniline	0.312	0.077	−0.608

Table 1. Continued

Model substance pair 'Drug/Metabolite'	k'_D	k'_M	T_M
R-O-R'→R-O-H T_M=0.00 to 0.06 (depending on R') (14)			
α-Naphthoxyacetic acid/α-Naphthol R'=CH_2-COOH	3.45	3.45	0.000
O-Phenylethylenglycol/Phenol R'=CH_2-CH_2-OH	1.38	1.43	0.014
Phenoxyacetic acid/Phenol R'=CH_2-COOH	1.24	1.43	0.063
R-NH_2→R-OH T_M=0.66 to 0.85 (15)			
L-Phenylalanin/L-3-Hydroxy-3-phenylpropionic acid	0.216	0.993	0.662
2-Phenylethylamine/2-Phenylethanol	0.262	1.68	0.807
α-Phenylethylamine/1-Phenylethanol	0.243	1.71	0.848
Benzylamine/Phenylmethanol	0.189	1.35	0.854
R-CH_2-NH_2→R-COOH T_M=0.74 to 0.94 (16)			
β-Phenylethylamine/Phenylacetic acid	0.262	1.43	0.738
Benzylamine/Benzoic acid	0.189	1.63	0.936
RR'CH-NH_2→R-CO-R' T_M=0.87 to 1.01 (17)			
Amphetamine/Benzylmethylketone	0.389	2.86	0.866
α-Phenylethylamine/Acetophenone	0.243	2.49	1.01
R-NH-R'→R-OH T_M=0.53 to 0.75 (depending on R') (18)			
N-*i*-Propylbenzylamine/Phenylmethanol R'=*i*-C_3H_7	0.400	1.35	0.529
N-Ethylbenzylamine/Phenylmethanol R'=C_2H_5	0.335	1.35	0.606
Methylbenzylamine/Phenylmethanol R'=CH_3	0.243	1.35	0.745
R-CS-R'→R-CO-R' T_M=−0.38 to −0.05 (19)			
Thiobenzamide/Benzamide	1.83	0.766	−0.377
Phenylthiourea/Phenylurea	0.716	0.643	−0.047
Ar-NO_2→Ar-NH_2 T_M=−1.41 to −0.50 (20)			
p-Nitrophenol/*p*-Aminophenol	1.97	0.077	−1.41
Nitrobenzene/Aniline	3.65	0.216	−1.23
p-Nitrobenzoic acid/*p*-Aminobenzoic acid	1.78	0.570	−0.495
o-Nitrobenzoic acid/*o*-Aminobenzoic acid[a]	1.07	1.14	0.029
Ar-X→Ar-H T_M=−0.96 to −0.04 (depending on X) (21)			
4-Iodaniline/Aniline	1.97	0.216	−0.960
4-Iodanisole/Anisole	11.65	3.97	−0.468
Iodbenzene/Benzene	12.79	4.56	−0.448
Bromobenzene/Benzene	8.36	4.56	−0.263
2-Chlorotoluene/Toluene	13.87	7.77	−0.251
Chlorobenzene/Benzene	7.24	4.56	−0.201
4-Chlorobenzaldehyde/Benzaldehyde	4.05	2.70	−0.176
4-Fluorophenylacetic acid/Phenylacetic acid	1.55	1.24	−0.098
4-Fluorobenzoic acid/Benzoic acid	1.79	1.63	−0.042
R-X→R-H T_M=−0.045 (21a)			
2-Chloroethylbenzene/Ethylbenzene	11.13	10.04	−0.045
R-CO-O-CH_3→R-CO-O-H T_M=−0.62 to −0.30 (22)			
Methylphenylacetate/Phenylacetic acid	5.99	1.43	−0.621
Phenylalanine methyl ester/Phenylalanine	0.400	0.131	−0.485
p-Hydroxymethylbenzoate/*p*-Hydroxybenzoic acid	1.55	0.551	−0.449
Methylbenzoate/Benzoic acid	4.07	1.63	−0.398
L-Tyrosine methyl ester/L-Tyrosine	0.200	0.100	−0.301
R-CO-O-C_2H_5→R-CO-OH T_M=−0.66 to −0.44 (23)			
p-Hydroxyethylbenzoate/*p*-Hydroxybenzoic acid	2.50	0.551	−0.656
Ethylphenylacetate/Phenylacetic acid	6.22	1.43	−0.638
Ethylbenzoate/Benzoic acid	6.72	1.63	−0.616
3-Phenylpropionic acid methyl ester/3-Phenylpropionic acid	9.46	2.14	−0.644
L-Tyrosine ethyl ester/L-Tyrosine	0.277	0.100	−0.442
R-O-CO-CH_3→R-OH T_M=−0.62 to −0.53 (24)			
2-Phenylethylacetate/2-Phenylethanol	6.97	1.68	−0.618
Benzylacetate/Phenylmethanol	5.44	1.35	−0.605

Table 1. Continued

Model substance pair 'Drug/Metabolite'	k'_D	k'_M	T_M
Phenylacetate/Phenol	4.15	1.22	−0.532
Ar-CO-NH_2→Ar-COOH T_M = 0.23 to 0.33 (25)			
p-Aminobenzamide/*p*-Aminobenzoic acid	0.339	0.570	0.226
Benzamide/Benzoic acid	0.766	1.63	0.328
Ar-CO-NRR'→Ar-COOH T_M = −0.28 to 0.51 (depending on R and R') (26)			
N,*N*-Diethylbenzamide/Benzoic acid R/R' = C_2H_5/C_2H_5	3.09	1.63	−0.278
N,*N*-Dimethylbenzamide/Benzoic acid R/R' = CH_3/CH_3	1.60	1.63	0.008
N-Benzoylmorpholine/Benzoic acid R/R' = $(-CH_2-CH_2)_2O$	1.39	1.63	0.069
N-Methylbenzamide/Benzoic acid R/R' = CH_3/H	1.21	1.63	0.129
Hippuric acid/Benzoic acid R/R' = CH_2-COOH/H	0.501	1.63	0.512
R-CO-NH-C_6H_5→R-COOH T_M = −0.61 to −0.48 (27)			
Phenylacetanilide/Phenylacetic acid	5.78	1.43	−0.606
p-Methoxybenzanilide/*p*-Methoxybenzoic acid	6.30	1.61	−0.593
Benzanilide/Benzoic acid	4.98	1.63	−0.481
Ar-NH-CO-CH_3→Ar-NH_2 T_M = −0.78 (28)			
p-Hydroxyacetanilide/*p*-Hydroxyaniline	0.466	0.077	−0.782
p-Ethoxyacetanilide/*p*-Ethoxyaniline	1.84	0.312	−0.772
R-COOH→R-H T_M = 0.20 to 0.74 (29)			
Tyrosine/Tyramine	0.100	0.158	0.199
Tryptophan/Tryptamine	0.239	0.400	0.224
2-Hydroxy-3-phenylpropionic acid/2-Phenylethanol	0.993	1.68	0.228
Hippuric acid/*N*-Methylbenzamide	0.501	1.21	0.382
α-Hydroxyphenyacetic acid/Phenylmethanol	0.543	1.35	0.396
α-Naphthoxyacetic acid/α-Methoxynaphthalene	3.45	14.61	0.627
Phenylacetic acid/Toluene	1.43	7.77	0.735

[a] Because of special structural interactions these pairs are excluded from the T_M regions given in the head line of each reaction.
[b] Because of the loss of two groups $\frac{1}{2}$ log (k'_M/k'_D) is given.

results, reaction 8: −0.11 to −0.33), for the *N*-demethylation of carboxylic amides −0.068 to −0.231 (our results, reaction 11, R" = CH_3: −0.12 to −0.20) and for the decarboxylation +0.323 to +0.643 (our results, reaction 29: +0.20 to +0.74). Besides the different experimental conditions, the different structure and the rather small number of compound pairs in [23] may explain the quantitative difference of the values.

A direct comparison of the results given in Table 1 with the functional group contributions τ_x for 30% acetonitril described by Smith et al. [20,21] and Schoenmakers et al. [19] is only possible for the hydroxylations (reactions 5 and 6 of Table 1) and the dehalogenation (reaction 21). In the other cases the chemical change is more complex, and only for some of them a description by two or more τ_x is possible, if the structural change is treated as a combination of single steps.

$$T_M = \sum \tau_{x,M} - \sum \tau_{x,D} \quad (2)$$

For example, the cleavage of the aromatic methoxy group (reaction 12) can be described as the combination of the OCH_3 removal and the addition of OH. Correspondingly, in the cleavage of the ethoxy group (reaction 13) a further CH_2 group must be taken into account.

$$T_{M(12)} = \tau_{OH} - \tau_{OCH_3} = -0.69 - 0.03 = -0.72 \quad (3)$$

$$T_{M(13)} = \tau_{OH} - \tau_{OCH_3} - \tau_{CH_2} = -0.69 - 0.03 - 0.35 = -0.97 \quad (4)$$

In Table 2 the T_M values of some model compounds of this investigation are compared with the data calculated according to Eq. (2) from the τ_x values of Smith et al. [20,21]. Unfortunately, no τ_{COOH} is

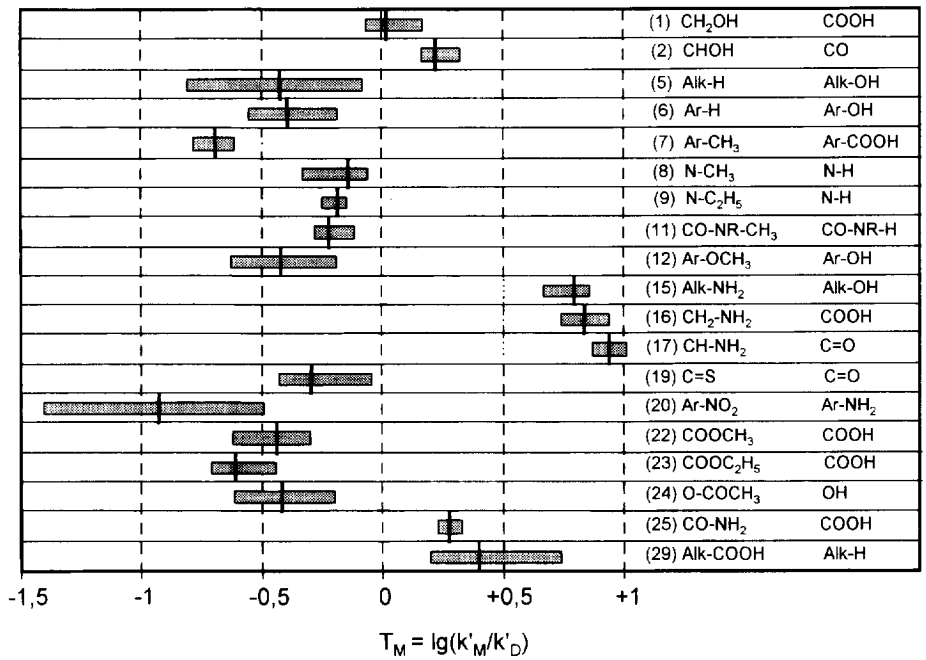


Fig. 1. Effects of some metabolization reactions on the reversed-phase HPLC retention characterized by the regions and mean values of $T_M = \log(k'_M/k'_D)$ of model substance pairs, Table 1, and real drug/metabolite pairs, Table 3.

Table 2
Comparison of the T_M values determined from model compounds (Table 1) and calculated from the functional group contributions τ_x published by Smith et al. [20,21] for 30% acetonitrile

React. No.	Substance pair	T_M (measured)	$T_M = \Sigma\tau_{x,M} - \Sigma\tau_{x,D}$ (calculated from Refs. [20,21])
2	Phenylmethanol/Benzaldehyde	**0.30**	τ_{CHO}-$\tau_{CH2OH} = -0.37 + 0.85 =$ **0.48**
5	Ethylbenzene/2-Phenylethanol	**−0.81**	$\tau_{OH,aliph.} =$ **−1.34**
6	Benzene/Phenol	**−0.50**	$\tau_{OH,arom.} =$ **−0.69**
8	*N*-Methylaniline/Aniline	**−0.16**	τ_{NH2}-$\tau^{a}_{NHCH3} = -0.75 + 0.23 =$ **−0.48**
12	Anisol/Phenol	**−0.44**	τ_{OH}-$\tau_{OCH3} = -0.69 - 0.03 =$ **−0.72**
13	4-Ethoxyaniline/4-Hydroxyaniline	**−0.60**	τ_{OH}-τ_{OCH3}-$\tau_{CH2} = -0.69 - 0.03 - 0.35 =$ **−0.97**
14	*O*-Phenylethylenglycol/Phenol	**0.01**	τ_{OH}-τ_{OCH3}-$\tau_{CH2OH} = -0.69 - 0.03 + 0.70 =$ **−0.02**
15	*β*-Phenylethylamine/2-Phenylethanol	**0.81**	τ_{OH}-$\tau_{NH2} = -0.67 + 0.78 =$ **0.11**
20	Nitrobenzene/Aniline	**−1.23**	τ_{NH2}-$\tau_{NO2} = -0.75 - 0.14 =$ **−0.61**
21	Chlorobenzene/Benzene	**−0.20**	$\tau_{Cl,aromatic} =$ **−0.36**
21	Bromobenzene/Benzene	**−0.26**	$\tau_{Br,aromatic} =$ **−0.54**
21a	2-Chloroethylbenzene/Ethylbenzene	**0.05**	$\tau_{Cl,aliphatic} =$ **−0.15**
28	*p*-Hydroxyacetanilide/*p*-Hydroxyaniline	**−0.78**	τ_{NH2}-$\tau_{NHCOCH3} = -0.75 + 0.86 =$ **0.13**

[a] τ values in 40% methanol.

found for the carboxylic group, which is a frequent product of metabolization.

It is seen that qualitatively the direction and the order of the metabolization effect on the retention can be estimated also from the τ_x values, if no amino group is involved and if there are no strong effects of the unchanged part of the molecule. Quantitatively, with the exception of the reactions (1), (15), (20) and (28) the calculated T_M values are more negative. For compound pairs without a protonation equilibrium (reactions 5, 21 and 21a) this may be caused by the ODS column used in the work of Smith et al. [20] deviating from the RP8 column in this investigation. In addition to this the difference in the pH between this investigation (pH 2.3) and the neutral mobile phase, in which, as a rule, the literature data were determined, seems to be important for all other reactions of Table 2, in which acidic or basic groups are removed, added, changed or liberated. This is also the reason for the very strong deviations particularly in the case of the formation or loss of the amino group (reactions 15, 20 and 28). At pH 2.3 the protonation equilibrium of the amino group is far more on the side of the weakly retained ammonium ion than in a neutral solution. Therefore, at pH 2.3 for reaction 15, where the amino group is removed, T_M is far more positive, and for reactions 20 and 28, where the amino group is formed or liberated, T_M is far more negative than calculated for neutral conditions.

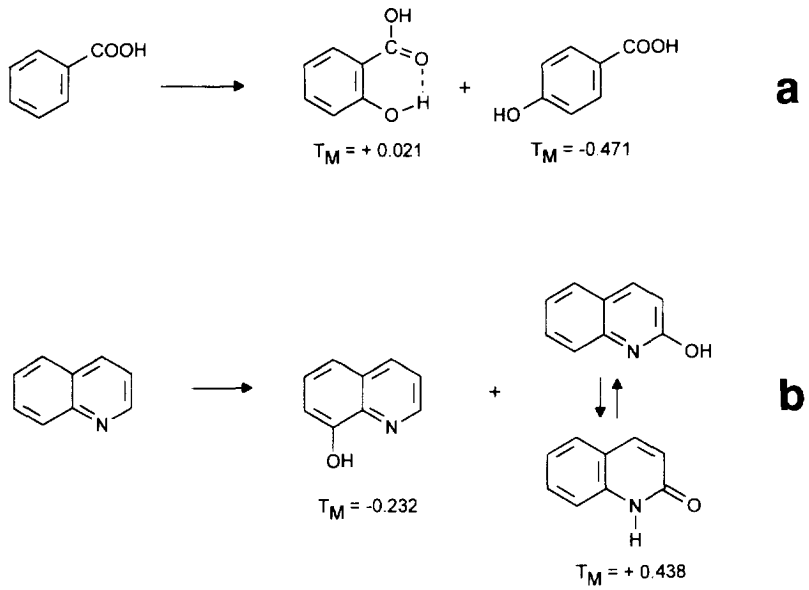
In addition to this influence of the experimental conditions the τ_x value depends strongly on the position and the structural environment to which it is linked, and since the number of the known τ_x values for different groups is still rather limited, a practical application of the prediction of T_M from τ_x is restricted to a minority of metabolization reactions.

The T_M regions in Table 1 and Fig. 1 for the reactions are in most cases relatively broad. The reason for this is that the effect of the metabolization is superimposed by an influence of the unchanged part of the molecule on T_M. This effect of the position of the functional group, of the complexity of the residual molecule and of steric, electronic or intramolecular hydrogen bond interactions was also described by several authors, and was reviewed by Smith [20]. It follows from the evaluation of our results that, in general, the effect of the addition, change or loss of a hydrophilic functional group on T_M is higher with a hydrophobic residual molecule than with a hydrophilic one. For example, T_M of the α-hydroxylation of ethylbenzene (−0.813) is more negative than that of the more hydrophilic phenylacetic acid (−0.421).

This effect of the hydrophilicity of the unchanged molecular part is particularly obvious in the case of the aromatic hydroxylation, where the addition of a second hydroxy group to phenol and α- or β-naphthol has a smaller effect than the hydroxylation of benzene or naphthalene. For the same reason the hydroxylation of basic compounds like phenylalanine ethyl ester, *N*-methylaniline or quinoline, which are protonated in the acidic mobile phase, has less negative T_M values than analogous non-basic compounds like phenylacetic acid, ethylbenzene or indol. Another example is the decarboxylation (reaction 29), which leads to smaller T_M for the hydrophilic amino acid tyrosine than for the more lipophilic phenylacetic acid.

Deviating from this general retention behaviour there are some model compound pairs the T_M values of which can be explained only by special intramolecular effects. For example, all hydroxylation products, which may form an intramolecular hydrogen bond like *o*-nitrophenol, salicylic acid or salicylic aldehyde (formula 5), have a much more positive T_M than the corresponding isomeric *m*- or *p*-products. The intramolecular formation of the hydrogen bond decreases the interaction and the stabilisation with the solvent molecules. Similarly the α-hydroxylation of *N*-heteroaromatic compounds like quinoline (formula 6) is characterised by much higher T_M values than the hydroxylation in other positions. Obviously, the tautomeric carbonylamide structure generally preferred is not *N*-protonated and, therefore, is not as hydrophilic as the other basic hydroxyquinolines. Generally, a deviating behaviour is to be expected, if the metabolization occurs in an *o*-position of another substituent (see formulae 5 and 6, Scheme 1, a and b).

Although there is a strong effect of the unchanged part of the molecule, the T_M regions of the different metabolization reactions obtained from the model compounds together with the general structural effects on k' shown above should be a helpful tool to distinguish for a given substance between two or



Scheme 1. Formulae 5 and 6.

more possible metabolites on the basis of the known metabolization behaviour. In ambiguous cases that pair of the various model compounds should be compared which has a structure most similar to the drug under investigation.

3.2. Verification of real drug/metabolite pairs

Since drugs in many cases provide a more complicated steric structure or more functional groups than the relatively simple model compounds chosen above, it was examined with some real drug/metabolite pairs whether the regions of T_M values are valid also in the practical application. The results are shown in Table 3 and the differences in the T_M regions are also included in Fig. 1.

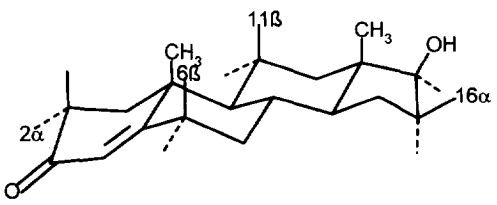
In most cases T_M of the drug/metabolite pairs is within or close to the limits found for the model compounds, but also for these compounds a strong effect of the unchanged part of the molecule was found as it is particularly obvious for the hydroxylation of saturated C-atoms (reaction 5 of Tables 1 and 2). If the unchanged molecule part is very lipophilic, as in the case of the steroids, the addition of the OH-group diminishes the retention time very strongly, whereas in the case of the more polar benzodiazepines the addition of the 3-OH-group leads to a smaller decrease of T_M. Also, different positions of the hydroxy group in the same molecule may lead to a very different shift of the retention time as shown for the four hydroxysubstituted testosterones (see structural formula 7), where the 6β- and 16α-hydroxymetabolites have a significantly more negative T_M than the 2α- and the 11β-isomers (see formula 7, Scheme 2). A similarly strong effect of the steric position of the OH-group of steroids was described by DiBussolo and Ness [24]. A possible explanation for the smaller interaction of the 2α- and 11β-isomers with the mobile phase could be that there is an intramolecular hydrogen bond between the 2α-OH and the 3-CO group, and that the 11β-OH-group is shielded by the two axial CH_3 groups in the positions 10 and 13.

In the case of the *N*-demethylation reaction (reaction 8 of Tables 1 and 3), which is very often found in drug metabolism, the T_M values are in most cases close to or slightly above the upper limit of the region obtained at the model compounds. As a reason, the larger size of the unchanged part of the molecules in comparison to the model compounds may lead to a smaller effect of the missing lipophilic methyl group on the solvation and on k'. The large structural difference between the model compounds and the real drug/metabolite pairs should be also the

Table 3
Capacity factors k' and $T_M = \log(k'_M/k'_D)$ of some drug/metabolite pairs

Drug/Metabolite	k'_D	k'_M	T_M	Model reaction Nr.[a]	T_M region
Estradiol/Estron	4.75	8.24	0.239	2	0.16 to 0.32
Prednisolone/Prednisone	1.32	1.53	0.066		
Testosterone/2α-Hydroxytestosterone	8.86	2.43	−0.562	5	−0.82 to −0.08
Testosterone/6β-Hydroxytestosterone	8.86	1.62	−0.739		
Testosterone/11β-Hydroxytestosterone	8.86	2.30	−0.586		
Testosterone/16α-Hydroxytestosterone	8.86	1.46	−0.782		
Estradiol/Estriol	4.75	1.45	−0.516		
Metamphetamin/Ephedrin	0.497	0.277	−0.254		
Delorazepam/Lorazepam	5.19	3.24	−0.20		
Diazepam/Temazepam	6.22	4.35	−0.156		
Flunitrazepam/3-Hydroxyflunitrazepam	5.33	3.04	−0.243		
Nordiazepam/Oxazepam	3.24	2.71	−0.077		
Alprazolam/α-Hydroxyalprazolam	4.30	2.47	−0.244		
Triazolam/α-Hydroxytriazolam	5.00	2.59	−0.286		
Salicylic acid/Gentisic acid	1.71	0.66	−0.415	6	−0.55 to −0.19
Metamphetamine/Pholedrine	0.497	0.219	−0.356		
Propafenone/5-Hydroxypropafenone	3.96	1.69	−0.371		
Δ^9-THC/Δ^9-THC-COOH[b]	7.32	2.38	−0.487	7	−0.91 to −0.61
Ephedrine/Norephedrine	0.277	0.235	−0.071	8	−0.33 to −0.11
Methadone/Normethadone	4.88	3.81	−0.107		
Tilidine/Nortilidine	1.40	1.10	−0.104		
Diphenhydramine/Nordiphenhydramine	2.40	1.99	−0.081		
Doxepine/Desmethyldoxepine	2.58	2.23	−0.063		
Amitriptyline/Nortriptyline	4.62	3.89	−0.075		
Clomipramine/Desmethylclomipramine	6.40	5.40	−0.073		
Imipramine/Desipramine	3.62	3.08	−0.070		
Clozapine/Desmethylclozapine	0.732	0.501	−0.165		
Metamphetamine/Amphetamine	0.497	0.389	−0.106		
3,4-Methylenedioxymethamphetamine/ 3,4-Methylendioxyamphetamine	0.462	0.366	−0.101		
Amiodarone/*N*-Desethylamiodarone[b]	3.66	2.50	−0.166	9	−0.25 to −0.15
Propafenone/*N*-Despropylpropafenone	3.96	1.48	−0.427	10	−1.40 to −0.37
Diazepam/Nordiazepam	6.22	3.24	−0.284	11	−0.30 to −0.12
Clobazam/Norclobazam	5.18	3.14	−0.217		
Flunitrazepam/Desmethylflunitrazepam	5.33	3.43	−0.191		
Coffeine/Theobromine	0.485	0.281	−0.237		
Codeine/Morphine	0.193	0.123	−0.196	12	−0.63 to −0.24
Hydrocodone/Hydromorphone	0.366	0.189	−0.287		
Thiopental/Pentobarbital	5.04	2.41	−0.321	19	−0.38 to −0.05
Parathione/Paraoxone	14.61	5.44	−0.429		
Flunitrazepam/7-Aminoflunitrazepam	5.33	1.09	−0.69	20	−1.41 to −0.50
Desmethylflunitrazepam/7-Aminodes-methylflunitrazepam	3.43	0.531	−0.811		
Cocaine/Benzoylecgonine	1.21	0.578	−0.322	22	−0.62 to −0.30
Methylsalicylate/Salicylic acid	5.26	1.71	−0.488		
Cocaethylene/Benzoylecgonine	2.10	0.578	−0.561	23	−0.66 to −0.44
Ethylsalicylate/Salicylic acid	8.77	1.71	−0.710		
Heroin/6-Monoacetylmorphine	0.758	0.408	−0.269	24	−0.62 to −0.53
6-Monoacetylmorphine/Morphine	0.408	0.166	−0.391		
17α-Hydroxyprogesterone acetate/ 17α-Hydroxyprogesterone	2.55	1.62	−0.198		
Prednisolone-21-acetate/Prednisolone	4.20	1.32	−0.503		
Acetylsalicylic acid/Salicylic acid[c]	1.12	1.71	0.182		

[a] Compare Table 1.
[b] Measured in a changed mobile phase with an acetonitrile–phosphate buffer ratio of 1.67:1.00 (v/v).
[c] Effect of the intramolecular hydrogen bond, cf. formula (5).



Scheme 2. Formula 7.

reason for the less negative T_M values of heroin and 6-monoacetylmorphine, which are protonated and, therefore, are relatively hydrophilic under the conditions of the measurement. Because of these differences between the model compounds and the real drugs in the practical applications for metabolite identification, the data of Table 3 as well as of Table 1 were used with the provision of the best structural similarity to the compound under investigation.

3.3. Examples of the application in intoxication cases

The T_M values proved to be a useful tool in order to support the identification of the metabolites in the HPLC–DAD investigation of intoxications with different drugs [25].

3.3.1. Diphenhydramine intoxication

Diphenhydramine cases are quite common in emergency and lethal intoxications [26]. Beside the drug two peaks with an almost identical UV spectrum are frequently found in the blood extracts with the following HPLC data:

Diphenhydramine: $k' = 2.40$.
Peak 1: $k' = 1.99$; $T_M = -0.081$.
Peak 2: $k' = 6{,}46$; $T_M = 0.429$.

The metabolization scheme of diphenhydramine [27,28] is shown in Fig. 2. The main metabolites are nordiphenhydramine formed by demethylation (reaction 8) and diphenylmethoxyacetic acid formed by desamination (removal of the $N(CH_3)_2$ group analogous to reaction 18) and subsequent oxidation to the carboxylic acid (reaction 1). In order to find out which peak belongs to which of the two main metabolites, the T_M values are predicted from the model substances:

Nordiphenhydramine: $T_{M.8} = -0.33$ to -0.11; because of the lipophilic character of the residual molecule a value at the positive side of this region is expected.

Diphenylmethoxyacetic acid: Several steps are necessary for the estimation of T_M. In order to get a more definite result not the whole region but substance pairs with a structure similar to the drug are used. In this case *N,N*-dimethylbenzylamine/*N*-methylbenzylamine/benzylamine and β-phenylethylamine/phenylacetic acid are chosen (reaction 16, cf. Table 1):

$$T_M = T_{M.8} + T_{M.8} + T_{M.16}$$
$$= -0.120 - 0.109 + 0.738 = 0.509$$

Comparison of these estimated data with the T_M values of the peaks leads to the result that the peak 1 should be nordiphenhydramine and the peak 2

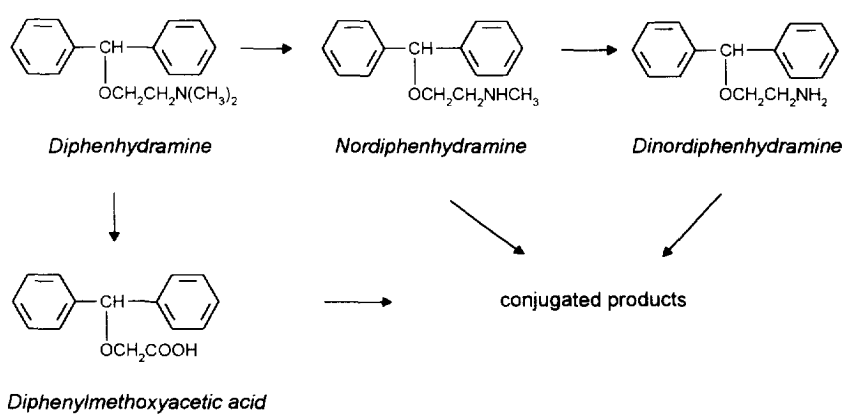


Fig. 2. Metabolization scheme of diphenhydramine according to Refs. [27,28].

diphenylmethoxyacetic acid. This was later confirmed via the authentic compounds.

3.3.2. Propafenone intoxications

Propafenone intoxications are up to now rather rare. In three cases beside the parent drug two peaks were observed in the chromatograms with the following data:

Propafenone: $k' = 3.96$.

Peak 1: $k' = 1.48$; $T_M = -0.427$; UV spectrum similar to the drug.

Peak 2: $k' = 1.69$; $T_M = -0.371$; UV spectrum different from the drug.

In the metabolization scheme [27] beside others *N*-despropylpropafenone and 5-hydroxypropafenone are described as the main metabolites (Fig. 3). For the prediction the reactions (10, *i*-propyl compounds) and (6, aromatic hydroxylation) are suitable. For the hydroxylation the substance pairs phenol/hydroquinone and acetophenone/*m*-hydroxyacetophenone (cf. Table 1) are relatively similar to the drug/metabolite structure.

N-Despropylpropafenone: $T_M = -0.46$ to -0.37.

5-Hydroxypropafenone: $T_M = -0.46$ to -0.24.

It follows from the comparison with the peak data that T_M of both peaks is in the region of the corresponding metabolization reactions. A discrimination between both by the T_M values is not possible because of the small difference between them. However, since in contrast to the removal of the *N*-propyl group the aromatic hydroxylation leads to a typical change of the UV spectrum, peak 2 can be attributed to the 5-hydroxymetabolite and peak 1 to the *N*-despropyl product.

This example shows that the applicability of the method as a single tool is limited, and that always a possible change of the UV spectrum should be considered.

3.3.3. Methaqualone intoxication

In this case the subject ingested an unknown amount of the drug. The chromatogram of the methylene chloride extract at pH 9.0 of the blood sample collected 17 h after ingestion is shown in Fig. 4. Beside the drug and the standard compound (MPPH) six peaks, M1–M6, can be attributed to methaqualone metabolites. The DAD-UV spectra corrected with respect to the background are shown in Fig. 5. The spectra of M2, M4 and M6 are sufficiently resolved for a detailed interpretation, whereas that of M1 and M5 are disturbed by noise, and that of M3 (not shown in Fig. 5) enabled observation of only a rough similarity to the drug spectrum.

According to the metabolization scheme of methaqualone [27,28] given in Fig. 6 the essential metabolization reactions are aromatic or aliphatic hydroxylations, and the metabolites in the decreasing order of the formation probability are 4′-hydroxymethaqualone, 2′-hydroxymethylmethaqualone and 3′-hydroxymethaqualone. The hydroxylation is in agreement with the T_M values, which are more

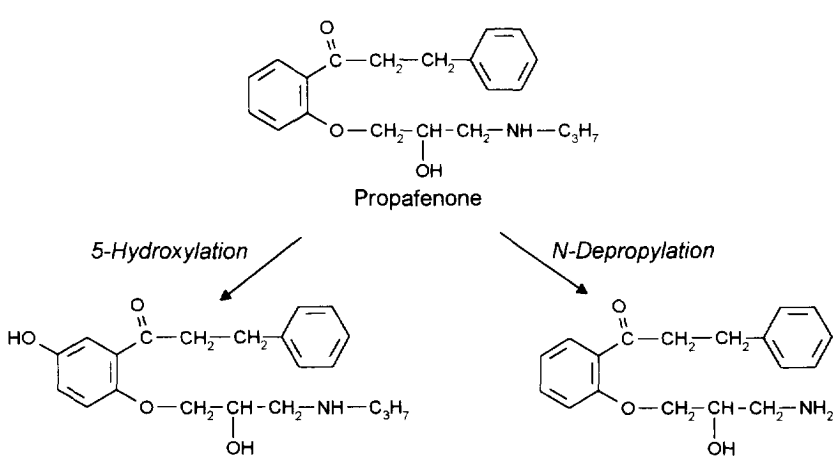


Fig. 3. Main metabolites of propafenone according to Ref. [27].

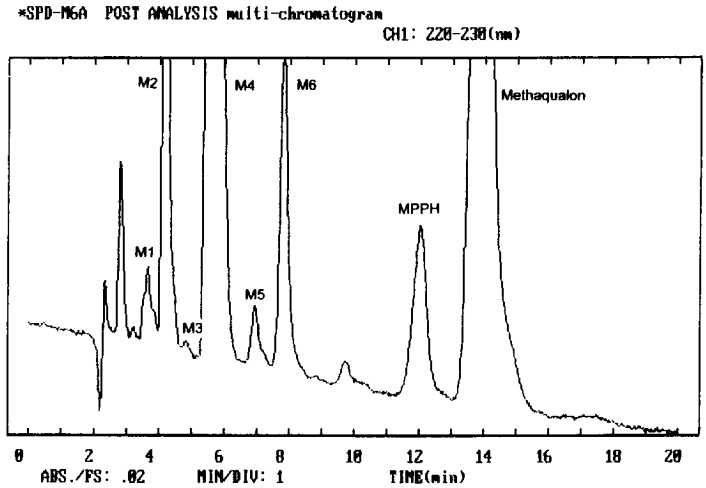


Fig. 4. HPLC–DAD chromatogram of a basic blood sample extract of a methaqualone intoxication.

negative than −0.3 for all metabolite peaks (Table 4). Since the spectrum is determined by the quinazolinone ring, the hydroxylation at the twisted 3-*o*-tolyl substituent or at the 2-methyl group should lead only to a minor alteration of the spectrum as it is found for the spectra given in Fig. 5. On the other

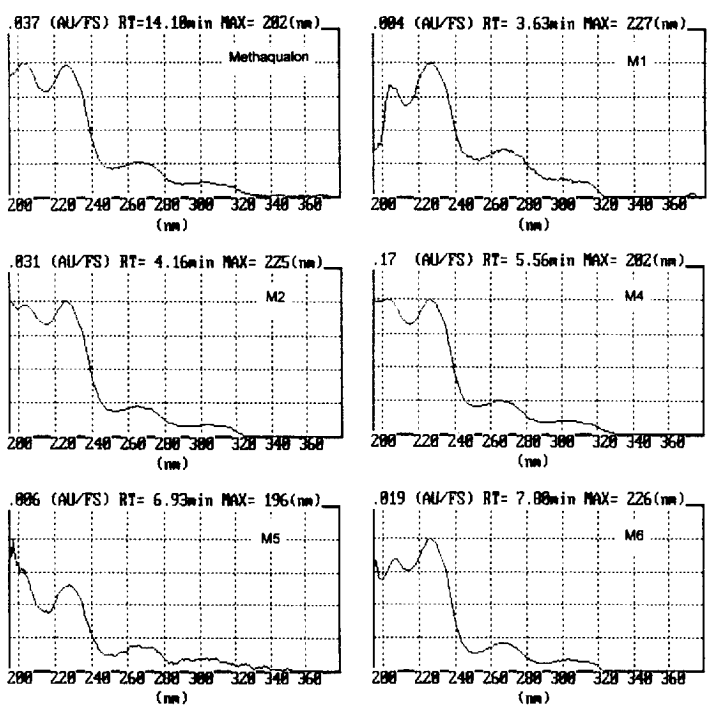


Fig. 5. DAD-UV spectra of methaqualone and five of its metabolites obtained from the chromatogram shown in Fig. 4. The spectral differences at short wavelengths may be caused by an incorrect background compensation at small concentrations.

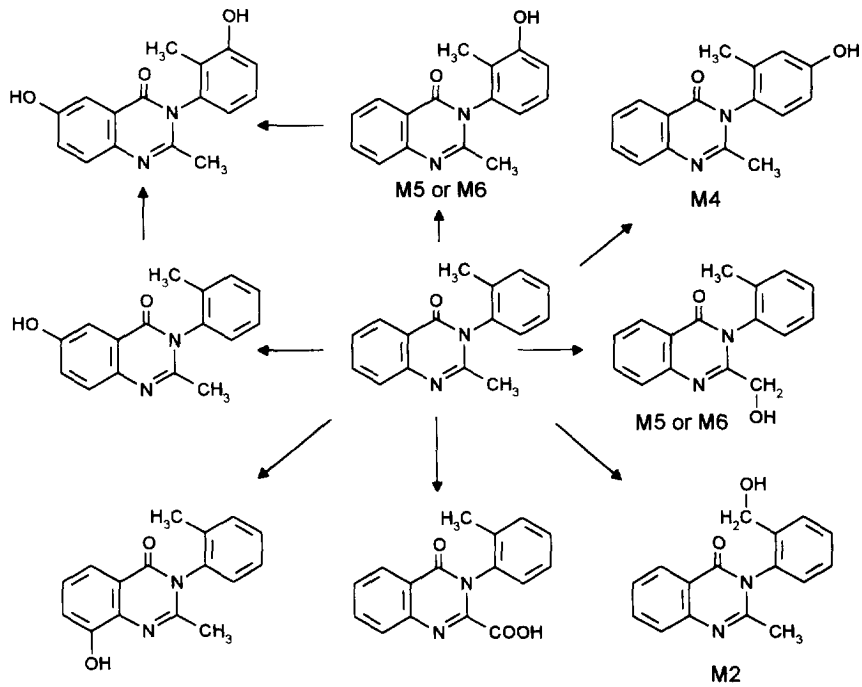


Fig. 6. Metabolization scheme of methaqualone according to Refs. [27,28].

hand, after hydroxylation at the benzene ring of the quinazoline molecule a stronger change of the spectrum should be observed.

From a closer consideration of the T_M values it should also partly be possible to distinguish between the hydroxylation products. A proposal for the peaks is given in Table 4. Since for the alkylbenzenes the hydroxylation in the side group always leads to a clearly more negative T_M than in the benzene ring (compare toluene and ethylbenzene in Table 1, reactions 5 and 6) the 2′-hydroxymethyl metabolite (attributed to M2) should have a more negative T_M than the 4′-hydroxy metabolite (M4). On the other hand, the 2-hydroxymethyl metabolite can be stabilised by an intramolecular hydrogen bond and should have a less negative T_M (M5 or M6). A less negative T_M for the 3′-hydroxymetabolite could be explained on the other hand by an ortho-effect as it is found to

Table 4
HPLC–DAD data of methaqualone and its metabolites

Substance	k'	T_M	Concentration (μg/ml)[a]	Structure proposal
Methaqualone	4.81	0.00	6.2	–
M1	0.385	−1.097	0.1	Not possible
M2	0.655	−0.866	1.5	2′-Hydroxymethylmethaqualone
M3	0.847	−0.755	0.01	Not possible
M4	1.19	−0.605	13	4′-Hydroxymethaqualone
M5	1.69	−0.454	0.1	2-Hydroxymethyl- or 3′-hydroxymethaqualone
M6	2.12	−0.356	0.65	2-Hydroxymethyl- or 3′-hydroxymethaqualone

[a] Because of the almost identical spectra the concentrations of the metabolites were estimated from the peak areas using the calibration factor of the unchanged drug (corrected by the quotient of the molecular masses, 1.064) as well as the extraction yield of the unchanged drug.

a smaller degree for the formation of the isomeric hydroxytoluenes from toluene (Table 1).

It follows from these examples that besides the similarity of the UV spectra also the shift of the retention time can contribute to the peak identification in toxicological analysis using HPLC–DAD, but, since the structure–k' relationship is very complicated, and since the T_M regions of the different metabolization reactions are rather broad and overlap each other, the results have only a provisional character. For an exact identification the comparison with the authentic substance or the use of more sophisticated methods like HPLC–MS are necessary.

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