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Characterization of a novel diclofenac metabolite in human urine by capillary gas chromatography–negative chemical ionization mass spectrometry

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Abstract

A sensitive analytical method was developed to characterize diclofenac metabolites in small amounts of body fluids. Desalted and lyophilized urine samples were extracted with supercritical carbon dioxide directly or after acidic hydrolysis. The extracts were derivatized with N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide. The derivatives were separated by capillary gas chromatography and identified by negative chemical ionization mass spectrometry. Full mass spectra were obtained at a level of $1 \cdot 10^{-9}$ g/ml. With direct extraction, the metabolites could be analysed in one step as open-chained acids and as (cyclic) oxindoles. By acidic hydrolysis the conjugates were transformed to the oxindoles. With both methods, a new main metabolite, [2-[(2,6-dichloro-4-hydroxy-3-methoxyphenyl)amino]phenyl]acetic acid, was identified. The mechanism of its formation is discussed.

Keywords: Diclofenac

1. Introduction

Almost two decades ago, the major metabolites of the anti-inflammatory drug diclofenac were isolated from urine by liquid chromatography. Spectroscopic and chemical methods served to elucidate their structures [1]. Aromatic hydroxylation and conjugation, primarily with glucuronic acid, were found to be predominant. Methods for the measurement of diclofenac and its phenolic metabolites in body fluids were later on reported by several authors (e.g., [2–8]). Some of these methods are based on GC, some others on HPLC. Often they comprise a cleavage of conjugates under strongly alkaline conditions (1–5 *M* NaOH, 70–80°C) prior to measurement.

For pharmacokinetic studies with diclofenac in humans, capillary gas chromatography (GC) combined with electron-capture detection (ECD) is a suitable approach [2–4,9]. In this procedure diclofenac and its open-chained phenolic metabolites are converted to the corresponding oxindoles by extractive alkylation. Oxindoles are also formed in another approach through dehydration and cyclization with pentafluoro-propionic anhydride [10], or with sulfuric acid and 2,2,2-trifluoro-ethanol [11]. The latter methods include a gas chromatography–negative ion chemical ionization mass spectroscopy

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(GC–NICI-MS) examination. In single ion monitoring mode the sensitivity lies between 2 and 200·10^{-12} g/ml of body fluid.

With the available methods it is possible to determine diclofenac and the known metabolites in a quantitative, reproducible and sensitive manner. However, these methods have not been designed to characterize complex metabolite mixtures in a qualitative way, or to detect unidentified products of biotransformation. We felt that such a more qualitative approach would be useful, because an important link is still missing in the metabolic pathways of diclofenac in humans (see Section 3.6).

Therefore, we decided to develop a sensitive and selective assay for the determination of diclofenac and its metabolites in body fluids. The new procedure should allow to measure open-chained acids and oxindoles in one step (which was not possible so far). GC–NICI-MS was the method of choice, because it provides both high separation power and structural information on individual GC peaks. Freeze-drying of samples and extraction with supercritical carbon dioxide should help to minimize problems of chemical instability and endogenous background. For the cleavage of the conjugates we chose acidic conditions. Further important steps of the procedure include: derivatization with N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide (MT-BSTFA) [12,13] at room temperature, separation on a tailor made glass capillary column and characterization of all metabolites by NICI-MS.

In single ion monitoring the sensitivity lies around 10^{-12} g/ml of body fluid.

2. Experimental

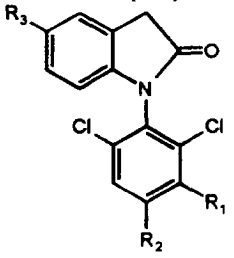
2.1. Reference compounds

Reference compounds related to diclofenac were synthesized as described previously [14]. The oxindoles used are listed in Table 1 and the open-chained acids are listed in Table 2.

2.2. Subject, dose and sample collection

The study was carried out in one healthy male volunteer aged 35, height 183 cm and weight 72 kg. The volunteer received a single oral dose of 100 mg of diclofenac sodium (Voltaren, Ciba-Geigy, Basel, Switzerland). The urine was collected in three fractions (0–12 h, 12–24 h and 24–48 h) after dosing.

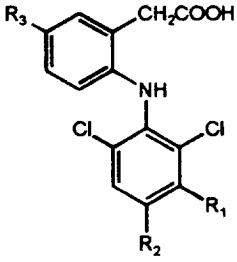
Table 1
Substituted N-phenyloxindoles



Reference compound[a]	R_1	R_2	R_3	Formula	Ring-closed form of metabolite
1	H	H	H	$C_{14}H_9Cl_2NO$	I
3	H	OCH_3	H	$C_{15}H_{11}Cl_2NO_2$	
7	H	OH	H	$C_{14}H_9Cl_2NO_2$	II
8	OH	H	H	$C_{14}H_9Cl_2NO_2$	IV
9	H	H	OH	$C_{14}H_9Cl_2NO_2$	III
12	OCH_3	OH	H	$C_{15}H_{11}Cl_2NO_3$	VII
14	OH	OCH_3	H	$C_{15}H_{11}Cl_2NO_3$	VI
18	OH	OH	H	$C_{14}H_9Cl_2NO_3$	
19	H	OH	OH	$C_{14}H_9Cl_2NO_3$	V

[a] Compound numbering according to the order of the reference compounds in Fig. 3.

Table 2
Substituted 2-(phenylamino)phenylacetic acids



Reference compound[a]	R_1	R_2	R_3	Formula	Metabolite
2	H	H	H	$C_{14}H_{11}Cl_2NO_2$	I
4	H	H	OCH_3	$C_{15}H_{13}Cl_2NO_3$	
5	H	OCH_3	H	$C_{15}H_{13}Cl_2NO_3$	
6	OCH_3	H	H	$C_{15}H_{13}Cl_2NO_3$	
10	H	H	OH	$C_{14}H_{11}Cl_2NO_3$	III
11	H	OH	H	$C_{14}H_{11}Cl_2NO_3$	II
13	OH	H	H	$C_{14}H_{11}Cl_2NO_3$	IV
15	OCH_3	OH	H	$C_{15}H_{13}Cl_2NO_4$	VII
16	OH	OCH_3	H	$C_{15}H_{13}Cl_2NO_4$	VI
17	H	OCH_3	OH	$C_{15}H_{13}Cl_2NO_4$	
20	H	OH	OH	$C_{14}H_{11}Cl_2NO_4$	V

[a] Compound numbering according to the order of the reference compounds in Fig. 3.

The samples were frozen at −20°C. The 0–12 h fraction was used for further examination.

2.3. Sample preparation

2.3.1. Solid phase extraction of non-hydrolysed urine

Three commercially available cartridges (prepacked with Chromabond C_{18}, Macherey-Nagel, Düren, Germany) were prewashed with 2 ml of methanol and twice with water (2×2.2 ml). Urine (5 ml) was applied to each cartridge by syringe injection, followed by washing with 6 ml of water. The metabolites were eluted with 6 ml of methanol, and the combined methanol eluates were concentrated in vacuo. The residue, a brown powder, was extracted with supercritical CO_2.

2.3.2. Acidic hydrolysis

Concentrated hydrochloric acid (3.6 ml) was added to 5-ml urine samples. The resulting 5 *M* HCl solution was stirred for 1 h at 80°C under argon atmosphere. The solution was cooled and lyophilized. The residue was dissolved in 5 ml of water. The solution was brought to pH 5–6 with 2 *M* $KHCO_3$ and was lyophilized. The residue, a brown powder, was subjected to extraction with supercritical carbon dioxide.

2.3.3. Analytical-scale supercritical fluid extraction (SFE)

Supercritical fluid extraction of the desalted and lyophilized urine was performed on a home-made extractor consisting of an extraction cell, a high-pressure syringe pump (Model 8500, Varian, Palo Alto, CA, USA), which has been modified for pressure control [15] and a gas chromatograph. The pressure of the supercritical extraction medium CO_2 was programmed by a Hewlett-Packard 75c microcomputer. The syringe pump was connected to the GC oven (Model 4160, Carlo Erba, Milan, Italy) via a stainless-steel transfer line.

The lyophilized urine samples were mixed with crystalline sodium chloride and transferred into the extraction cell. The cell was placed into a GC oven, heated to the extraction temperature of 100°C and pressurized with 35 MPa CO_2 for 2 h. The SFE flow-rates of about 5 ml/min CO_2 (measured at the

pump) were controlled using pieces of fused silica tubing of various length with 0.025 mm I.D., at the outlet of the system as restrictors. The extracted metabolites were collected by inserting the heated restrictor outlet into a vial containing about 1 ml of acetonitrile. Prior to the GC–MS analyses, the extracts were derivatized.

2.3.4. Derivatization

For the derivatization of diclofenac and its metabolites, aliquots of one third of the SFE extracts were carefully vacuum centrifuged. The dry residue was dissolved in a mixture of 50 μl of acetonitrile and 50 μl of MTBSTFA. The reaction mixture was kept at room temperature for 30 min and the excess of MTBSTFA was removed by concentrating the solution to dryness at room temperature under vacuum. The residue was dissolved in acetonitrile to a final volume of 20 μl. An aliquot of 1 μl was used for GC–MS analysis.

2.4. Instrumentation

2.4.1. Gas chromatography

The gas chromatographic separation was performed using a 4160 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a constant flow/constant pressure regulator (CP/CF 516, Carlo Erba) and a 20 m×0.3 mm glass capillary column coated with SOP-50 (CH_3O-terminated poly(diphenyl/dimethyl)siloxane copolymer) or SOP-50-TF (CH_3O-terminated poly(diphenyl/3,3,3-trifluoropropylmethyl)siloxane copolymer [16]. All samples were introduced by cold on-column injection. The GC oven was temperature-programmed from 40°C to 350°C with 4°C/min. Carrier gas was hydrogen.

2.4.2. Gas chromatography–mass spectrometry

The separation equipment already described above was linked by a high temperature interface [17] to a 4600 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA). The ionization technique employed was negative chemical ionization (NICI) with methane as a buffer gas and a mixture of CH_4/N_2O as reagent gas [18]. The ion source temperature was 150°C. The transfer line was kept at 350°C. The scan range was m/z 236–700.

3. Results and discussion

3.1. Supercritical fluid extraction

Analytical-scale supercritical fluid extraction is an attractive alternative to conventional solvent extraction (e.g., [19]). The amount of liquid solvent can be

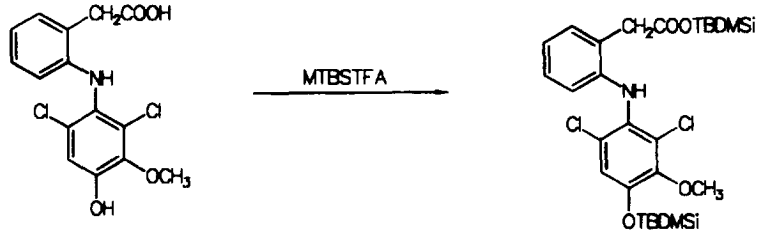


Fig. 1. Silylation of [2-[(2,6-dichloro-4-hydroxy-3-methoxyphenyl)amino]phenylacetic acid.

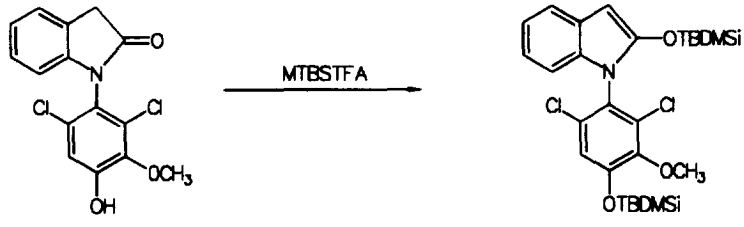


Fig. 2. Silylation of 1-(2,6-dichloro-3-methoxy-4-hydroxyphenyl)oxindole.

drastically reduced, the recoveries are comparatively good and the extraction time is short. The solvent strength of a supercritical fluid can easily be controlled by pressure and temperature.

Diclofenac and its metabolites could be extracted from lyophilized urine very selectively (see Section 3.5). The amount of urine needed could be reduced to 1–5 ml. Owing to the good solubility of diclofenac in pure supercritical carbon dioxide, the addition of modifiers was not necessary.

3.2. Derivatization

The main criteria for the derivatization of diclofenac and its metabolites were: (a) The derivatization reaction should take place under neutral conditions to avoid side reactions such as oxindole formation, and should be performed at room temperature to avoid thermally induced oxindole formation; (b) the derivatives should be volatile, should provide characteristic mass spectra at high sensitivity and should be resistant against hydrolysis.

Thus, MTBSTFA was selected as an appropriate silylating agent for the transformation of diclofenac and its metabolites to the corresponding TBDMSi derivatives. For the open-chained acids, the reaction followed the scheme shown below (e.g., silylation of reference compound 15; Fig. 1).

In the case of the oxindole derivatives, enolisation occurred followed by introduction of a second TBDMSi-group (e.g., silylation of reference compound 12; Fig. 2).

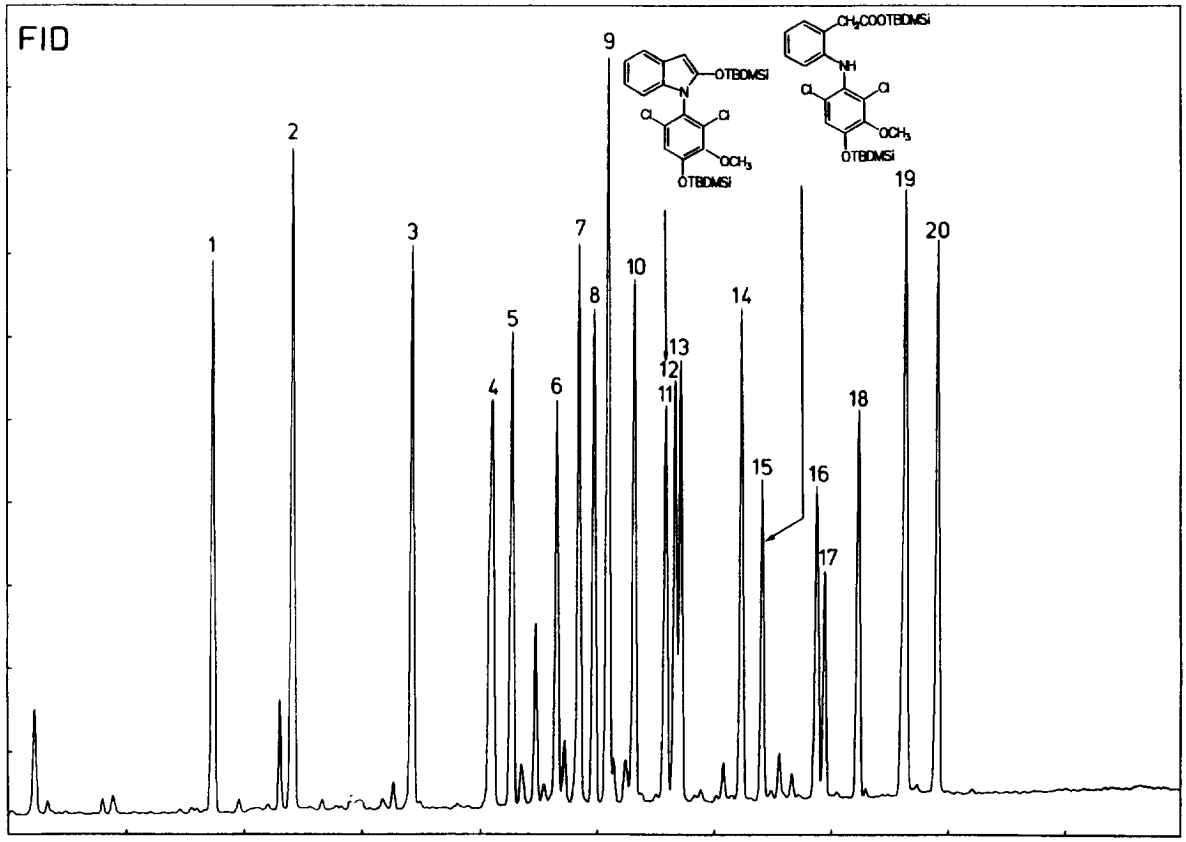


Fig. 3. FID chromatogram of all reference compounds after derivatization with MTBSTFA. The peak numbers correspond to the list of compounds given in Table 1 and Table 2. Column: 20 m×0.3 mm glass capillary coated with SOP-50-TF (CH_3O-terminated poly(diphenyl)/3,3,3-trifluoropropylmethyl)siloxane copolymer [16]. Temperature: 40°C to 350°C with 4°C/min. Carrier gas: hydrogen.

3.3. Optimization of the GC method

All available hydroxy/methoxy substituted reference substances of diclofenac were separated as TBDMSi derivatives on a diphenyl/dimethyl and on a diphenyl/3,3,3-trifluoropropylmethyl substituted polysiloxane phase, published recently [16]. On both phases not only all silylated diclofenac acids, but also the corresponding oxindoles could be base line separated.

However, in practice the best results could be obtained on the trifluoro-propylmethyl substituted phase. The trifluoropropyl group, which is a strong electron acceptor, interacts with free electrons of the carbonyl groups. As a result, the TBDMSi-open-chained acids showed a unique shift to higher retention values, and the TBDMSi-oxindoles were eluted as clearly separated groups (Fig. 3). This retention behavior is advantageous, if diclofenac similar metabolite pattern have to be classified in complex biological matrices. The flame ionization detection (FID) chromatogram of all reference substances, i.e., open-chained acids and oxindoles, is shown in Fig. 3.

3.4. Optimization of the MS ionization method

The success of a given GC–MS method depends, among other things, on the selection of the mass spectrometric ionization method. In GC–MS we can choose between electron impact (EI) and chemical ionization (CI).

In EI- and CI/CH_4 mode, all hydroxy/methoxy substituted references showed intensive molecular ions. The EI spectra are characteristic and most of the reference isomers could be identified by EI. In CI/CH_4 mode, the mass spectra, e.g., of the important hydroxy-substituted metabolites, showed only minor differences. Therefore, particularly in biological matrices, the unambiguous identification of isomeric metabolites was only possible in combination with the accompanying GC retention values. Unfortunately, the sensitivity of EI and CI/CH_4 was only 10^{-8} g/ml of TBDMSi-diclofenac-acid or

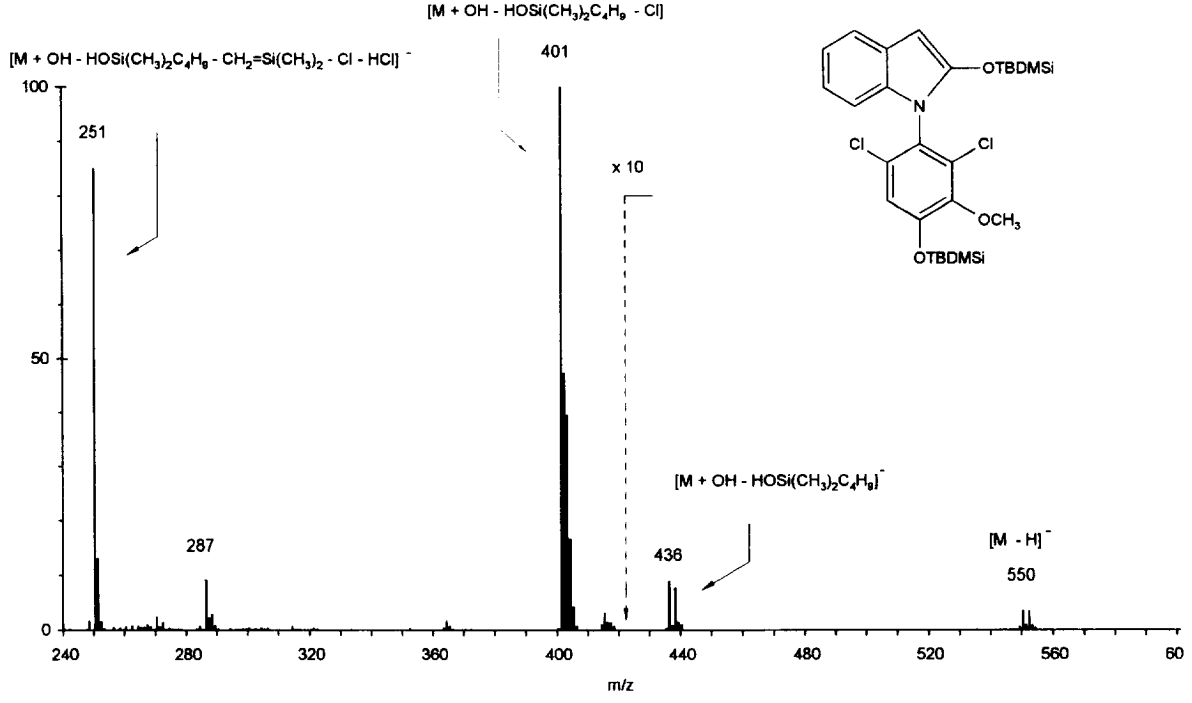


Fig. 4. Partial negative CI/CH_4/N_2O mass spectrum (*m/z* 236–700) of 1-(2,6-dichloro-3-methoxy-4-*tert.*-butyldimethylsilyloxyphenyl)-2-*tert.*-butyldimethylsilylindole.

-oxindole, and was hence unsatisfactory for the detection of trace amounts.

Significantly better sensitivity was obtained with negative chemical ionization. Both electron capture chemical ionization (ECCI) with CH_4 as a buffer gas and chemical ionization, using a mixture of CH_4/N_2O as reagent gas (formation of OH^-, [18]), were performed. In either ionization mode the formation of characteristic, dominant fragment ions, important for the identification of trace compounds, followed a uniform reaction scheme representative for all metabolites. Unfortunately, the negative CI mass spectra were interfered by intensive background ions up to *m/z* 230. For this reason the spectra were recorded over a limited scan range from *m/z* 235–700, as shown in Fig. 4 and Fig. 5.

In negative CI, only weak $[M-H]^-$ were observed. Nevertheless, all TBDMSi indoles showed dominant characteristic fragment ions at $[M+OH-HOSi(CH_3)_2C_4H_9]^-$, $[M+OH-HOSi(CH_3)_2C_4H_9-Cl]^-$, $[M+OH-HOSi(CH_3)_2C_4H_9-CH_2=Si(CH_3)-C_4H_9-Cl]^-$, $[M+OH-HOSi(CH_3)_2C_4H_9-HCl-Cl]^-$, $[M+OH-HOSi(CH_3)_2C_4H_9-CH_2=Si(CH_3)-C_4H_9-HCl-Cl]^-$, $[M^--CH_2=Si(CH_3)C_4H_9-Cl]^-$ or $[M^--2CH_2=Si(CH_3)C_4H_9-Cl]^-$. The TBDMSi acid esters showed, in addition, characteristic fragments at $[M^--CH_3OSi(CH_3)_2C_4H_9-Cl]^-$ and $[M+OH-2HOSi(CH_3)_2C_4H_9-Cl]^-$. Analogous nucleophilic substitutions of esters by OH^- or NH_2^- ions were reported independently [20,21].

The molecular mass information of unknown diclofenac metabolites could be ascertained reliably by extrapolation from these fragment ions. The diagnostically important fragment ions of all presently known diclofenac metabolites are listed in Table 3 and Table 4.

While the ECCI spectra gave no molecular ions, the negative CI spectra showed weak $[M-H]^-$ ions (Fig. 3 and Fig. 4). Nevertheless, in practice the electron capture mode was preferred, since Brønsted bases like OH^- reduce the life-time of the filament of the ion source significantly. The CI/OH^- mode was only occasionally used to verify the extrapolated molecular weights.

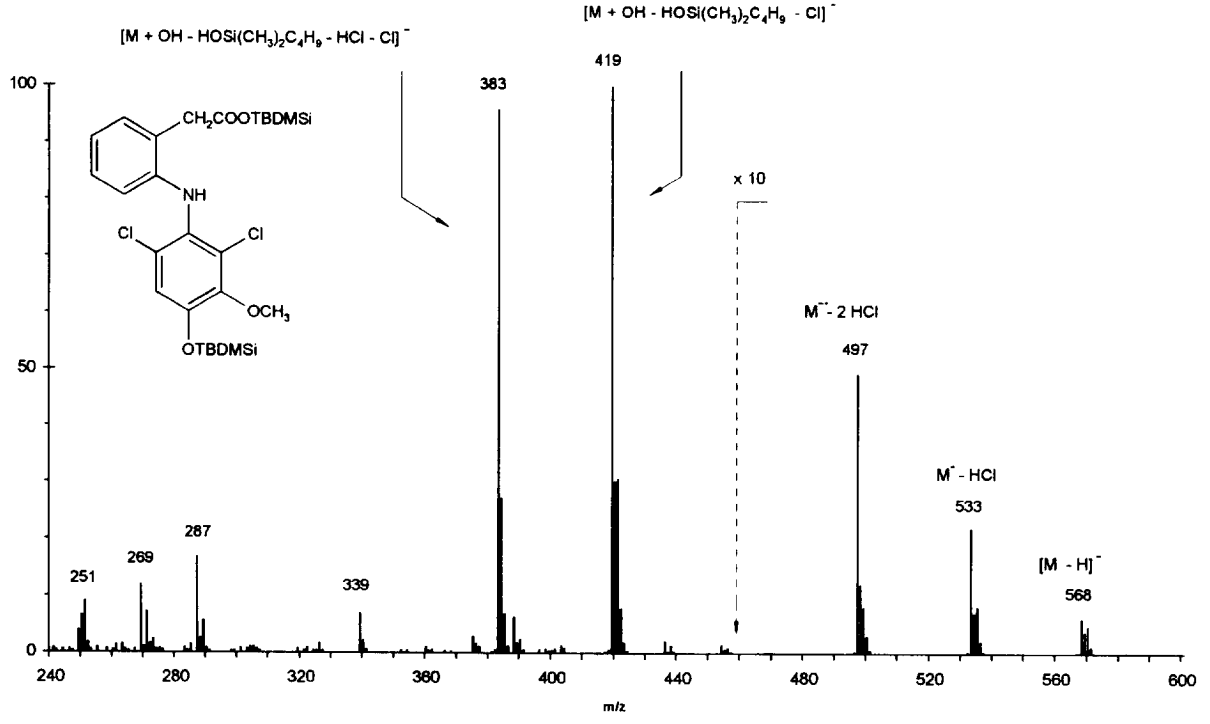


Fig. 5. Partial negative CI/CH_4/N_2O mass spectrum (*m/z* 236–700) of [2-(2,6-dichloro-3-methoxy-4-*tert.*-butyldimethylsilyloxy)-amino-]phenyl acetic acid-(*tert.*-butyldimethylsilyl) ester.

Table 3
Principal ions in the ECCI mass spectra of TBDMSi-acid esters of diclofenac metabolites II–VII[a]

Metabolite	II 4′-Hydroxy-diclofenac	III 5-Hydroxy-diclofenac	IV 3′-Hydroxy-diclofenac	V 4′,5-Dihydroxy-diclofenac	VI 3′-Hydroxy-4′-methoxy-diclofenac	VII 4′-Hydroxy-3′-methoxy-diclofenac
$[M+OH-HOSi(CH_3)_2C_4H_9-Cl]^-$	389(100)	389(100)	389(100)	519(100)	419(30)	419(100)
$[M^- -CH_3OSi(CH_3)_2C_4H_9-Cl]^-$					388(100)	
$[M+OH-HOSi(CH_3)_2C_4H_9-HCl-Cl]^-$	353(45)	353(44)		483(42)	383(30)	383(96)
$[M+OH-2HOSi(CH_3)_2C_4H_9-Cl]^-$	257(23)	257(46)				

[a] The ion abundances (%) are given in parentheses and are relative to the base peak (the relative abundances of M^- or $[M-H]^-$ ions are negligible in allcases).

The sensitivity of ECCI and negative CI was about 100 times higher than in positive CI or EI. For all examined silylated diclofenac metabolites, full spectra could be obtained at a level of 10^{-9} g/ml. The mass spectra of all reference compounds were characteristic and the isomeric diclofenac metabolites could be unequivocally classified only by means of negative CI–MS data (see Table 3 and Table 4).

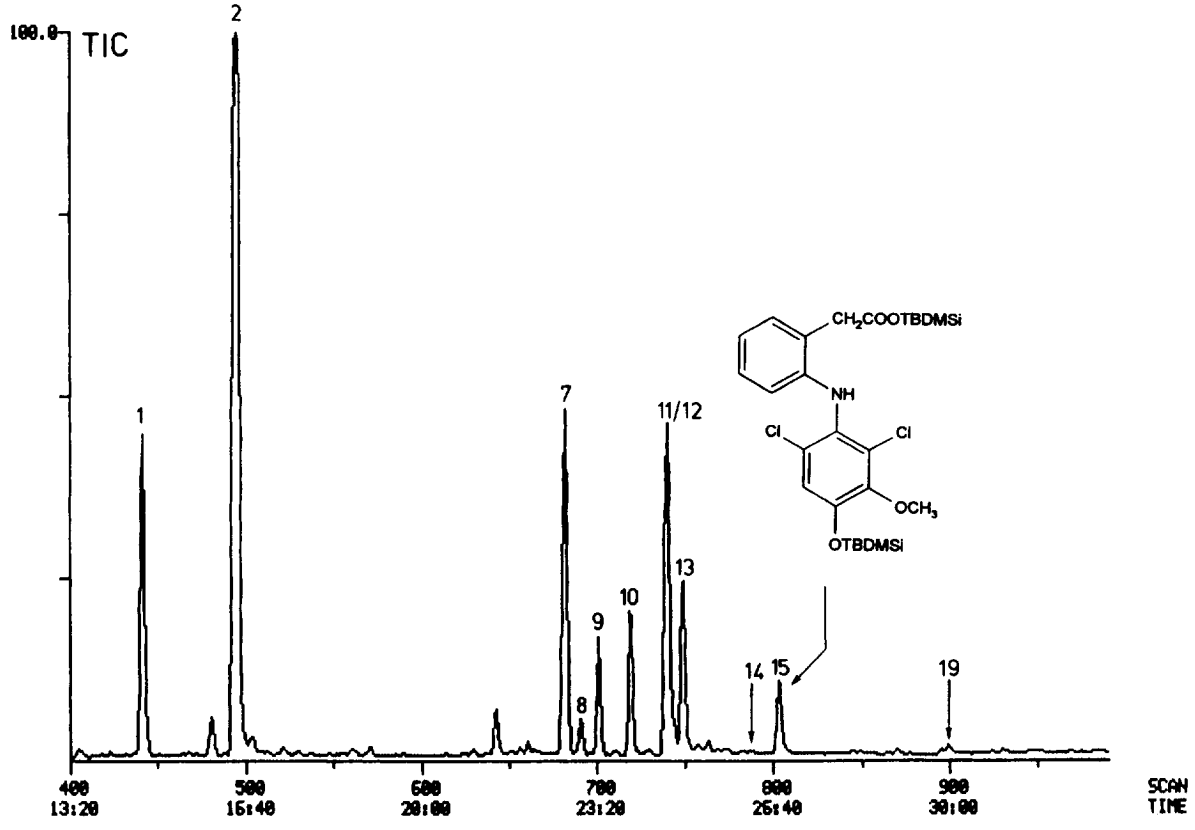


Fig. 6. TIC chromatogram of a urine sample (0–12 h) before acidic hydrolysis. The peak numbers correspond to the list of compounds given in Table 1 and Table 2. Column: 20 m×0.3 mm glass capillary coated with SOP-50-TF (CH_3O-terminated poly(diphenyl)/3,3,3-trifluoropropylmethyl)siloxane copolymer [16]. Temperature: 40°C to 350°C with 4°C/min. Carrier gas: hydrogen.

Table 4
Principal ions in the ECCI mass spectra of TBDMSi-oxindole ethers of diclofenac metabolites II–VII[a]

Metabolite	II 4′-Hydroxy-oxindole	III 5-Hydroxy-oxindole	IV 3′-Hydroxy-oxindole	V 4′,5-Dihydroxy-oxindole	VI 3′-Hydroxy-4′-methoxy-oxindole	VII 4′-Hydroxy-3′-methoxy-oxindole
$[M+OH-HOSi(CH_3)_2C_4H_9-Cl]$	371(100)		371(49)	501(100)		401(100)
$[M^- -CH_2=Si(CH_3)C_4H_9-Cl]$		372(100)	372(59)	502(96)	402(100)	
$[M+OH-HOSi(CH_3)_2C_4H_9-CH_2=Si(CH_3)C_4H_9-Cl]$	257(59)			387(43)		
$[M+OH-HOSi(CH_3)_2C_4H_9-HCl-Cl]$			335(19)			
$[M^- -2\ CH_2=Si(CH_3)C_4H_9-Cl]^-$			258(100)		288(30)	
$[M+OH-HOSi(CH_3)_2C_4H_9-CH_2=(CH_3)C_4H_9-HCl-Cl]^-$				351(25)		251(85)

[a] The ion abundances (%) are given in parentheses and are relative to the base peak (the relative abundances of M^- or $[M-H]^-$ ions are negligible in all cases).

3.5. GC–MS analysis of unconjugated diclofenac metabolites and of metabolites after acidic hydrolysis in urine

The total ion current (TIC) chromatogram of a desalted sample of lyophilized urine collected 0–12 h post-dose, extracted with supercritical carbon dioxide and derivatized with MTBSTFA, is shown in Fig. 6. All presently known diclofenac metabolites were detected, together with a new metabolite (VII, see Table 2), as open-chained acids and/or the corresponding cyclic forms. For the first time, metabolite VI (minor peak 14), previously only found in plasma samples [4], could be traced in human urine as well.

The TIC chromatogram of a urine sample, treated with HCl prior to supercritical fluid extraction and derivatization (Fig. 7), showed only the ring-closed derivatives. The acidic cleavage of the conjugated metabolites led quantitatively to the corresponding oxindoles. All metabolites known from previous studies could again be detected. The new metabolite (VII, see Table 1) was identified as major product.

The molecular mass of the new metabolite VII could be ascertained by mass spectra (see Fig. 4 and Fig. 5). The structures of the open-chained compound and the respective oxindole were confirmed by comparison of the MS and GC data with those of the silylated reference substances. They were identified as 2-[(2,6-dichloro-4-*tert.*-butyldimethyl-

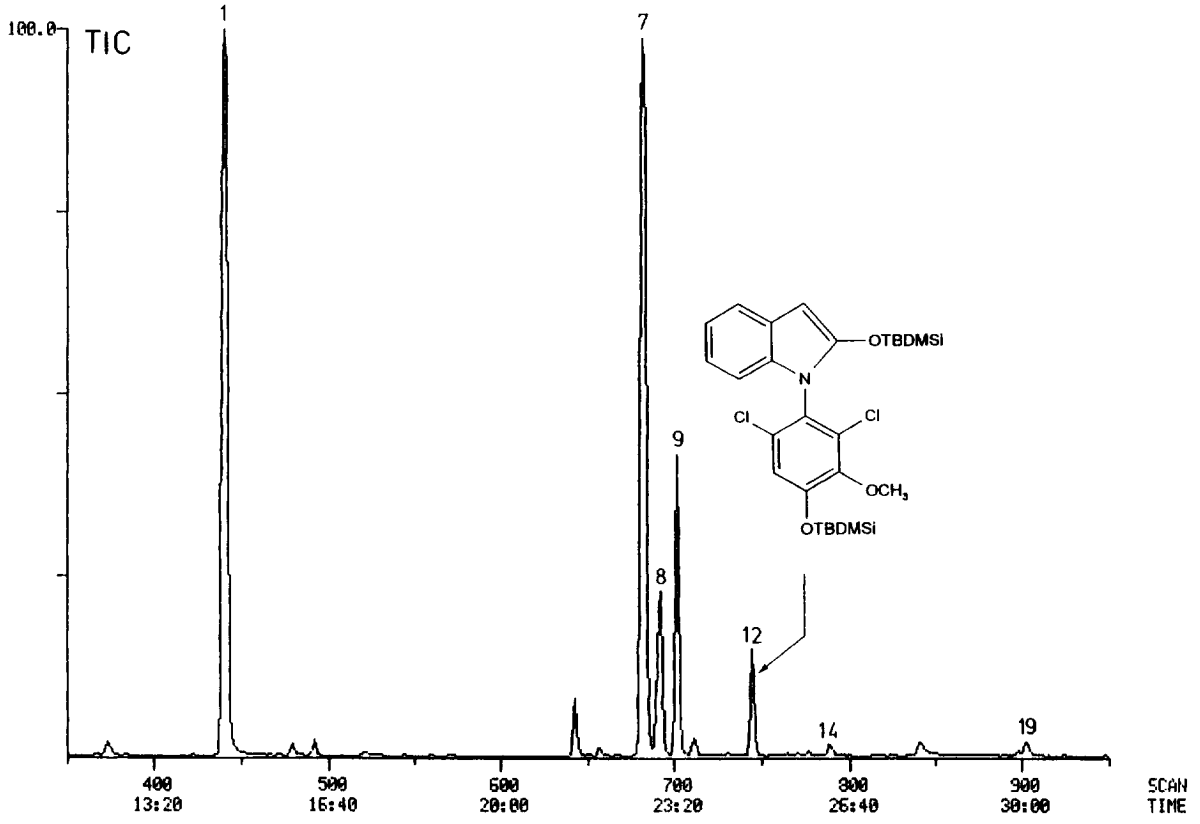


Fig. 7. TIC chromatogram of a urine sample (0–12 h) after acidic hydrolysis. The peak numbers correspond to the list of compounds given in Table 1 and Table 2. Column: 20 m×0.3 mm glass capillary coated with SOP-50-TF (CH_3O-terminated poly(diphenyl)/3,3,3-trifluoropropylmethyl)siloxane copolymer [16]. Temperature: 40°C to 350°C with 4°C/min. Carrier gas: hydrogen.

silyloxy-3-methoxy-phenyl)amino]phenyacetic acid (*tert.*-butyldimetylsilyl) ester and 1-(2,6-dichloro-3-methoxyphenyl)-2-(*tert.*-butyldimethylsilyloxy)indole.

3.6. Mechanism of the formation of diclofenac metabolites

From our earlier studies it is known that diclofenac (I; for the assignment of metabolite numbers to chemical structures see Fig. 8) is eliminated from the human body by extensive metabolism. Oxidative attacks at various positions of the phenyl rings being predominant [1,4]. The end products of these pathways include monofunctional phenols (metabolites II, III, IV) and bifunctional phenols or derivatives thereof (V, VI). Metabolite VII detected in the present study belongs to the latter group of compounds. It is noteworthy that all diclofenac metabolites, including VII, show only marginal anti-inflammatory and analgesic activities in animal models [14,22,23]

After we had identified the 3′-hydroxy-4′-methoxy derivative (VI) in a previous investigation [4], we considered the 4′-hydroxy-3′-methoxy isomer (VII) as a missing link at that time, from a theoretical point of view. From independent studies [24] it is indeed known that catechols carrying an amino function *para* to one of the hydroxyl groups preferentially undergo *meta*-methylation (as in metabolite VII), *para*-methylation being less important.

The true mechanism by which the phenolic metabolites of diclofenac are formed remains to be explored. Based on published examples on aromatic hydroxylation, however, we wish to propose a hypothetical pathway leading to single or twofold substitution in the chlorinated ring of diclofenac (Fig. 9). Oxidative attack at the second phenyl ring will follow the same basic mechanism, although in

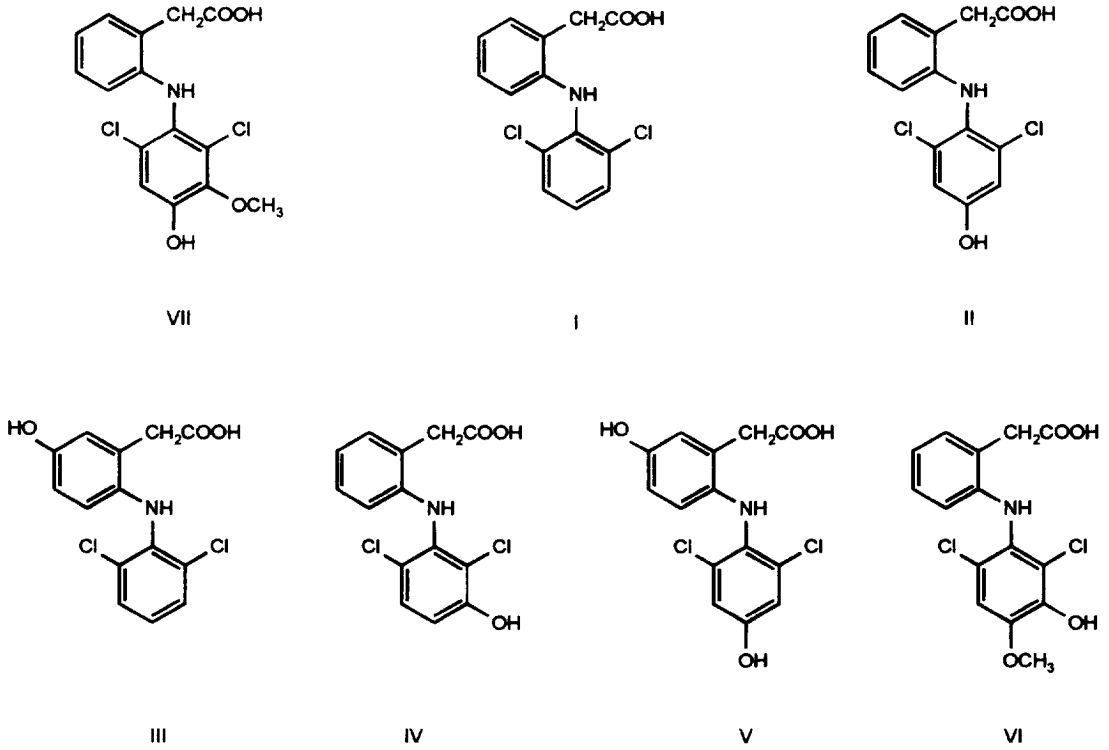


Fig. 8. Structures of diclofenac (I) and six human metabolites: 4′-hydroxy-diclofenac (II); 5-hydroxy-diclofenac (III); 3′-hydroxy-diclofenac (IV); 4′,5-dihydroxy-diclofenac (V); 3′-hydroxy-4′-methoxy-diclofenac (VI); and 4′-hydroxy-3′-methoxy-diclofenac (VII).

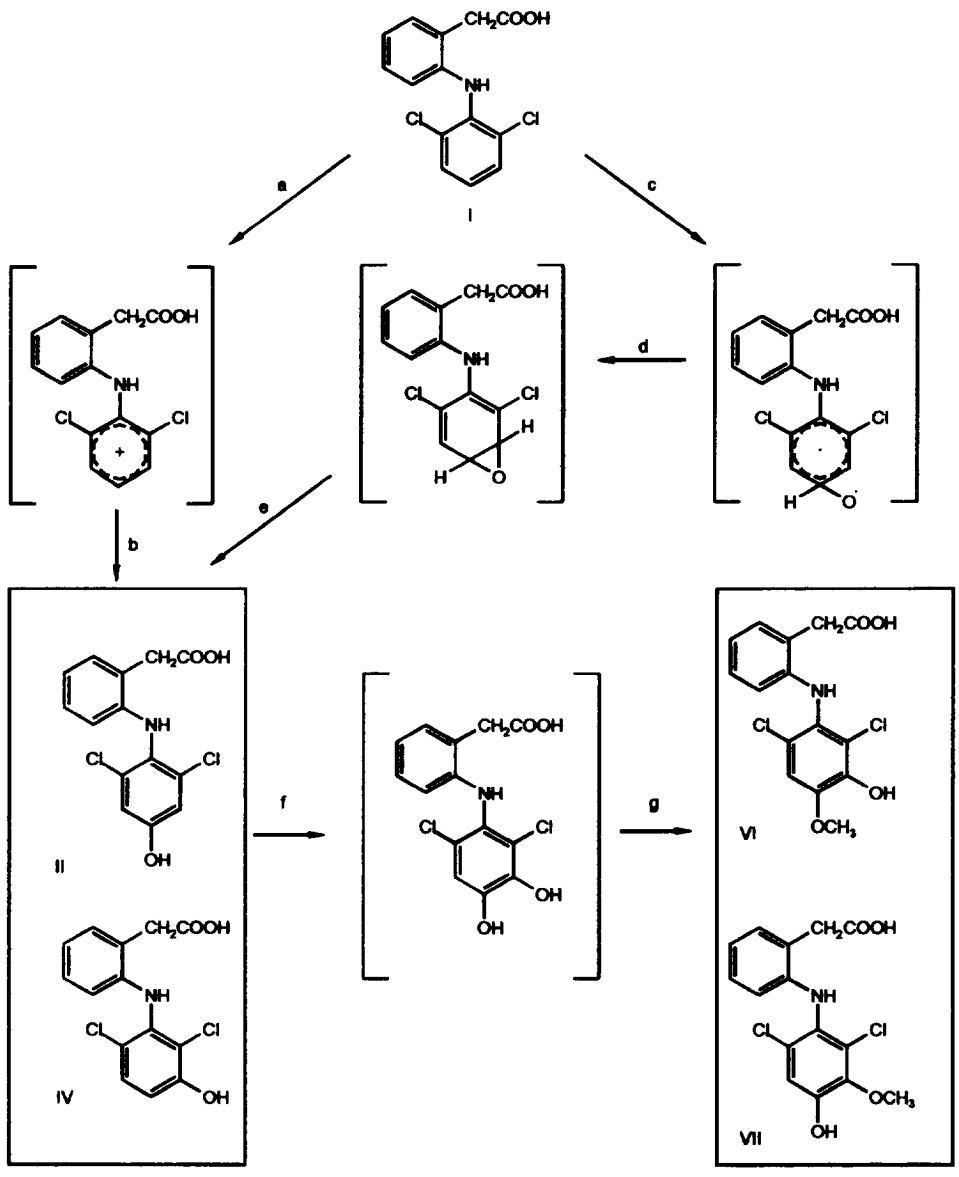


Fig. 9. Proposed mechanism of the oxidative attack at the chlorinated ring of diclofenac (the intermediates in parentheses are hypothetical).

this case only single substitution occurs (metabolite V; see Fig. 8).

The first tangible products in Fig. 9 are the monofunctional phenols II and IV. In accordance with known mechanisms [25,26], reaction (a) is one possible pathway of initial oxidation. Removal of an electron leads to a cation which yields the phenol by water-assisted deprotonation (b). Another conceivable way is reaction (c), resulting in a biradical which rearranges first to an arene oxide (d) and then to a phenol (e). Part of the phenolic metabolites II and IV undergoes hydroxylation in *ortho*-position (f). The resulting catechol is finally transformed to the hydroxy-methoxy compounds VI and VII.

Catechols may also be formed directly from arene oxides, with a dihydrodiol as an intermediate (reaction not shown in Fig. 9). This direct pathway is primarily observed in simple aromats, like bromo-

benzene. With more complex molecules, like phenytoin, catechols arise from stepwise oxidation [27].

The essential reactions in Fig. 9 are catalysed by hepatic enzymes. An isoenzyme (CYP2C) of the cytochrome P450 complex is responsible for the initial oxidative attack [27]. The ultimate conversion of the catechol intermediate into metabolites VI and VII is mediated by a catechol-O-methyltransferase, as can be inferred from general knowledge [24,28,29].

From the new findings we may conclude that metabolite VII represents the hitherto missing link in the metabolic pathways of diclofenac in humans.

4. Conclusion

The analytical procedure, consisting of acidic hydrolysis of the urine samples, supercritical fluid extraction of the lyophilized residues, transformation of the metabolites to the respective TBDMSi derivatives, separation and identification of the single compounds by means of GC-negative CI–MS resulted in the characterization of a hitherto unknown metabolite (VII) of diclofenac. The analytical method is very sensitive and is suitable for the analysis of samples with only minute levels of metabolites, as e.g., in plasma after topical application of diclofenac. It is likewise suitable, when only small sample volumes are available such as with synovial fluid or aqueous humour.

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