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Gemfibrozil and its oxidative metabolites: quantification of aglycones, acyl glucuronides, and covalent adducts in samples from preclinical and clinical kinetic studies

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Abstract

A gradient reversed-phase HPLC analysis for the direct measurement of gemfibrozil (GEM) and four oxidative metabolites in plasma and urine of humans and in tissue homogenates of rats was developed. The corresponding acyl glucuronides and the covalently bound protein adducts (in protein precipitates) were determined after liberation from the respective conjugates via alkaline hydrolysis. The limits of detection for the covalent adducts in human plasma are: 10 ng ml^{-1} (GEM), 20 ng ml^{-1} (M1), 0.5 ng ml^{-1} (M2, M4), and 5 ng ml^{-1} (M3). The method was validated with respect to selectivity, recovery, linearity, precision, and accuracy. It has been applied to the analysis of preclinical and clinical studies. Pharmacokinetic profiles of gemfibrozil, its metabolites, and covalent adducts in human plasma and rat tissue homogenates are given.

Keywords: Gemfibrozil; Aglycones; Acyl glucuronides

1. Introduction

Gemfibrozil, a fibrate lipid-regulating agent, is clinically effective in reducing serum cholesterol and triglyceride levels, decreasing LDL and increasing HDL levels [1]. Apart from new HMG CoA reductase inhibitors (e.g., atorvastatin), gemfibrozil is still a drug of choice in the treatment of hyperlipidaemias involving raised triglyceride levels [2] and has been effective in reducing the incidence of coronary heart disease (by 34%) [1]. Extensive phase-I metabolism yields at least four oxidative metabolites – three hydroxylated products (M1, M2, M4) and one dicarboxylic acid (M3) – and subsequent conjugation with glucuronic acid leads to the five respective acyl glucuronides (Fig. 1) [3–6]. Renal excretion is the most important elimination pathway for the respective carboxylic acids as well as such glucuronides in man. Accordingly, a total of 60–70% of a

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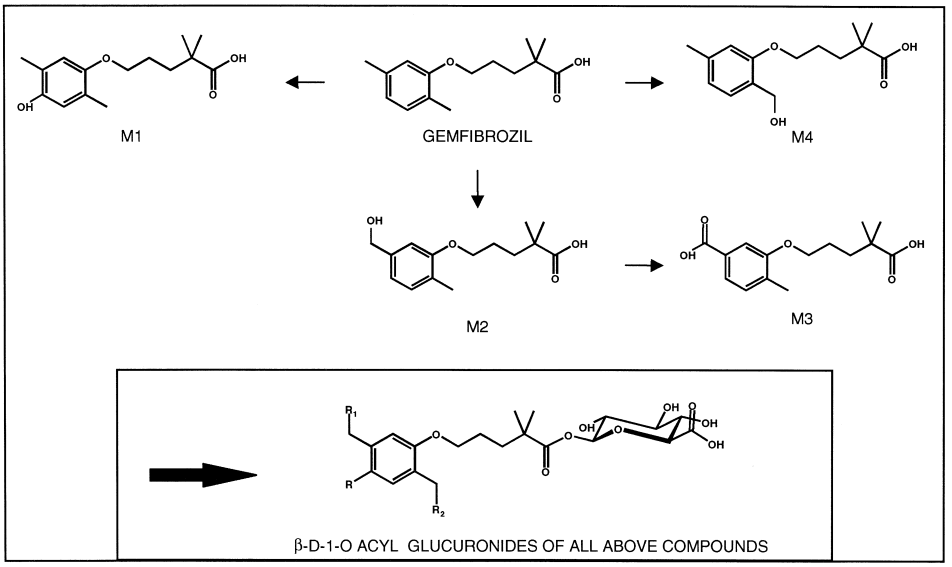

Fig. 1. Biotransformation of gemfibrozil: metabolite structures.

gemfibrozil dose are found in urine, while only about 6% are recovered in faeces following peroral administration [4,5]. As to be expected for lower molecular weight compounds, to which, upon glucuronide formation, almost 200 molecular weight units are added, considerable species differences were detected with respect to the extent of renal and biliary/faecal excretion [7,8]. In rats, further oxidative metabolites were identified in rat urine [9], including a diol metabolite (both ring methyls hydroxylated) and the product of its further metabolism, the acid–alcohol derivative (ortho ring methyl hydroxylated, meta ring methyl further oxidized to acid), as well as ether and acyl (=ester) glucuronides of the same metabolite.

Acyl glucuronides are reactive intermediates which undergo typical chemical reactions such as hydrolysis, isomerization, and covalent binding to endogenous compounds. Hydrolysis of the ester glucuronides at physiological pH leads to back-formation of the aglycones and may be responsible for a potential increase of aglycone concentrations, also ex vivo/in vitro, i.e., after blood or urine sampling. Since the hydrolysis rate is temperature and pH-dependent, post-sampling degradation can be minimized by immediate cooling and pH-stabilization (pH 3–4) [10,11], which is essential for the correct determination of aglycone and glucuronide concentrations. Isomerization – i.e., migration of the acyl residue from the C-1 position to the C-2, C-3, and C-4 positions of the glucuronic acid ring – leads to the generation of β-glucuronidase-resistant regioisomers. Like hydrolysis, it is pH-dependent and can hence be avoided. Covalent binding has been demonstrated for many acyl glucuronide-forming drugs in vivo [3,12–17], in vitro studies have verified the role of the acyl glucuronides in the formation of such covalent adducts [3,15–19], which were proven to be formed via two different mechanisms, i.e., by nucleophilic displacement and condensation of the rearranged acyl glucuronide isomers with lysine residues (e.g. Lys-159), as shown for the acyl glucuronide of benoxaprofen [17,20].

In addition to pH-dependent isomerization and hydrolysis, tissue homogenates include a further complicating source of instability, which is hydrolysis via lysosomal hydrolytic enzymes that are released upon the homogenization process and exhibit their highest activity at acidic pH, i.e. at the pH level where nonenzymatic hydrolysis and isomerization are minimized.

Analytical methods are described in literature for the quantification of gemfibrozil in plasma (HPLC) [21,22], for gemfibrozil and its main metabolite M3 in plasma and urine (GC [23], HPLC [24]), and for the respective glucuronides (after hydrolysis).

Another method, which was developed for the simultaneous determination (HPLC) of gemfibrozil and M1-M4 in plasma and urine requires the use of two detectors and two integrators as well as different stationary and mobile phases for the different biophases (plasma, urine) [4]. Moreover, it was only validated for fairly high plasma concentrations, so that the determination of the usually low concentrations of M2 and M4 was not possible. Furthermore, in the current studies, the respective procedure was expanded to include compounds released from the respective acyl glucuronides and covalent protein adducts.

Thus, aim of the present investigations was (a) to establish a direct gradient HPLC method for the simultaneous determination of gemfibrozil and M1–M4 in concentration ranges relevant for pharmacokinetic studies, and to validate the assay for the measurement of gemfibrozil, its major phase-I and phase-II metabolites, and the respective covalent adducts in different biophases (human plasma and urine, various rat tissues) as well as (b) to apply the methods to the elucidation of the pharmacokinetics of gemfibrozil, all its metabolites, and adducts in humans and rats.

2. Experimental

2.1. Chemicals and reagents

Gemfibrozil, its metabolites M1–M4 [M1: 5-(4-Hydroxy-2,5-dimethyl-phenoxy)-2,2-dimethyl-pentanoic acid; M2: 5-(5-Hydroxymethyl-2-methyl-phenoxy)-2,2-dimethyl-pentanoic acid; M3: 3-(4-Carboxy-4-methyl-pentyloxy)-4-methyl-benzoic acid; M4: 5-(2-Hydoxymethyl-5-methyl-phenoxy)-2,2-dimethyl-pentanoic acid], and the structural analogues C-2 [4-(2,5-Dimethyl-phenoxy)-2,2-dimethyl-butyric acid] and C-5 [7-(2,5-Dimethyl-phenoxy)-2,2-dimethyl-heptanoic acid] were kindly provided by Parke Davis (Ann Arbor, Michigan, USA). Bumetanide and piretanide were provided by Boehringer Ingelheim/Thomae (Biberach/Riss, Germany) and Cassella (Frankfurt, Germany), respectively, β-glucuronidase and β-glucuronidase/arylsulfatase were obtained from Boehringer–Mannheim (Mannheim, Germany). 1,4-Saccharolactone (SAL) and phenylmethyl sulfonyl fluoride (PMSF) were from Sigma Chemicals. All other chemicals were from E. Merck (Darmstadt, Germany) and had the highest analytical grade.

2.2. Gradient HPLC analysis

The HPLC system consisted of a Merck–Hitachi AS-4000 autosampler (E. Merck, Darmstadt, Germany), a Jasco PU-980 HPLC pump (Groβ-Zimmern, Germany), a Jasco LG-980-02 ternary gradient unit with a Gastorr GT-103 degasser, and a Shimadzu RF-551 fluorescence detector (Kyoto, Japan). The separation was performed on two different columns, a Zorbax ODS 5 μm, 150×4.6 mm (Du Pont, Wilmington, Delaware, USA) and a Spherisorb ODS II 3 μm, 125×4.6 mm (E. Merck, Darmstadt, Germany) were used. The mobile phase consisted of two separate eluents, of which eluent A was a mixture of acetonitrile and 10 m*M* acetate buffer pH 4.7 (3:7) and eluent B was acetonitrile. Gradient elution was carried out stepwise as listed in Table 1.

Table 1
Stepwise gradient elution of gemfibrozil, its phase-I metabolites, and the internal standards bumetanide and C-2 and respective detector settings (eluent A = mixture of acetonitrile and 10 m*M* acetate buffer pH 4.7 (3:7), eluent B = acetonitrile)

Time	Flow rate		Mobile phase	Detector settings Sensitivity, attenuation
	Zorbax	Spherisorb		
0–16 min	1.5 ml min^{-1}	1.2 ml min^{-1}	100% eluent A	0–10 min
16–24 min	2.0 ml min^{-1}	1.6 ml min^{-1}	linear change from A: B = 55%: 45% to A: B = 50%: 50%	LOW, GAIN×4 10–17 min HIGH, GAIN×16
24–26.5 min	2.0 ml min^{-1}	1.6 ml min^{-1}	A: B = 50%: 50%	17–25 min
26.5–29.5 min	1.5 ml min^{-1}	1.2 ml min^{-1}	100% eluent A	LOW, GAIN×1

Fluorescence detection was achieved at 300 nm excitation and 380 nm emission wavelength with respect to bumetanide and M3 (piretanide: 315/450 nm). Thereafter, the wavelength was switched to 283 nm excitation and 315 nm emission for the detection of M2, M4, M1, C-2, and gemfibrozil. The assay was carried out at room temperature.

2.3. Preparation of calibration and quality control standards

Calibration curves for gemfibrozil and M1–M4 in plasma, urine, and rat tissue homogenates were constructed by adding known quantities of the compounds to the respective blank samples. As all acyl glucuronide containing samples those blank materials were pH-adjusted (see Section 2.5). Calibration curves for the covalent binding assay were constructed from washed blank plasma and rat tissue homogenate spiked with gemfibrozil and its phase-I metabolites before alkaline hydrolysis. In the course of assay validation with tissue homogenates known amounts of analytes were added at least 2 h prior to sample work-up to ensure sufficient diffusion into cell fragments that were possibly still present in the homogenates.

Blank plasma, urine and tissue homogenates contained those stabilizing agents that are usually immediately added to samples to prevent hydrolysis of labile ester glucuronides (phosphoric acid or buffer for pH3–4 adjustment; PMSF (0.4 m*M*) and SAL (16 m*M*) to block hydrolytic enzymes in tissues; see below).

Slopes and intercepts were generated by weighted ($1/\text{concentration}^2$) least squares regression. Characteristics of the calibration curves for gemfibrozil and M1–M4 are included into Tables 3–5. Linearity of the respective curves was always confirmed by separate calculation of three different segments (low, intermediate, and high concentration range). Quality control (QC) specimens were prepared similarly to the calibration samples using separate stock solutions. In each case, three batches of QCs were prepared at upper and center concentrations as well as at concentrations close to the limits of quantification.

2.4. Method validation

Prior to the analysis of a larger number of samples obtained in a preclinical and a clinical study, method validation was performed in accordance with the summary of the conference on 'Analytical methods validation: Bioavailability, bioequivalence, and pharmacokinetic studies', which provided guidelines for kinetic studies in humans and animals [25,26]. The quality of the method was verified with respect to selectivity, limits of detection and quantification, recovery, linearity, as well as accuracy and precision. According to [26] requirements for precision and accuracy included a general level of acceptance of $\leq$15% except at the LOQ where $\leq$20% were acceptable.

Selectivity was verified with six independent blank samples of plasma, urine, and pooled rat tissue homogenates. The limit of detection was determined at a signal-to-noise ratio of 3, the limit of quantification is defined as lowest analyte concentration that can be measured with a stated level of confidence, in practice it is the lowest concentration in the calibration curve [21]. Recovery relates the amounts of added compounds that are found after extraction to the amount of added compounds prior to extraction; analyses were performed at upper, lower, and intermediate concentrations. Linearity of the method was confirmed over the entire concentration range with spiked samples of plasma and urine, linearity of the covalent binding assay was examined with washed and hydrolyzed blank plasma. Precision (relative standard deviation, RSD) refers to the variability within one sample run (intra-day) and between sample runs (inter-day), accuracy (relative error, RE) focuses on the differences between nominal and measured concentrations within one sample run as well as between sample runs. Precision and accuracy were established by repeated determination of plasma, urine, and covalent binding samples using standard mixtures of gemfibrozil and M1–M4, intra-day and inter-day variations were calculated at upper, lower, and intermediate concentrations.

The respective concentrations were adjusted to the expected concentration range in kinetic studies and different for aglycones, glucuronides and adducts. For plasma aglycones the levels included (gemfib-

rozil, 1.2 (lower)–4.0 (intermediate)–15.0 μg ml^{-1} (upper); M1, 0.06–0.2–1.0 μg ml^{-1}; M2, 0.1–0.5–1.0 μg ml^{-1}; M3, 0.48–1.6–8.0 μg ml^{-1}; M4, 0.1–0.5–1.0 μg ml^{-1}) were different from those evaluated for acyl glucuronides determinations (gemfibrozil, 1.5–3.0–7.5 μg ml^{-1}; M1, 0.15–0.3–0.75 μg ml^{-1}; M2, 0.01–0.02–0.04 μg ml^{-1}; M3, 1.47–2.93–7.34 μg ml^{-1}; M4, 0.01–0.02–0.04 μg ml]$^{-1}$ and for the determinations of adduct levels (gemfibrozil, 50–100–200 ng ml^{-1}; M1, 80–150–300 ng ml^{-1}; M2, 20–40–80 ng ml^{-1}; M3, 60–120–240 ng ml^{-1}; M4, 20–40–80 ng ml^{-1}). Regarding the acyl glucuronides validation, spiking was performed prior to glucuronide cleavage, for the adduct assay spiking was performed after the protein precipitation and washing steps.

Due to the limited availability of rat blank tissue material, it was not appropriate to validate all analyzed rat matrices regarding the validation parameters mentioned above. In preliminary studies, stabilized tissue homogenates were found to be largely comparable with human or rat plasma with respect to all validation parameters.

Uniformity of the analytical conditions during method validation and study analysis was ensured by in-process control. For that purpose, each sample run in the human and animal studies contained two independent calibration curves and consisted of 20% of quality control specimens (QCs) in the above mentioned concentration ranges.

2.5. Sample stabilization and analysis

To minimize hydrolysis and isomerization of the acyl glucuronides, plasma, urine, and rat tissue samples were adjusted to pH 3–4. For that purpose, to 4.0 ml of plasma 40 μl of phosphoric acid (42.5%) were added, 5.0 ml of urine were buffered with 2.0 ml of phosphate buffer pH 7.4 (KH_2PO_4) and stabilized with 80 μl of phosphoric acid (42.5%). The samples of the rat tissues (heart, liver, kidney, duodenum, jejunum, ileum, colon, urinary bladder, skeletal muscle, and adipose tissue) were homogenized in 2 ml g^{-1} of ice-cold phosphate buffer pH 4.0 containing 1.15% of potassium chloride. All samples were stored at −20°C immediately after stabilization.

For the determination of aglycone and glucuronide levels, it may also be necessary to include esterase and glucuronidase inhibitors in the case of tissue homogenate samples (final concentrations: 0.4 m*M* PMSF, 16 m*M* SAL), in order to prevent glucuronide hydrolysis which would decrease glucuronide levels and increase aglycone concentrations in the samples.

Aglycones – plasma, tissues: To a 10 ml borosilicate glass culture tube were added 252.5 μl of acid-stabilized plasma sample (⇔250 μl sample) or 500 μl of homogenized rat tissue (calibrator, QCA, or unknown), 250 μl of sodium chloride (5%) (rat tissue: 500 μl of NaCl 20%), 20 μl of bumetanide (882 μg ml^{-1}) and C-2 (31.25 μg ml^{-1}), and 5.0 ml of a mixture of cyclohexane and ethylacetate (8:2). Samples were immediately vortex-mixed, mixed on a horizontal shaker for 10 min, centrifuged (2500 g for 10 min at −10°C), and 4.0 ml of the organic layer evaporated under vacuum. The residue was dissolved in 100 μl of eluent A, 30 μl were injected onto the column. Aglycones – urine: Urinary samples were treated in a similar way as plasma samples. To 710 μl of stabilized urine sample (⇔500 μl sample) 50 μl of piretanide (667 μg ml^{-1}) and C-5 (5.3 mg ml^{-1}) were added and the mixture extracted with cyclohexane/ethylacetate (8:2). Following evaporation of the organic layer the sample was reconstituted in 200 μl of mobile phase, 20–50 μl of which were injected.

Urine may also be analyzed by direct injection (710 μl stabilized sample; addition of internal standard solutions (see above) and 290 μl acetonitrile; centrifugation). 50 μl of the supernatant may be injected onto the column.

Acyl glucuronides: Alkaline glucuronide hydrolysis was performed by adding 200 μl NaOH (1 *M*) and 250 μl of sodium chloride (5%) (rat tissue: 500 μl of NaCl 20%) to 252.5 μl of stabilized plasma sample (⇔250 μl sample) or 500 μl of homogenized rat tissue. After an incubation period of 60 min at 40°C the mixture was acidified with 200 μl HCl (1 *M*), 50 μl of bumetanide (250 μg ml^{-1}) and C-2 (26 μg ml^{-1}) was added, and the extraction was performed as described above. The evaporation residue was dissolved in 200 μl eluent A, 50 μl were injected onto the column. Stabilized urine samples were treated similarly, 710 μl (⇔500 μl sample)

were used, the internal standard solution contained bumetanide (2.5 mg ml^{-1}) and C-2 (260 μg ml^{-1}), the residue was dissolved in 500 μl of eluent A, and 50 μl were injected onto the column. Acyl glucuronide concentrations were calculated as the difference of aglycone and total (aglycones+acyl glucuronides) concentrations and are expressed as equivalents of the respective aglycones.

Covalent adducts: Covalent binding of gemfibrozil and M1–M4 was determined as described by, e.g., Smith et al. (1986). To 505 μl of stabilized plasma sample (⇔500 μl sample) or 1000 μl of homogenized rat tissue, 1 ml of an ice-cold mixture of acetonitrile and ethanol (2:1) was added to precipitate protein. After vortex-mixing and centrifugation (2500 *g*, 5 min, 0°C) the supernatant was discarded and the remaining protein pellet then washed 15 times with 1 ml aliquots of a mixture of methanol and ether (3:1) to remove unbound and reversibly bound compounds. The residual pellet, containing irreversibly bound gemfibrozil and metabolites, was incubated with 1 ml KOH (0.25 *M*) at 80°C for 60 min to release the analytes from protein. The mixture was then adjusted to pH 3 with 50 μl of phosphoric acid (42.5%), and after addition of 500 μl of sodium chloride (20%) and 50 μl of bumetanide (125 μg ml^{-1}) and C-2 (1.26 μg ml^{-1}) the extraction of liberated gemfibrozil and M1–M4 was carried out as described above. The residue was dissolved in 200 μl of eluent A and 50 μl were injected onto the column. Protein present after alkaline treatment was measured by the Biuret method [27] with human serum albumin as standard for the protein assay. Adduct concentrations are calculated as equivalents of the respective aglycones.

2.6. Enzymatic acyl glucuronide hydrolysis

Enzymatic deglucuronidation was carried out with 200 μl urine, 20 μl phosphoric acid (8.5%), 300 μl buffer (pH 5), and 50 μl of β-glucuronidase at 37°C for 2 h. Two different enzyme preparations were tested: (1) β-glucuronidase (E.C. 3.2.1.31, 20 U ml^{-1}, *Escherichia coli*) and (2) β-glucuronidase/arylsulfatase (E.C. 3.2.1.31–E.C. 3.1.6.1, 5.5 and 2.6 U ml^{-1}, *Helix pomatia*).

2.7. Long-term sample stability

Aglycone concentrations were monitored for storage stability [26]. No decreases of concentrations were detected up to 12 months of storage, with or without pH stabilization. Acyl glucuronides present in ex-vivo samples did show instability unless stabilized, leading to increased aglycone levels and decreased glucuronide concentrations. Adduct levels were found to be unaffected under sample storage conditions (pH stabilized samples).

2.8. Pharmacokinetic studies

The developed HPLC method was applied to the evaluation of the kinetics of gemfibrozil, its phase-I and phase-II metabolites, and covalent adducts in humans and rats. In a clinical trial, plasma and urine samples of young hyperlipidaemic patients who received a single gemfibrozil dose (900 mg, p.o.) were analyzed. In an animal study samples of rat tissues (plasma, heart, liver, kidney, duodenum, jejunum, ileum, colon, urinary bladder, adipose tissue and skeletal muscle) obtained from rats who were administered gemfibrozil (150 mg kg^{-1} i.p.; $n=3$ for each time point) for 14 days were analyzed with respect to aglycone, acyl glucuronide, and covalent adduct concentrations.

3. Results and discussion

3.1. Chromatography

The separation of gemfibrozil, M1–M4, and the used internal standards carried out under the conditions described above yielded appropriate peak separations as depicted in the representative chromatograms shown in Fig. 2. The determined retention times, capacity factors (k'), and resolution factors (R_s) of the compounds are listed in Table 2. Baseline separation was achieved on both, the Zorbax and the Spherisorb column, although the Spherisorb showed an improved performance regarding peak resolution. The obtained resolution factors were constantly higher than 3, which demonstrates the excellent separation of all analytes. Flow pro-

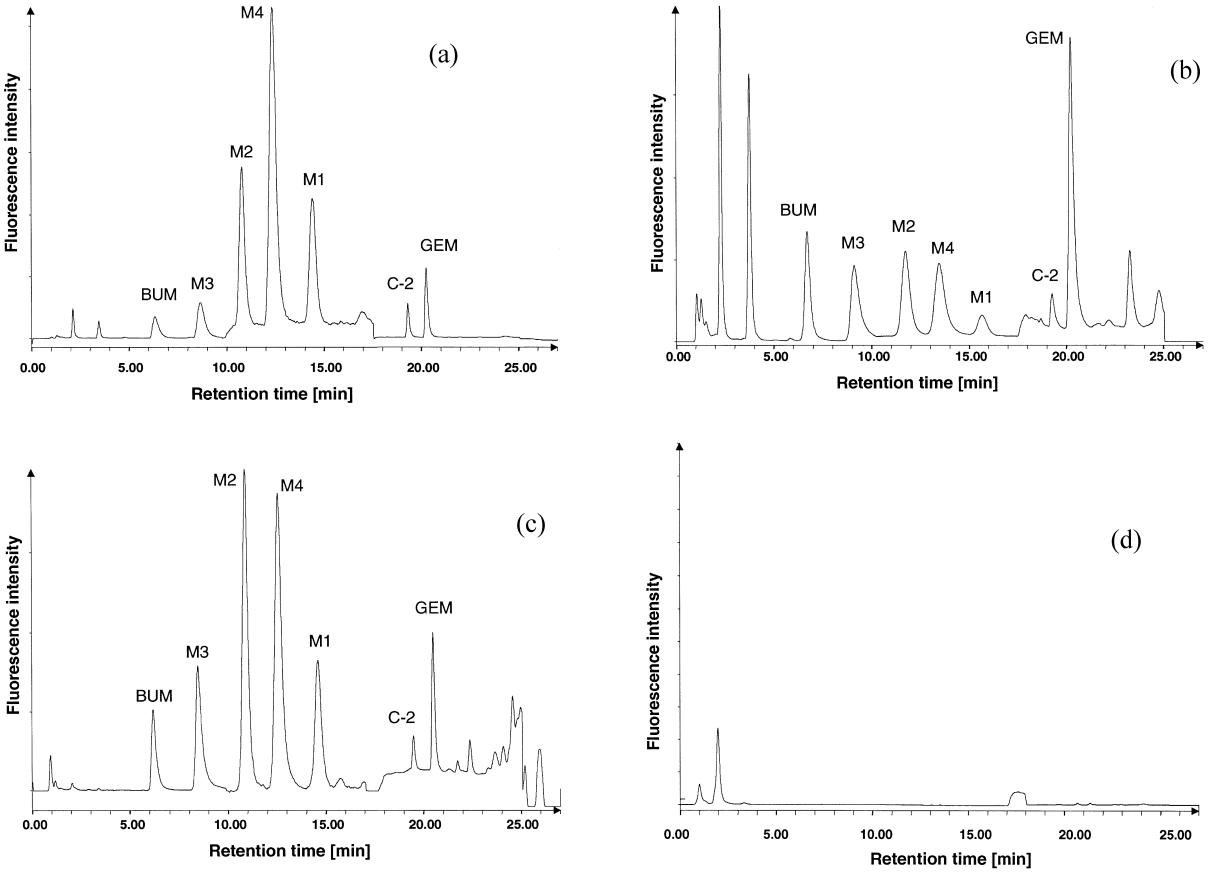

Fig. 2. (a) Typical chromatogram obtained under the described chromatographic conditions from human plasma after oral administration of a single gemfibrozil dose (900 mg) to a young hyperlipidaemic patient: internal standard bumetanide (BUM), M3 (9.2 μg ml^{-1}), M2 (0.8 μg ml^{-1}), M4 (0.02 μg ml^{-1}), M1 (0.8 μg ml^{-1}), internal standard C-2, and gemfibrozil (GEM, 1.2 μg ml^{-1}). (b) Typical chromatogram obtained under the described chromatographic conditions from human urine after oral administration of a single gemfibrozil dose (900 mg) to a young hyperlipidaemic: internal standard bumetanide (BUM), M3 (6 μg ml^{-1}), M2 (0.3 μg ml^{-1}), M4 (0.3 μg ml^{-1}), M1 (2.3 μg ml^{-1}), internal standard C-2, and gemfibrozil (GEM, 5 μg ml^{-1}). (c) Typical chromatogram obtained under the described chromatographic conditions from washed and hydrolyzed rat plasma proteins after i.p. administration of 100 mg kg^{-1} d^{-1} to rats: internal standard bumetanide (BUM), M3 (9 μg ml^{-1}), M2 (0.2 μg ml^{-1}), M4 (0.2 μg ml^{-1}), M1 (1.4 μg ml^{-1}), internal standard C-2, and gemfibrozil (GEM, 12 μg ml^{-1}). (d) Chromatogram obtained from blank human plasma after alkaline hydrolysis for adduct cleavage under the described chromatographic conditions. Peaks: internal standard bumetanide (BUM), M3, M2, M4, M1, internal standard C-2, and gemfibrozil (GEM).

gramming permitted the elution of all substances within approx. 20 min, which is considered to be fast for the elution of seven analytes. The fluorescence detector was time-programmed to switch wavelengths during elution, since the excitation and emission wavelengths of the maximum fluorescence intensities differed between gemfibrozil (Ex 283 nm/Em 315 nm), M1 (295/330 nm), M3 (300/340 nm), and M2 and M4 (280/308 nm). The optimum performance was achieved when the used detector was set at Ex 300 nm and Em 380 nm for the determination of the internal standard bumetanide and M3 (piretanide: 315/450 nm) and to detect all other substances thereafter at 283/315 nm.

Matrix-specific interfering peaks that required alterations of the mobile phase composition were not

Table 2
Retention times (t_B), capacity factors (k'), and resolution factors (R_S) for the HPLC separation of gemfibrozil, its oxidative metabolites, and the internal standards bumetanide and C-2 in order of elution[a]

Compound	t_B (min)	k'	R_S
t_0	1.0		
Piretanide	3.9	2.9	
Bumetanide	6.1	5.1	10.2
Metabolite 3	8.4	7.4	5.2
Metabolite 2	10.8	9.8	4.9
Metabolite 4	12.5	11.5	3.0
Metabolite 1	14.6	13.6	3.5
C-2	19.4	18.4	11.6
Gemfibrozil	20.4	19.4	3.8

[a] Resolution factors were calculated as $1.177 \cdot (t_{R(2)} - t_{R(1)})/(b_{0.5(1)} + b_{0.5(2)})$, where t_r represents the retention time of the respective peak and 0.5 the half-width of a peak.

found in any case, particularly when sample work-up included an extraction step. Therefore, only minor differences were observed in the chromatograms of blank samples of the various tissues or the different sample pretreatments necessary to quantify the glucuronide and protein conjugates.

3.2. Method validation

The results of the validation process are summarized in Tables 3–5 and demonstrate the methods' applicability to the analysis of preclinical and clinical kinetic studies.

Due to limited availability of blank rat tissues it was not feasible to validate the analyzed rat matrices regarding all validation parameters mentioned above. No differences were observed between human and rat plasma regarding selectivity and recovery, and the validation results for tissue homogenates (except recovery) were similar to those for plasma. Therefore, the complete applicability of the analytical method to rat blood and tissue samples was assumed on the basis of experiments performed with a limited amount of blank matrices.

3.2.1. Selectivity

No interfering peaks were detected in any of the investigated matrices, consequently the selectivity of the assay for gemfibrozil, M1–M4, and the internal standards was affirmed. LC–MS–MS investigations, performed according to [28] to demonstrate the absence of detectable amounts of alkaline-cleavable sulfates or glycosides, confirmed that acyl glucuronides were the only relevant phase-II metabolites.

Spiking plasma samples with aglycones and isolated glucuronides (Spahn-Langguth et al., unpublished) revealed that none of the analytes was detectable when taken through the precipitation, washing, and hydrolysis steps.

Only when stability studies were performed with isolated glucuronide fractions and the respective solutions were assayed without extraction slight modifications of the mobile phase composition were necessary (Spahn-Langguth et al., unpublished).

3.2.2. Recovery – aglycones

Mean ($n=6$) extraction efficiency values for plasma (Tables 3–5) in the studied range of concentrations were 80–90% for the analytes (81% (GEM), 79% (M1), 86% (M2), 90% (M3), 88% (M4), and 92%), and 90% for the internal standards bumetanide and C-2, respectively. Recovery rates for the extraction of urine (Table 3) were higher than for plasma and always exceeded 94%. The efficiency of the extraction of various rat tissue homogenate hydrolysates (Table 5) was in part less than for plasma, but since the recovery values were constant, they were still sufficient for a reliable determination of gemfibrozil and metabolites in rat tissues. When recoveries were estimated with spiking performed after covalent binding washes and when the compounds were taken through adduct hydrolysis and extraction steps, the respective values were not significantly different from those given in Table 5.

3.2.3. Limits of detection and quantification – aglycones

Limits of detection for gemfibrozil and M1–M4 in the plasma, urine, and covalent binding assays are summarized in Tables 3–5. Limits of quantification of the compounds in plasma were lower for the oxidative metabolites than for parent gemfibrozil. Different values for the plasma, urine, and covalent binding assay are a result of distinct sample preparations and detector settings.

Table 3

	Gem	M1	M2	M3	M4
(A)					
Aglycones: Quantification of gemfibrozil and its oxidative metabolites in human plasma. (All values are expressed as mean±SD, $n=6$.)					
Calibration curves					
concentration range [μg ml]$^{-1}$	0.1–42.0	0.0032–1.5	0.0002–0.15	0.18–18.3	0.0001–0.15
correlation coefficient	0.9998	0.9990	0.9991	0.9997	0.9998
slope	0.24	2.01	0.39	0.26	1.08
intercept	0.008	−0.003	−0.022	−0.008	0.014
Recovery rates (%)	81.4±1.0	79.4±1.5	85.7±1.5	90.2±1.3	88.4±0.7
Intra-day precision (% RSD)					
upper,	0.55	1.0	0.5	7.7	0.5
intermediate, and	2.2	0.2	1.5	1.6	3.5
lower concentration range	4.3	1.1	1.1	0.68	2.5
Intra-day accuracy (% RE)					
upper,	−2.1	0.8	−0.9	−2.1	0.2
intermediate, and	−1.2	1.8	−2.6	−5.3	−2.2
lower concentration range	−5.0	3.5	−3.0	3.0	−0.3
Inter-day precision (% RE)					
upper,	5.8	3.8	3.5	5.5	4.9
intermediate, and	2.5	4.7	5.2	3.1	6.2
lower concentration range	2.5	5.0	5.9	1.8	5.8
Inter-day accuracy (% RE)					
upper,	−3.5	4.8	−3.8	2.0	−1.6
intermediate, and	−4.8	−1.0	−2.3	−4.5	1.2
lower concentration range	−6.4	3.8	0.8	−6.9	1.4
Limit of detection [ng ml]$^{-1}$					
signal-to-noise ratio=3	50	20	0.5	10	0.5
(B)					
Conjugates: Quantification of the glucuronides of gemfibrozil and its oxidative metabolites in human plasma. (All values are expressed as mean±SD, $n=6$; Interday precision and accuracy data as well as detection limits do not differ from those given above.)					
Calibration curves					
concentration range [μg ml]$^{-1}$	0.24–20	0.172–1.2	0.003–0.11	0.18–18.3	0.003–0.01
correlation coefficient	0.9994	0.9990	0.9991	0.9995	0.9998
slope	0.733	0.001	0.013	0.196	0.014
intercept	−0.002	−0.056	−0.022	−0.009	−0.021
Recovery rates (%)	80.8±1.2	73.1±1.2	85.7±1.5	88.2±1.1	85.4±0.8
Intra-day precision (%RSD)					
upper,	2.7	3.3	3.4	2.2	2.0
intermediate, and	2.0	2.7	2.8	3.6	3.5
lower concentration range	1.7	5.2	4.3	2.0	3.5
Intra-day accuracy (%RE)					
upper,	−2.0	3.4	−0.4	2.0	−0.9
intermediate, and	−0.2	1.6	−3.8	−1.8	−2.0
lower concentration range	−5.3	0.8	−1.4	−6.2	−0.3
(C)					
Tissue homogenates-extraction recoveries: Determination of gemfibrozil and its oxidative metabolites in rat tissue homogenates yielded similar validation data as found with plasma. Only extraction recoveries were different. (Means±SD, $n=6$.)					
Recovery rates (%)					
Heart	85.4±4.2	80.8±0.6	93.8±1.6	90.3±2.4	91.0±1.5
Liver	77.2±3.0	50.3±7.5	54.4±2.5	57.9±7.9	58.6±2.1
Kidney	60.0±5.2	51.2±5.6	58.1±4.3	48.9±4.6	59.5±2.9
Intestine	68.0±1.5	56.9±5.0	65.3±5.1	65.1±3.9	64.5±5.1
Skeletal muscle	71.2±7.3	43.6±1.6	63.9±3.2	63.0±3.0	63.7±2.2

Table 4
Quantification of gemfibrozil and its oxidative metabolites in human urine (All values are expressed as mean±SD, $n=6$)

	Gem	M1	M2	M3	M4
Calibration curves					
concentration range [μg ml]$^{-1}$	0.5–80	0.5–20.1	0.03–3.9	1.8–60.5	0.1–4.0
correlation coefficient	0.9993	0.9964	0.9963	0.9978	0.9953
slope	0.077	0.018	0.142	0.056	0.156
intercept	0.014	−0.003	−0.001	0.002	−0.004
Recovery rates (%)	96.2±1.9	98.0±1.1	99.6±0.2	99.5±0.7	99.0±0.4
Intra-day precision (% RSD)					
upper,	3.8	7.3	3.1	3.8	3.2
intermediate, and	2.3	3.5	4.7	4.5	3.2
lower concentration range	3.5	5.9	3.5	3.1	5.2
Intra-day accuracy (% RE)					
upper,	1.1	6.8	5.3	2.3	4.2
intermediate, and	0.3	5.4	5.9	−1.4	4.0
lower concentration range	−2.2	3.3	2.6	−6.3	1.0
Inter-day precision (% RE)					
upper,	2.5	4.5	2.8	6.0	3.1
intermediate, and	4.5	3.3	2.8	2.3	2.5
lower concentration range	2.7	2.3	3.6	1.4	2.3
Inter-day accuracy (% RE)					
upper,	−3.6	1.1	1.4	−1.3	1.5
intermediate, and	2.5	−1.6	−1.9	−4.6	1.1
lower concentration range	1.1	−6.0	−2.9	−4.8	−2.2
Limit of detection [ng ml]$^{-1}$					
signal-to-noise ratio=3	100	100	10	50	100

3.2.4. Linearity

Correlation coefficients of the calibration curves of all compounds (spiked samples) in the plasma, urine, and covalent binding assay were always better than 0.995. Since the single-range values for the slope never varied more than 5% from the slope over the whole range, the method can be considered linear for all analytes and all matrices.

Representative calibration curves for the respective analytes were as follows: Gemfibrozil, $y = 0.0220x + 0.2763$ ($r^2 = 0.9995$); M1, $y = 0.0064x - 0.0970$ ($r^2 = 0.9996$); M2: $y = 0.1238x + 0.0500$ ($r^2 = 0.9993$); M3, $y = 0.0020x + 0.0267$ ($r^2 = 0.9999$); M4, $0.1148x + 0.0739$ ($r^2 = 0.9981$).

3.2.5. Precision and accuracy

In plasma, urine, and in the covalent binding assay relative standard deviations never exceeded 8% ($n=6$ in each case) and relative errors were also always below 8% ($n=6$ in each case), which demonstrates the excellent quality of the HPLC method and its suitability for the quantification of gemfibrozil and its metabolites.

3.2.6. Stability

Aglycones, acyl glucuronides and adducts were stable in acidified plasma and urine samples from preclinical and clinical studies. In order to stabilize ester glucuronides in tissue homogenates and prevent post-sampling increases of aglycone levels, addition of inhibitors of hydrolytic enzymes must be considered.

Analytes incubated at protein hydrolysis conditions according to the above protocol did not show any sign of instability.

3.3. Acyl glucuronide cleavage

In the present investigations the efficacy of different cleavage procedures was analyzed. As was to be expected, alkaline glucuronide cleavage was more efficient than enzymatic hydrolysis. It was complete – i.e., yield was maximal – already after 30 min and

Table 5
Quantification of covalent protein binding of gemfibrozil and its oxidative metabolites in plasma (and tissue homogenates) [a]

	Gemfibrozil	M1	M2	M3	M4
Calibration curves					
concentration range [ng ml]$^{-1}$	24–1175	52–780	5–80	14–2850	5–84
correlation coefficient	0.9995	0.9971	0.9983	0.9990	0.9970
slope	0.021	0.006	0.127	0.002	0.120
intercept	−0.072	−0.091	−0.013	−0.009	−0.012
Intra-day precision (%RSD)					
upper,	3.8	3.0	3.3	2.7	3.5
intermediate, and	3.9	4.9	3.2	4.8	3.0
lower concentration range	3.7	4.6	4.3	4.6	3.5
Intra-day accuracy (%RE)					
upper,	1.6	−1.5	−1.2	−3.2	0.8
intermediate, and	0.6	−2.7	−3.8	−4.8	−2.6
lower concentration range	−0.6	1.1	−4.1	−4.6	−1.8
Inter-day precision (%RE)					
upper,	4.9	6.7	2.9	5.7	3.6
intermediate, and	3.4	5.4	2.8	4.9	5.4
lower concentration range	2.4	3.5	4.7	3.9	2.0
Inter-day accuracy (%RE)					
upper,	3.2	−7.7	−5.1	−1.8	−1.8
intermediate, and	5.2	−2.0	−6.3	−4.0	−2.0
lower concentration range	2.7	2.6	−4.8	−4.8	−2.5
Limit of detection [ng ml]$^{-1}$					
signal-to-noise ratio = 3	10	20	0.5	5	0.5

[a] Blank washed protein pellets were spiked and then treated in the same way as the samples of the study. Data given was obtained from plasma, preliminary validation data including recovery rates achieved for all rat tissue homogenates were in a comparable range. (All values are expressed as mean ± SD, $n = 6$.)

incubation for up to 4 h did not lead to any further increase of concentrations of released aglycones.

The investigated β-glucuronidase preparations differed very much regarding their hydrolytic activity. With a pure β-glucuronidase preparation only 30% (29.6 ± 15.8%) of the value of alkaline treatment was reached on average, while β-glucuronidase/arylsulfatase was much more efficient and yielded ca. 80% (78.5 ± 8.4%) of the yield obtained via alkaline cleavage. Neither by increased amounts of β-glucuronidase (up to 100 μl), nor by extended incubation periods (up to 16 h) the extent of enzymatic hydrolysis yields were enhanced. Glucuronide data obtained via alkaline cleavage represent the sum of the β-1-O-acyl glucuronides and their positional isomers. To assure the absence of acyl sulfate ester mixed anhydrides or other conjugates (e.g. glycosides), urine samples obtained in the clinical study were analyzed by LC–MS–MS. Only acyl glucuronides of gemfibrozil and M1–M4 were detected in these investigations performed under the conditions described in the previous publication of Dahms et al. [28], i.e., it was not possible to detect other alkaline-cleavable metabolites, which confirms the selectivity of this part of the assay.

Because of its superior performance, alkaline cleavage was applied to the analysis of the glucuronides in the preclinical and clinical studies. This does not lead to any lack of assay specificity, since isomeric ester glucuronides are known to be β-glucuronidase-resistant but labile to base hydrolysis. Since isomerization is usually of little relevance in vivo and isomeric conjugates are measured in very low concentrations only in ex-vivo plasma samples and those urine samples, in which isomers were formed in the urinary tract because the urine was not sufficiently acidic, the sum of ester glucuronide isomers of each analyte was regarded as most reasonable approach.

Repetitive cleavage and work-up ($n = 8$) of a plasma sample obtained from a clinical study yielded relative standard deviations of 3.2% for gemfibrozil

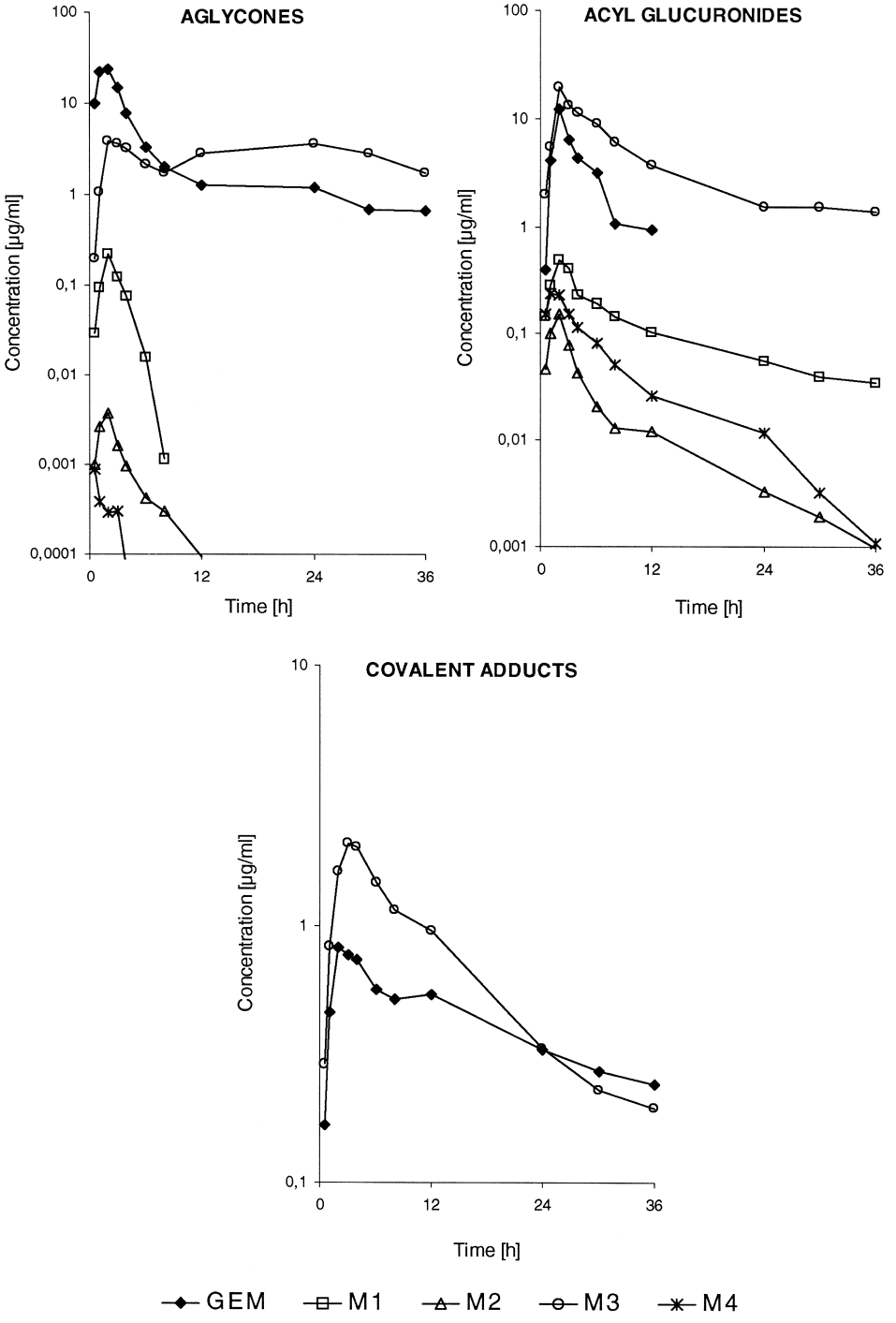

Fig. 3. Plasma concentration–time profiles of gemfibrozil and M1–M4, the corresponding acyl glucuronides, and covalent protein adducts after oral administration of a single gemfibrozil dose (900 mg) to young hyperlipidaemic patients.

(5.15 μg ml^{-1}), 6.1% for M1 (0.313 μg ml^{-1}), 5.9% for M2 (0.062 μg ml^{-1}), 2.7% for M3 (7.38 μg ml^{-1}), and 4.4% for M4 (0.041 μg ml^{-1}).

3.4. Covalent binding assay

The precision of the covalently binding assay was determined by repetitive ($n=8$) determination of protein adduct concentrations of gemfibrozil and M1–M4 in samples obtained from clinical studies. Relative standard deviations were 7.8% (gemfibrozil; 751 ng ml^{-1}), 7.2% (M3; 521 ng ml^{-1}), and 8.0% (M4; 52 ng ml^{-1}) in a sample from a healthy volunteer, where M1 and M2 adducts were not detectable, and 11.3% and 8.4% for M1 and M2 adducts in an elderly patient (M1; 23 ng ml^{-1}, M2; 15 ng ml^{-1}), which confirms the high quality of the method.

The protein washing procedure was studied with respect to the efficacy in removing noncovalently bound substances and regarding adduct and protein loss during the procedure. Noncovalently bound substances were removed effectively, neither gemfibrozil nor metabolites remained detectable after washing the protein pellet 12 times. Since the pellets were washed 15 times in the preclinical and the clinical studies, they are considered free of noncovalently bound substances. Adduct loss was calculated from protein concentrations in untreated plasma and after the washing procedure. The differences were sufficiently small with a mean adduct loss value of 19.6±2.1% ($n=66$), which was accounted for by normalization of adduct concentrations on the basis of the residual protein fraction.

Table 6
Excretion balances (% of dose) of gemfibrozil and its phase-I and phase-II metabolites in young hyperlipidaemic patients after oral administration of a single gemfibrozil dose (900 mg)[a]

	Urinary recovery (% of dose)	
	Aglycones	Acyl glucuronides
Gemfibrozil	0.04±0.01	34.1±8.9
Metabolite 1	0.02±0.01	5.0±1.7
Metabolite 2	–	1.1±0.2
Metabolite 3	9.6±2.4	15.1±2.9
Metabolite 4	–	0.6±0.1
Total	9.6±2.4	55.9±11.5

[a] Amounts excreted into urine extrapolated to infinity were calculated as $Ae_{0\rightarrow\infty} = Ae_{0\rightarrow t_{last}}/(1-e^{-\lambda z \cdot t_{last}})$. (All values are expressed as mean±SD; $n=3$.)

3.5. Pharmacokinetic studies

The plasma concentration-time profiles for gemfibrozil, its phase-I and phase-II metabolites, and covalent adducts in six young hyperlipidaemic patients are shown in Fig. 3, urinary excretion balances are listed in Table 6. The concentration–time profiles of the covalent adducts of gemfibrozil and M3 in rat tissues are given in Fig. 4.

4. Conclusions

A sensitive, reliable and accurate analytical method has been established and validated to investigate the kinetics of gemfibrozil, its phase-I and phase-II metabolites, and covalent protein adducts in humans and animals. The suitability of the method to measure pharmacokinetic studies has been confirmed by the application of the method on a preclinical study with rats and clinical trials with hyperlipidaemic and elderly patients. However, it needs to be emphasized again that a complex number of different matrix-specific and analyte-specific processes may complicate sample handling and data interpretation. E.g., tissue homogenates may contain esterases and β-glucuronidases usually present intracellularly in lysosomes. Such enzymes readily hydrolyze acyl glucuronides, especially at acidic pH, thus resulting in artificially high concentrations of aglycones and artificially low concentrations of glucuronide conjugates. This may be particularly important in tissue homogenates with high exposure to glucuronide conjugates (liver and kidney).

The methods and data presented here again demonstrate the complexity possibly involved in the assay of compounds forming acyl glucuronides and adducts, particularly when closely related compounds need to be quantified simultaneously (e.g., aglycone stereoisomers and/or additional carboxylic acid phase-I metabolites) as discussed previously [29]. For gemfibrozil and its four phase-I metabolites (=5 aglycones) five acyl glucuronides are formed, for each of which four positional isomers are pos-

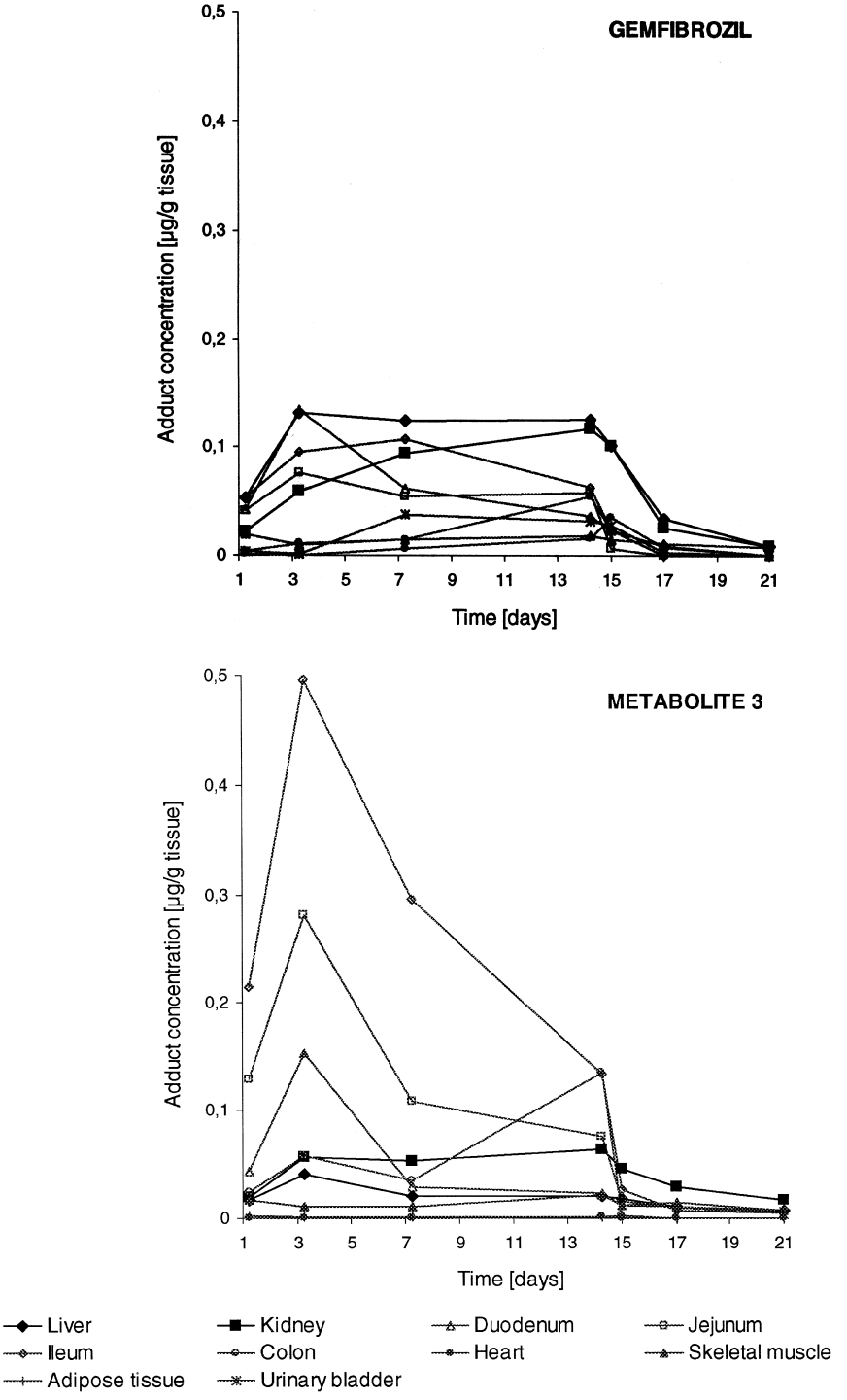

Fig. 4. Tissue concentrations of the covalent protein adducts of gemfibrozil (G) and M3 after chronic administration of gemfibrozil (150 mg kg^{-1} d^{-1}, i.p.) to rats.

sible (not yet accounting for isomerism within the carbohydrate moiety). Hence, although direct and specific assays for acyl glucuronides and their isomeric conjugates are also feasible, the summation of all isomers of one aglycone appears to be a reasonable and practicable approach in the case of routine analysis of gemfibrozil conjugates.

In a similar way as the indirect glucuronide quantification the alkaline cleavage procedure of protein precipitates for the determination of adduct levels is still lacking generally applicable alternatives. In a recent article of Qui et al. [20], in which tryptic digests were subject to tandem-MS analysis when studies were performed to establish the structure and specific binding sites of adducts of benoxaprofen glucuronides and human serum albumin (generated in the presence and absence of sodium cyanoborohydride to study the mechanism of covalent binding). For the same compound and a closely related structure we had previously proposed a direct adduct assay, since both compounds exhibit excellent fluorescent properties. However, the general problem with respect to the measurement of adduct levels in vivo is the low ''epitope'' density, which prevented the establishment of any practicable alternative to alkaline protein cleavage.

General aspects that need to be considered with respect to alternative adduct assays are the different binding mechanisms and adduct types (for the imine mechanism positional isomers may occur in the glucuronide segment [11]) that were demonstrated and the fact that – although albumin appears to be the major binding protein – different and heterogenous proteins may be involved, where the actual ''binding site'' at the protein may be specific or not. Tryptic digests with formation of short-chain segments of the macromolecule with a drug or metabolite attached to them are feasible, yet careful mechanistic investigations with respect to the acyl glucuronide as well as the macromolecule are a prerequisite for the development of and is a prerequisite for the generation of reference compounds for such an assay.

Recent developments in pharmacokinetic research focus on the identification of transporters involved in drug absorption and disposition. In this respect investigation of glucuronide transport is an important subject as well, where acyl glucuronide assays are one important prerequisite [30,31].

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