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Note

Assay method for product formation in in vitro enzyme kinetic studies of uridine diphosphate glucuronyltransferases: 2-arylpropionic acid enantiomers*

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In enzyme kinetic studies, direct quantification of the product formation should be the method of choice and preferred to measuring the decrease of substrate concentration, which may result in considerable analytical errors in the determination of the rate of formation, especially when the substrate-to-product ratio is high. Conjugates with D-glucuronic acid formed by uridine diphosphoglucuronyltransferases (UDPGTs) in the presence of uridine diphosphoglucuronic acid (UDPGA) are hydrophilic derivatives of drugs, which can easily be separated from the parent compound by chromatographic methods [1, 2].

Formation of metabolites by UDPGTs will result in diastereomeric conjugates, if the substrate is diastereotopic and occurs as a mixture of the two possible enantiomers. Performing chiral derivatization chemically has usually one major aim, which is a reliable and simple method for enantiomer quantification. For this purpose the availability of derivatization reagents with a very high degree of enantiomeric purity is desirable. Glucuronic acid, the coupling component in one of the major biotransformation pathways in mammals, is an oxidation product of D-glucose and occurs in its D-form only. Therefore, it represents an intrinsic optically active component with a high enantiomeric purity. This permits the direct

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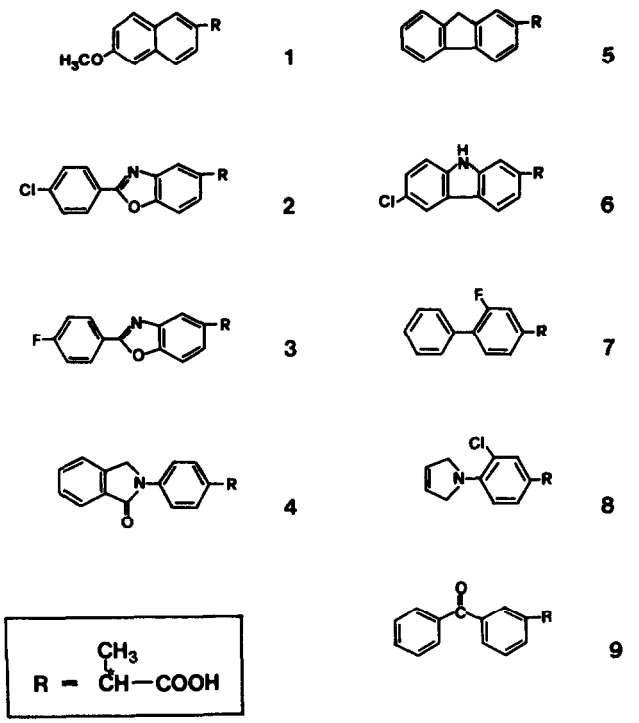

Fig. 1. Chemical structures of the investigated 2-arylpropionic acids: 1 = naproxen; 2 = benoxaprofen; 3 = flunoxaprofen; 4 = indoprofen; 5 = cicloprofen; 6 = carprofen; 7 = flurbiprofen; 8 = pirprofen; 9 = ketoprofen.

stereospecific separation and determination (e.g. in plasma and urine) of the diastereomeric products formed from the substrate by metabolizing enzymes [3]. The direct determination of diastereomeric glucuronides has been described, e.g. for oxaprotiline [4], oxazepam [5], propranolol and 4-hydroxypropranolol [6] and amobarbital [7].

In enzyme kinetic studies (with e.g. liver microsomes) of the enantiomers of 2-arylpropionic acids, different rates of product formation were observed between the two enantiomers, when they were investigated separately or as racemates [8, 9]. This paper describes a direct method for the simultaneous study of both enantiomers of several 2-arylpropionic acids (Fig. 1), e.g. in enzyme kinetic studies with UDPGTs, which can include in vitro glucuronidation studies with different organs and species, and furthermore the investigation of interferences between the enantiomers of a drug.

EXPERIMENTAL

Chemicals

R/S-, *R*- and *S*-flunoxaprofen were provided by Ravizza (Muggio, Italy), *R/S*-benoxaprofen and *R/S*-indoprofen by Eli Lilly (Bad Homburg, F.R.G.) and Farmitalia Carlo Erba (Freiburg, F.R.G.), respectively, before being withdrawn

from the market. *R*- and *S*-naproxen were obtained from Syntex (Palo Alto, CA, U.S.A.), *R*/*S*-flurbiprofen from Thomae (Biberach, F.R.G.), *R*/*S*-cicloprofen from Squibb (Princeton, NY, U.S.A.), pirprofen from Ciba-Geigy (Wehr, F.R.G.) and ketoprofen from Bayer (Wuppertal, F.R.G.). *R*/*S*-carprofen was provided by Hoffmann-La Roche (Basel, Switzerland). *R*- and *S*-benoxaprofen were prepared via formation of diastereomeric derivatives of α-methylbenzylamine, preparative high-performance liquid chromatography (HPLC) and subsequent hydrolysis, as described earlier [10]. *R*- and *S*-carprofen were separated via formation of diastereomeric salts [11]. Tetrabutylammonium (TBA) hydrogensulphate was purchased from Janssen Chimica (Beerse, Belgium). Solvents were HPLC grade and from E. Merck (Darmstadt, F.R.G.). Magnesium chloride, Trizma base, phenylmethylsulphonyl fluoride, saccharic acid 1,4-lactone and UDPGA (ammonium salt), which were used for incubation of UDPGT (EC 2.4.1.17) and 2-arylpropionic acids, as well as commercially available UDPGT (Type III, bovine) were obtained from Sigma (Munich, F.R.G.). The pH 7.4 phosphate buffer was prepared by adjusting the pH of a 0.155 *M* solution of sodium dihydrogenphosphate to 7.4 by adding a 0.103 *M* solution of disodium hydrogenphosphate (310 mosm/l).

UDPGTs

A preparation of rat liver microsomes was used as source for UDPGTs. Furthermore, the applicability of commercially available UDPGT for enzyme kinetic studies was tested.

To prepare liver microsomes, fresh livers obtained from White Wistar rats were homogenized with 3 volumes of pH 7.4 phosphate buffer. The homogenate was centrifuged at 10 000 *g* for 20 min. The supernatant was centrifuged at 100 000 *g* for 60 min, resuspended and centrifuged again. The final pellet was suspended and homogenized in pH 7.4 phosphate buffer. All the above processes were carried out at 0–4 °C on ice. Microsomal protein was determined using the Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, U.S.A.). UDPGA was dissolved in distilled water. Stock solutions of 2-arylpropionic acids were prepared in methanol or dimethylsulphoxide [20 mg/ml, except for benoxaprofen (10 mg/ml) and indoprofen (4 mg/ml)].

Formation of glucuronic acid conjugates from different 2-arylpropionic acids

The incubation mixture contained 1 mg of microsomal protein per ml, 50 m*M* Tris–HCl buffer (pH 7.4), 10 m*M* magnesium chloride, 10 m*M* 1,4-saccharolactone, 1 m*M* phenylmethylsulphonyl fluoride, 0.04% Triton X-100, 10 m*M* UDPGA and 0.1–0.4 m*M* substrate in a final volume of 250 μl. The mixture was preincubated at 37 °C for 10 min before the reaction was started by addition of UDPGA. After 20 min the reaction was stopped by cooling the tubes to 0 °C. The samples were then prepared for HPLC analysis as described below.

Equipment and chromatographic conditions

A Knauer HPLC gradient system (consisting of two HPLC pumps 64 and a programmer 50 B, Knauer, Berlin, F.R.G.) with a 10-μl or a 50-μl sample loop

(Rheodyne, Cotati, CA, U.S.A.) and a Beckman Ultrasphere ODS column (25 cm×0.46 cm, 5 μm particle size) as stationary phase was equipped with a Shimadzu fluorescence monitor RF 530 (Shimadzu, Kyoto, Japan) for measurement of the intrinsic fluorescence of the molecules, and a Knauer variable-wavelength HPLC spectrophotometer for measuring the UV absorbance. The mobile phase was acetonitrile–TBA buffer (10 m*M*) pH 2.5 (28:72, v/v, mobile phase I) for all the compounds except indoprofen, where a decrease of the stronger eluent was necessary to achieve a satisfactory separation. The eluent used in this case was acetonitrile–TBA buffer (8 m*M*) pH 2.5 (23:77 v/v, mobile phase II). The flow-rate was 1.5 ml/min.

The composition of the gradient versus time depended on the retention times of the diastereomeric conjugates. Once the conjugates had been resolved, i.e. after the appearance of both peaks, mobile phase I or II was maintained without changes for an additional 3–5 min. The stronger eluent was then increased stepwise to wash out the excess substrate, which was retained longer than the conjugates, in the following way: For 2 min the mobile phase (I or II) was mixed with 20%, then for 5 up to 10 min with 60% of acetonitrile, the duration being dependent on the lipophilicity of the substrate (e.g. 10 min for benoxaprofen). Thereafter, the initial mobile phase I or II was allowed to equilibrate for 5–10 min before the next sample was injected. As several compounds with completely different chromophoric properties were investigated, different modes of detection had to be applied. Fluorescence measurements were performed with flunoxaprofen (excitation 305 nm, emission 355 nm), benoxaprofen (313/365 nm), carprofen (285/350 nm) and indoprofen (275/433 nm). UV absorption was used to detect flurbiprofen (255 nm) and naproxen (285 nm), both of which also have fluorescent properties, ketoprofen (255 nm), pirprofen (265 nm) and cicloprofen (238 nm).

Sample preparation

A 50-μl volume of sample (resulting from incubation with microsomes) was pipetted into 50 μl of acetonitrile in a microcentrifuge tube. It was diluted with mobile phase I (or II for indoprofen) to a final volume of 500 μl. The mixture was vortexed briefly and centrifuged, and the supernatant was directly injected into the chromatographic system. The injection volume was 50 μl for UV detection and 10 μl when the intrinsic fluorescence of the aglycone was measured.

HPLC resolution

The resolution of the diastereomeric conjugates was characterized by calculation of the separation and resolution factors, α and R, respectively. Peak parameters were calculated as follows: capacity, $k' = (t_p - t_0)/t_0$; separation factor, $\alpha = k'_2/k'_1$; resolution, $R = 2(t_2 - t_1)/(w_1 + w_2)$ where t_0 is the retention time of the unretained compound, t_p is the retention time of the peak (t_1 of peak 1, t_2 of peak 2), and w_1 and w_2 are the widths of peaks 1 and 2 in min.

Stability of the conjugates (pH dependent)

The stability of the flunoxaprofen glucuronides, extracted from incubation mixtures, was investigated. The relative decrease of the glucuronide concentra-

tion was determined for both the *S*- and the *R*-glucuronide during incubation in 50 m*M* Tris–HCl buffer at 37°C. From the apparent first-order rate of decrease a half-life was calculated for both glucuronides.

Determination of the initial rates of formation of diastereomeric glucuronides

For two of the investigated compounds, flunoxaprofen and naproxen, the rate of formation over 2 h was investigated in order to define the time period in which the rate of formation is approximated by a straight line.

Reproducibility

The coefficients of variation (C.V.) were determined for the analytical procedure by performing a series of analyses ($n=8$) from one sample resulting from an incubation experiment with microsomes, and for the formation of glucuronides in the incubation mixture. For this purpose two series of samples (0.4 and 0.2 m*M* substrate) were prepared and incubated simultaneously at 37°C for 10 min ($n=8$). From such experiments the C.V. for the sample preparation and chromatographic analysis as well as for the analysis of enzyme kinetic activity could be characterized. The C.V. values were determined for flunoxaprofen and naproxen.

RESULTS

The procedure described here is suitable for studies of the enantioselectivity of glucuronide formation from 2-arylpropionic acids. The enzymatic activity of UDPGTs and their stereoselectivity may vary between different species [9, 12] and between different organs within one species due to the occurrence of isoenzymes. Using different amounts of commercially available bovine UDPGT the maximum rates of glucuronide formation were found to be very low compared with rat liver microsomes, resulting in very low yields, which were below the detection limits for some of the compounds. Since significantly higher rates of glucuronide formation were found when washed microsomes were used, the investigations were performed with microsomes only.

The assay method allows direct measurement of the formation of the diastereomeric D-glucuronides of several 2-arylpropionic acids. Table I lists the chromatographic resolution parameters of the diastereomeric conjugate pairs for the nine 2-arylpropionic acids investigated. The separation and resolution factors given in this table, as well as the example depicted in Fig. 2, demonstrate that a good resolution of the *R*- and *S*-conjugate was accomplished with mainly one standard solvent system. Only indoprofen, as one of the most hydrophilic compounds from this group, required a more polar mobile phase. All the peaks characterized in the table disappeared almost completely when treated with β-glucuronidase at pH 5. When the two enantiomers were available (flunoxaprofen, benoxaprofen, naproxen, indoprofen) and could therefore be incubated separately, the elution order was always *S*-glucuronide before *R*-glucuronide. From this it was assumed that the elution order *S* before *R* is a general rule for the

TABLE I

HPLC RESOLUTION OF D-GLUCURONIC ACID CONJUGATES OF SEVERAL 2-ARYLPROPIONIC ACIDS

Compound	k'_1	k'_2	α	R
Flunoxaprofen	40.8	44.2	1.08	2.00
Flurbiprofen	36.4	40.0	1.10	2.02
Naproxen	11.8	12.8	1.08	1.59
Ketoprofen	10.0	11.2	1.12	2.11
Indoprofen*	8.6	8.9	1.03	0.30
	(27.7)	(29.2)	(1.05)	(1.54)
Pirprofen	13.5	14.4	1.06	1.45
Benoxaprofen	57.5	63.0	1.10	2.39
Carprofen	42.2	46.7	1.11	2.20
Cicloprofen	27.4	31.0	1.14	2.27

*Because of insufficient resolution under "standard conditions", a modified mobile phase was applied, the resolution parameters of which are given in parentheses.

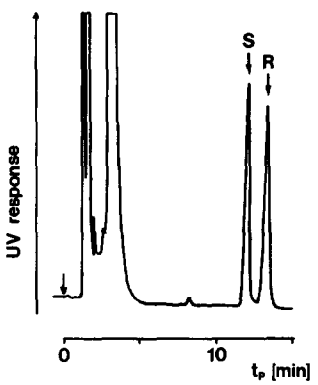

Fig. 2. HPLC resolution of the (diastereomeric) conjugates of ketoprofen enantiomers with D-glucuronic acid.

glucuronides of this class of drugs in such reversed-phase systems. A very recent article by El Mouelhi et al. [13] confirmed this finding.

As expected, the partition coefficients of the aglycones (taken from the literature) seem to correlate with the k' values of the conjugates. Carprofen and benoxaprofen, the compounds with the highest partition coefficients, exhibited the highest retention times, whereas the compounds with the lowest partition coefficients had shorter retention times (ketoprofen, indoprofen). Interestingly, the difference in k'_1 between benoxaprofen and flunoxaprofen was comparably high, namely 17, although the chemical structures are closely related. They differ in one substituent only: the chlorine atom of benoxaprofen is substituted by a fluorine atom in the flunoxaprofen molecule. A study of the time course of the apparent rate of formation for the model compounds (flunoxaprofen and naproxen) showed that the linear range for flunoxaprofen is comparable with that observed

TABLE II

RATIOS OF *S*-GLUCURONIDE TO *R*-GLUCURONIDE OBTAINED FOR SEVERAL 2-ARYLPROPIONIC ACIDS [AT 0.4 m*M* SUBSTRATE (0.1 m*M* FOR INDOPROFEN)]

Incubation of racemic compound with rat liver microsomes and UDPGA, as described in Experimental.

Compound	Ratio
Flunoxaprofen	0.53
Flurbiprofen	0.88
Naproxen	1.49
Ketoprofen	0.97
Indoprofen	0.36
Pirprofen	0.51
Benoxaprofen	0.38
Carprofen	0.43
Cicloprofen	0.62

for naproxen [6]. Apparent linear behaviour of the curve was found within 30 min for both the *S*- and the *R*-enantiomers.

Under the conditions described (rat liver microsomes), the initial rates of formation were 2.3 nmol/mg protein per min for the glucuronide of the *S*- and 4.3 nmol/mg protein per min for the glucuronide of the *R*-enantiomer. As acyl glucuronides are known to be unstable under certain conditions, the stability of the conjugates of *R*- and *S*-flunoxaprofen was tested under the conditions applied for their formation. The glucuronide of the *R*-enantiomer turned out to be less stable under these conditions. At pH 7.4 its half-life was 4.5 h, compared with 8 h for the glucuronide of the *S*-enantiomer. pH-dependent decomposition based on these half-lives does not seem to have a relevant influence on the observed yields for *R* and *S* at a maximal incubation time of 10–15 min. However, the observed rates of formation must be defined as apparent rates owing to their reversibility by chemical and enzymatic hydrolysis.

The variability of the results was investigated by determining the C.V. values for the analytical procedure, including sample preparation and chromatography, with *R*/*S*-flunoxaprofen. The C.V. values for the conjugates in two incubation mixtures (containing 0.4 and 0.2 m*M* *R*/*S*-flunoxaprofen as substrate) were 2.6% (*S*) and 4% (*R*) for the higher and 2.9% (*S*) and 3.9% (*R*) for the lower substrate concentration. The variability of the rate of formation within one set consisting of nine different incubation tubes was 7.8% for both isomers of flunoxaprofen and 6.9% for both isomers of naproxen.

For the glucuronides of different 2-arylpropionic acids, the different *S*/*R* ratios obtained (Table II) clearly show that the ratio is not uniform for a certain class of drugs. Under the conditions described, the *S*/*R* ratios of the apparent initial rates of formation of diastereomeric glucuronides from racemic 2-arylpropionic acids ranged from 0.36 (indoprofen) to 1.5 (naproxen) at substrate concentrations in the range of K_m.

CONCLUSION

It appears again that making use of the high enantiomeric purity of an optically active natural compound seems to be a very useful approach in enantiospecific

drug analysis. Chiral derivatization applying biochemical (enzymatic) processes with endogenous enantiomerically pure coupling components [14] or using products from such processes for derivatization (as sugar derivatives [15] or configurationally stable amino acid derivatives [16]) usually provides more reliable results because of the high enantiomeric purities of such compounds and their derivatives, so subsequent mathematical corrections on the basis of impurities are not necessary.

D-Glucuronic acid conjugates of 2-arylpropionic acid enantiomers turned out to be derivatives, which can easily be resolved by HPLC, e.g. refs. 3–8 and 13. The recent paper of El Mouelhi et al. [13] also shows a resolution of the diastereomeric glucuronides of ibuprofen, naproxen and benoxaprofen. The direct determination of the diastereomeric glucuronides, formed from diastereotopic substrates by UDPGTs, is possible without any extraction or chemical derivatization procedure and will therefore provide more reliable data in enantioselective glucuronidation of such drugs in vitro as well as in vivo, since the procedure can readily be adopted for biological materials other than incubation media [3].

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