Journal of Chromatography, 383 (1986) 51–60
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3327

LIQUID CHROMATOGRAPHIC ASSAY FOR THE MEASUREMENT OF GLUCURONIDATION OF ARYLCARBOXYLIC ACIDS USING URIDINE DIPHOSPHO-[U-^{14}C] GLUCURONIC ACID

C. HAMAR-HANSEN, S. FOURNEL* and J. MAGDALOU

Centre du Médicament, Unité Associée au CNRS No. 597, Université de Nancy I, 30 Rue Lionnois, 54000 Nancy (France)

J.A. BOUTIN

Department of Pharmacology and Experimental Therapeutics, Johns Hopkins School of Medicine, Baltimore, MD 21250 (U.S.A.)

and

G. SIEST

Centre du Médicament, Unité Associée au CNRS No. 597, Université de Nancy I, 30 Rue Lionnois, 54000 Nancy (France)

(First received April 3rd, 1986; revised manuscript received July 11th, 1986)

SUMMARY

A general method for the assay of UDP-glucuronosyltransferase activity towards arylcarboxylic acids (clofibric acid, 1- and 2-naphthylacetic acid) using UDP-[U-^{14}C]glucuronic acid in liver microsomes is described. The ^{14}C-labelled glucuronide was separated by high-performance liquid chromatography, identified by hydrolysis by β-glucuronidase, characterized by laser desorption mass spectrometry and quantified by scintillation counting. The coefficient of variation of the enzyme activity for the inter-assay repeatability was below 4.5%. As little as 2.5 nmol of the arylcarboxylic acid glucuronides could be detected and precisely quantified. The method was applied to the determination of the apparent kinetic constants for glucuronidation of the acids. Clofibric acid was the best substrate for UDP-glucuronosyltransferase (V_{max}/K_M, the ratio of the maximum initial velocity and the Michaelis–Menten constant, is 12.3). The two isomers, 1- and 2-naphthylacetic acids, were transformed at a similar rate. However, they exhibited different enzymatic affinities, as the K_M values were 1.0 mM and 5.6 mM for 1- and 2-naphthylacetic acid, respectively. This indicates that the spatial organization of the substrates played a critical role in this acyl glucuronoconjugation.

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INTRODUCTION

UDP-glucuronosyltransferase (EC 2.4.1.17) is a membrane-bound enzyme exhibiting a very broad substrate specificity. This protein is able to link glucuronic acid to a great variety of structurally different molecules, such as phenols and carboxylic acids [1]. The formation of ester glucuronides has been extensively studied in the past few years [2] and this is relevant for arylcarboxylic acids. The latter compounds are of major therapeutic and economic importance, as hypolipidaemic, anti-inflammatory and diuretic drugs, herbicides and plant hormones.

These acids are principally excreted as amino acid or sugar conjugates [3], and UDP-glucuronosyltransferase plays a crucial role in their detoxification and elimination. The study of the glucuronidation of such acids is complicated by numerous difficulties. The formation of ester glucuronides in rat liver microsomes is characterized in vitro by its extremely low rate. Moreover, acyl glucuronides are known to be quite unstable, either undergoing facile hydrolysis to glucuronic acid and the aglycone, or intramolecular rearrangement at mild alkaline pH [4]. Thus, direct evaluation of the glucuronidation reaction after acidic [5] or enzymatic [6] hydrolysis of the glucuronide is not possible; neither a general spectrophotometric method, such as that of Mulder and Van Doorn [7] and Colin-Neiger et al. [8], nor a method based on the disappearance of substrate [9] can be used because of their lack of sensitivity. The conjugation of clofibric acid by UDP-glucuronosyltransferase has been particularly studied. Analysis of ester glucuronide was performed either by gas chromatography coupled to mass spectrometry [10] or by high-performance liquid chromatography (HPLC) coupled to fast atom bombardment [11, 12]. However, these techniques cannot be used in routine work. The enzyme activity can also be measured with radiolabelled aglycones [13]; this procedure is limited by the availability of the radioactive material. Conversely, labelled UDP-glucuronic acid (UDPGA) provides a general method for measurement of conjugation reactions after separation of the glucuronide by thin-layer [14] or column chromatography [15–17]. These two methods could not be used in our assay because of the lability of the acyl glucuronides and the limit of sensitivity.

This paper reports the development of a direct, sensitive and accurate method for the evaluation of glucuronidation of arylcarboxylic acids in liver microsomes. The enzyme reaction was performed using UDP-[U-^{14}C] glucuronic acid ([^{14}C] UDPGA) for the quantitation of the product after separation by HPLC. This method has been successfully used for the determination of apparent kinetic constants of UDP-glucuronosyltransferase activity towards clofibric acid and 1- and 2-naphthylacetic acid, and can be extended to a large number of aglycones.

EXPERIMENTAL

Chemicals

UDPGA (disodium salt) was provided by Boehringer (Mannheim, F.R.G.) and [^{14}C] UDPGA (specific acitivity 260 μCi/mmol) was purchased from

Amersham (Les Ulis, France). Clofibric acid [2-(4-chlorophenoxy)-2-methylproprionic acid] and 1- and 2-napthylacetic acid were supplied by Ega Chemie (Strasbourg, France). Acetonitrile (HPLC grade) was obtained from BDH (Poole, U.K.). Triton X-100 and digitonin were purchased from Sigma (St. Louis, MO, U.S.A.). Scintran EX scintillation cocktail was provided by BDH and trifluoroacetic acid by Merck (Darmstadt, F.R.G.).

Incubation conditions

The microsomes were prepared from the livers of male Wistar rats (180—200 g) according to Hogeboom's method [18], and their protein content was measured by the technique of Lowry et al. [19]. For the assay of UDP-glucuronosyltransferase the following mixture was used: 75 m*M* Tris–HCl (pH 7), 3.5 m*M* UDPGA, 0.8 μCi [^{14}C]UDPGA, 600 μg of microsomal protein, and 7.5 m*M* magnesium chloride, in a total volume of 500 μl. Before the assay, activation was achieved by incubating the microsomes for 20 min in ice, with various concentrations of digitonin. The reaction was started by addition of the carboxylic acid dissolved in a constant volume of 20 μl of dimethyl sulphoxide (0.25—2.5 m*M* final concentration). After incubation in a shaking water-bath at 37°C for 15 min, the reaction was stopped and the protein precipitated by the addition of 50 μl of 0.2 *M* hydrochloric acid. The protein was then pelleted by centrifugation at 4000 *g* for 3 min. Control reactions without carboxylic acid were run simultaneously.

HPLC conditions

An aliquot (50—100 μl) of the supernatant was injected into a Waters Model 6000A HPLC system equipped with a U6K injector. A reversed-phase column pre-packed with LiChrosorb RP-18 (Hibar RT 250-4, 7 μm, Merck) was used with a continuous flow-rate of 1.0 ml/min. The mobile phase was acetonitrile–trifluoroacetic acid–water, 48:0.08:152 (v/v/v) when conjugation of the two naphthylacetic acids was investigated, and 55:0.08:145 (v/v/v) for glucuronidation of clofibric acid. The separated products were monitored by a UV detector (Waters Model 480) set at 233 and 283 nm for clofibric acid and naphthylacetic acids, respectively. The apparatus was coupled to a Spectra-Physics Model SP4100 computing integrator (San Jose, CA, U.S.A.).

Quantification of the glucuronides

The eluent fractions (0.5 min) were collected into 25-ml plastic scintillation vials, 10 ml of scintillation cocktail were added and the radioactivity was estimated with a liquid scintillation spectrometer (Beckman, Model LS 1801, Irvine, CA, U.S.A.). Under these conditions, the counting efficiency was 94%, and the background corresponding to 0.5-min eluent mobile phase in 10 ml of scintillation liquid was 60 dpm.

The quantity of glucuronide synthesized was calculated from the specific activity of the labelled conjugate. The peak of radioactive glucuronide was collected in four fractions. The amount of carboxylic acid glucuronide formed was calculated by subtracting the radioactivity obtained in controls from that of the assay values in the corresponding collected fractions and then dividing this value by the total counts eluted from the column. The specific activity of

carboxylic acid glucuronide was then calculated by multiplying the amount of glucuronide formed by the UDPGA added in the incubation mixture; 100% of the radioactivity was recovered after elution.

Laser desorption mass spectrometry

For the characterization of the glucuronidė of 1-napthylacetic acid by mass spectrometry (MS), rabbit liver microsomes immobilized on active Sepharose were used according to the method of Lehman et al. [20]. This preparation, incubated overnight under the conditions described above, generated sufficient material for analysis by laser desorption MS. At the end of the incubation time the beads were removed by filtration under vacuum and washed with 20 ml of distilled water. The filtrate was then passed through a reverse C_{18} Sep-Pak cartridge (Millipore, Waters Assoc., Milford, U.S.A.) [21]. After elution by methanol and separation by HPLC the glucuronide was analysed in a mass spectrometer, CVC (Rochester, U.S.A.) Model 2000 time-of-flight (TOF) with a 2-m flight-tube and modified for laser desorption as described previously [22]. The laser was a Tachito (Needham, MA, U.S.A.) Model 215 A CO_2 laser with a pulse width of 10 ns. Digitized spectra were transferred to a Le Croy 3500 computer (Alexandria, DC, U.S.A.) via a parallel interface. Twenty-five real-time spectra, each produced by a single laser pulse, were summed to generate the mass spectrum. The mass assignments were made using the times of flight of the alkali ions Na^+, K^+ and Cs^+ to obtain the calibration peaks; the masses of interest were obtained using the equation $t = aM^{1/2} + B$, in which t is the time of flight, M is the mass of the compound, and a and B are constants depending of the mass spectrometer and determined by using Cs^+ as reference according to the method of Van Breemen et al. [22].

RESULTS AND DISCUSSION

Analytical method

Under the experimental procedure described, the retention time of 1-naphthylacetic acid glucuronide was 16 min (Fig. 1). Under the same conditions, 2-naphthylacetic acid glucuronide was eluted after 20 min and clofibric acid glucuronide after 18 min. In all three cases, the free aglycone was eluted after 60 min. Identification of the peak of glucuronide detected by UV spectrometry was confirmed by simultaneous detection of radioactivity.

The collected glucuronide could be totally hydrolysed by incubation with β-glucuronidase (10 000 U) in 0.2 *M* acetate buffer (pH 5.0) for 16 h at 37°C. Similar results were obtained with 1- and 2-naphthylacetic acid and clofibric acid (results not shown).

Base-catalysed acyl migration of ester glucuronides of drugs, especially clofibric acid, has been previously reported [23]. This intramolecular transesterification occurs mainly at mild alkaline pH values higher than pH 7 [4], and also when methanol or any nucleophilic solvent is used [24]. This could result from nucleophilic substitution during acyl rearrangement [12, 25]. By contrast, acetonitrile has been shown to enhance the stability of acyl glucuronides [26], especially at low pH.

Our experimental conditions, pH of incubation (pH 7), immediate acidifica-

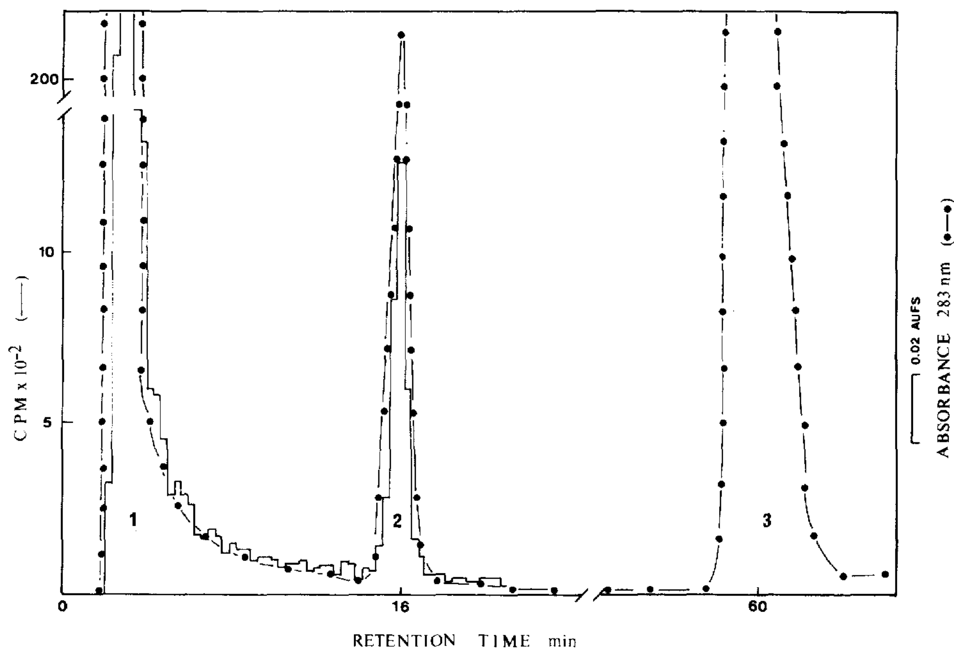

Fig. 1. Chromatogram of the HPLC resolution of the reaction products after incubation of rat liver microsomes with 1-naphthylacetic acid and UDP-[U-^{14}C]glucuronic acid. Peaks: 1 = UDP-glucuronic acid; 2 = 1-naphthylacetic acid glucuronide; 3 = 1-naphthylacetic acid. The mobile phase was acetonitrile—trifluoroacetic acid—water (48:0.08:152, v/v/v), and the flow-rate 1.0 ml/min for 24 min and 1.2 ml/min thereafter.

tion of the mixture at the end of the incubation time (pH < 4) and refrigeration of the samples, the HPLC solvent (acetonitrile) was chosen to minimize the risk of hydrolysis and acyl shift. Indeed, only one isomer of the glucuronide was detected, and it was found that it was not hydrolysed during the HPLC separation since 94% was recovered after collection and the second HPLC analysis.

The validity of the method was assessed by the inter-assay repeatability. Six incubations of 1-naphthylacetic acid (1 m*M* final concentration) were prepared and injected on the same day under the conditions previously described. The results are presented in Table I. The coefficient of variation (C.V.) was below 4.5% after determination of the ratio of radioactivity associated with the glucuronide peak and the total radioactivity collected from the column. The detection limit of the method was 2.5 nmol glucuronide quantified in the incubation mixture.

Four different scintillation cocktails were tested under the same conditions. An aliquot of [^{14}C]UDPGA was first counted in 10 ml in each scintillator, 0.5-min mobile phase was then added and the samples were counted immediately and several days after. A 30% decrease of the initial radioactivity was observed with Scintran T (BDH), which contains toluene, between the second and the sixth day. The radioactivity thereafter gradually increased to the starting value after the twelfth day and was stable until the twenty-sixth day. By contrast, no change in the radioactivity counting was observed with Scintran EX (BDH), PCS II (Amersham) or MP (Beckman), which contain

TABLE I

INTER-ASSAY REPEATABILITY FOR GLUCURONIDATION OF 1-NAPHTHYLACETIC ACID

Assay No.	Radioactivity (dpm)			Specific acitivity (nmol/min/mg of protein)
	Glucuronide peak	Total eluted	Ratio*· ($\times 10^3$)	
1	908	151 373	6.0	0.83
2	821	141 000	5.9	0.80
3	1061	167 377	6.3	0.87
4	804	137 946	5.8	0.80
5	882	147 345	6.0	0.82
6	1077	166 709	6.5	0.89
Mean ± S.D.				0.83 ± 0.04
Coefficient of variation (%)				4.5

*Ratio represents glucuronide peak divided by total eluted.

xylene and pseudocumene, respectively. Scintran EX was then used throughout the study to avoid the chemiluminescence produced by toluene.

Laser desorption mass spectrometric characterization of the acyl glucuronide of 1-naphthylacetic acid

As previously described for other acyl-linked glucuronides [22], the laser desorption spectra produced molecular ions as the alkali-metal cation adducts ($M + K^+$), ($M + Na^+$) and ($M + Cs^+$). The major ion of 1-naphthylacetic acid was ($M + K^+$) (*m/e* 401) and two smaller peaks ($M + Na^+$) (*m/e* 384) and ($M + Cs^+$)

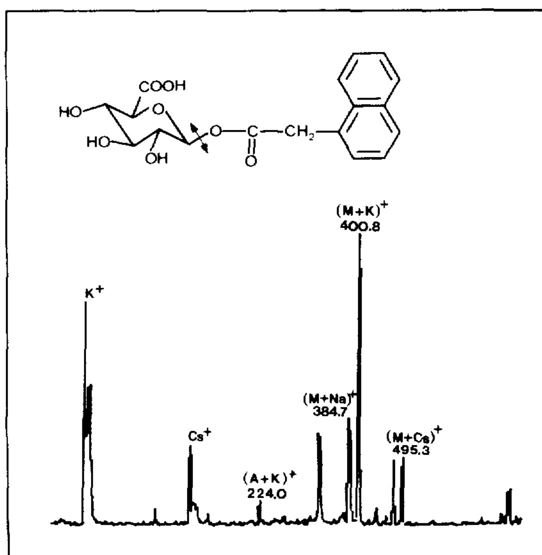

Fig. 2. Laser mass spectrum of 1-naphthylacetic acid glucuronide formed by rabbit liver microsomes. M = 1-naphthylacetic acid glucuronide; A = 1-naphthylacetic acid. Time resolution is 10 ns by channel.

(*m/e* 495) were detected (Fig. 2). A minor peak corresponding to the free aglycone ($A + K^+$) (*m/e* 224) was also observed (Fig. 2).

Determination of enzymatic activity

Optimal conditions for the assay. The pH for measurement of the enzyme activity was set at 7.0 throughout the study. No forms resistant to β-glucuronidase have been detected at this pH for clofibric acid glucuronide [4] and specific activity was optimal at this pH (results not shown).

As the enzyme exhibits latency in intact microsomes [27], it was necessary to remove the permeability barrier exerted by the membrane with activators such as detergents. Among the detergents available to activate the enzyme, Triton X-100 was unable to produce maximal activation. In contrast, maximal activation of UDP-glucuronosyltransferase was achieved with a digitonin—protein weight ratio of 1.0 (Fig. 3): at this concentration of the detergent, the activity was enhanced 100% over control. The same amount of digitonin has been used for the determination of UDP-glucuronosyltransferase activity towards clofibric acid [14] or bilirubin [28]. It seems that digitonin is specific in activating the enzyme form involved in the formation of ester glucuronides. Nevertheless, it has been suggested by Burchell and Blanckaert [29] that the form of the enzyme involved in bilirubin glucuronidation would be different from the one that metabolizes clofibric acid.

The activity was linear with time, at least for 30 min (Fig. 4), and was a function of the protein concentration up to 1 mg in the incubation mixture (Fig. 5).

Determination of apparent kinetic constants for glucuronidation of clofibric acid and 1- and 2-naphthylacetic acid in rat liver microsomes. Table II indicates the values of K_M (Michaelis—Menten constant) and V_{max} (maximum initial velocity) for glucuronidation of the arylcarboxylic acids. Clofibric acid showed the best affinity with a K_M value (95 μM) considerably lower than those calculated for the other compounds (mM range). The two isomers 1- and 2-

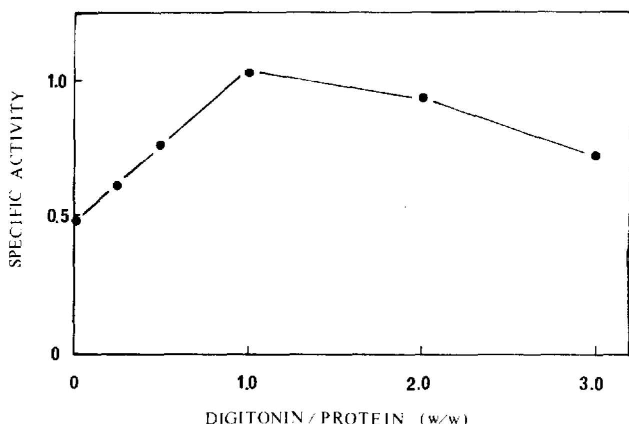

Fig. 3. Activation by digitonin of rat liver UDP-glucuronosyltransferase towards 1-naphthylacetic acid. The concentrations of 1-naphthylacetic acid and UDP-glucuronic acid were 1.0 and 4.5 mM, respectively. The amount of protein in the incubation mixture was 0.60 mg. The specific activity is expressed in nanomoles of glucuronide formed per minute per milligram of protein.

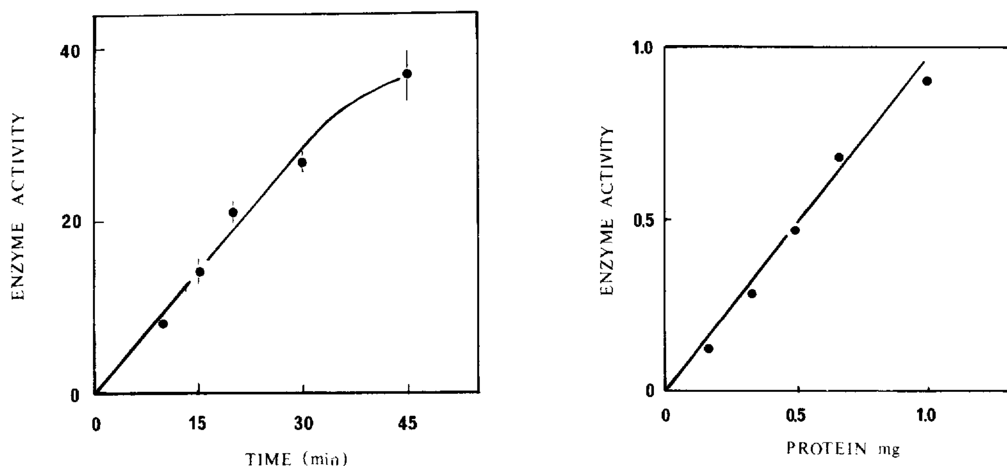

Fig. 4. Effect of incubation time on rat liver UDP-glucuronosyltransferase activity towards 1-naphthylacetic acid. The enzyme activity was linear up to 30 min under the following conditions: activated microsomes (0.60 mg of protein) at a digitonin/protein ratio of 1.0 (w/w); concentrations of aglycone and UDP-glucuronic acid, 1.0 and 4.5 m*M*, respectively. The enzyme activity is expressed in nanomoles of glucuronide formed per milligram of protein.

Fig. 5. Effect of microsomal protein concentration on rat liver UDP-glucuronosyltransferase activity towards 1-naphthylacetic acid. The assays were performed with activated microsomes at a ratio digitonin/protein ratio of 1.0 (w/w); incubation time, 30 min; concentrations of aglycone and UDP-glucuronic acid, 1.0 and 4.5 m*M*, respectively. The enzyme activity is expressed in nanomoles of glucuronide formed per minute in the incubation mixture.

TABLE II

APPARENT KINETIC CONSTANTS FOR GLUCURONIDATION OF ARYLCARBOXYLIC ACID BY RAT LIVER MICROSOMES

These parameters were calculated by linear regression analysis.

Arylcarboxylic acid	V_{max} (nmol/min/mg of protein)	K_M (m*M*)	V_{max}/K_M (μl/min/mg of protein)
Clofibric acid	1.17	0.095	12.31
1-Naphthylacetic acid	2.29	1.00	2.29
2-Naphthylacetic acid	2.18	5.60	0.39

naphthylacetic acid, were conjugated at similar rates. However, the K_M value of 1-naphthylacetic acid was five times less than that of 2-naphthylacetic acid. The analysis of the data V_{max}/K_M showed that clofibric acid is the best substrate for UDP-glucuronosyltransferase.

These results are in good agreement with those of Odum and Orton [13], who reported that the ratio V_{max}/K_M for the conjugation of clofibric acid was 9.25 compared with 12.3 μl/min/mg protein reported by us. The V_{max} value or glucuronidation of 1-naphthylacetic acid is similar to that found by Dixon et al. [2] but the K_M value reported by these authors was lower ($0.061 \cdot 10^{-3}$ *M*). This discrepancy can probably be explained by the different methodologies

used, as well as other experimental conditions, notably the pH of incubation (6.2 compared with 7.0 in this work).

It is interesting to note that 1-naphthylacetic acid is a better substrate than its 2-isomer when considering their kinetic constants (K_M/V_{max}). These data confirm that geometric features are of crucial importance for the orientation of the aglycone in the active site, as we previously demonstrated for the 4- and 7-hydroxylated coumarins [30]. Similar results were obtained for the glucuronidation of morphine [31] or 3- and 17-hydroxysteroids [32], but this example provides the first evidence for the regioselectivity of acylglucuronide formation.

CONCLUSION

The present method, using the common co-substrate radiolabelled UDPGA, does not require any glucuronide standard and it can be easily generalized to other aglycones. It provides a useful tool for investigation of the properties of UDP-glucuronosyltransferase(s) conjugating carboxylic groups. This is of particular interest since, at present, there is little knowledge of these substrates compared with phenols. Finally, the high sensitivity of the assay allows its use not only for animal liver microsomes, but also for the study of conjugation in limited amounts of human tissue.

ACKNOWLEDGEMENTS

We are grateful to Dr. J. Caldwell (Department of Pharmacology, St. Mary's Hospital, Medical School, London, U.K.) for advice and stimulating discussion. A part of this work has been performed in the laboratory of Dr. C. Fenselau (Department of Pharmacology and Experimental Therapeutics, Johns Hopkins School of Medicine, Baltimore, MD, U.S.A.); the help of Dr. R.J. Cotter and Dr. J. Honovitch in laser desorption mass spectrometry is acknowledged. The secretary help of Mrs. G. Junger and Miss F. Rakotozafy is also acknowledged as well as the skilful technical assistance of Miss S. Detty and Mrs C. Lafaurie. This work was supported by grants from the Centre National de la Recherche Scientifique (UA No. 597) and from the European Economic Community (STI, 04 6JC).

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