RADIOCHEMICAL ASSAY OF UDP GLUCURONYLTRANSFERASE (*p*-NITROPHENOL)

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1. Introduction

UDP glucuronyltransferase (UDPglucuronate glucuronyltransferase, EC 2.4.1.17) catalyzing the glucuronide formation of foreign compounds is firmly bound to the membranes of endoplasmic reticulum. The kinetics of the UDP glucuronyltransferase has therefore been studied with only crude preparations. These kinetic studies have been hampered by the lack of an accurate and sensitive method for determining enzyme activity. *p*-Nitrophenol is very often used as an aglycone, because its spectrophotometric quantitation is rapid and simple [1]. It has also been widely used in recent detailed kinetic studies of this enzyme [2–8]. This method has, however certain disadvantages, since the consumption of the substrate is measured and not the formation of the product, which is preferable in kinetic studies. The absorption spectrum of *p*-nitrophenyl glucuronide and free aglycone have different absorption maxima (glucuronide at 312 and aglycone at 400 nm, respectively), and this has been utilized in the direct measurement of UDP glucuronyltransferase activity [9]. This method is, however, rather insensitive. In kinetic studies low substrate concentrations should also be used due to their physiological relevance. With the old spectrophotometric methods mentioned above this has been very difficult.

In the present paper we describe a radiochemical method for UDP glucuronyltransferase determination with increased accuracy and sensitivity. ^{14}C-Labeled *p*-nitrophenol, which is now commercially available, was used as a glucuronyl acceptor substrate.

2. Experimental

2.1. *Isolation of microsomes*

Male Wistar rats about three months old were used for the enzyme preparation. The animals were killed by a blow on the head and bled. The liver was excised, cooled in ice cold 0.25 M sucrose and homogenized in 0.25 M sucrose in a volume four times the fresh weight of the tissue. After centrifugation at 10 000 *g* for 10 min the microsomal fraction was separated from the supernatant by spinning for 60 min at 105 000 *g*. The microsomal pellet was resuspended into isotonic sucrose in a final conc. corresponding to 1 g of liver per ml. This microsomal suspension was used in the enzyme assays.

2.2. *Incubation technique*

The reaction mixture in the radiochemical UDP glucuronyltransferase (*p*-nitrophenol) determination consisted of 85 μl of 0.35 mM *p*-nitrophenol (E. Merck AG, Darmstadt, West-Germany) in 0.5 M phosphate buffer with 10 mM dipotassium EDTA, pH 7.0 [10] and 15 μl of aqueous [2,6-^{14}C]*p*-nitrophenol (16.7 mCi/mmole, International Chemical and Nuclear Corporation, Irvine, Calif., USA) corresponding to 40 nCi per sample unless otherwise stated. UDPglucuronic acid (UDPGlcUA) (98%, ammonium salt, Sigma Chemical Company, St. Louis, Mo., USA) was dissolved in *p*-nitrophenol phosphate buffer and each sample also had a corresponding blank without added UDPGlcUA. The final concentration of UDPGlcUA

was 4 mM unless otherwise stated. The reaction was initiated by adding 50 μl of liver microsomal suspension. After the incubation (5–20 min) the reaction was stopped by adding 0.7 ml of 3% w/v aqueous trichloroacetic acid.

2.3. *Extraction procedure*

In the next step 0.7 ml of 1.0 M Tris-maleate buffer, pH 6.0 was added after letting the tubes stand for about 10 min. The precipitated protein was separated by centrifugation (1500 *g*, 10 min) and the final pH before the subsequent ether extraction was about 5.3. The buffering improved the stability of the extraction system and promoted the separation of *p*-nitrophenol and its glucuronide. The unconjugated *p*-nitrophenol was separated from the more water soluble *p*-nitrophenyl glucuronide by extracting the solution three times with 5 ml of diethyl ether (ad narcosin, Orion Oy, Helsinki, Finland). The separation of the aqueous and organic phases from one another after each shaking was accelerated by centrifuging (1000 *g* for 2 min). One ml of the aqueous phase was then pipetted into a counting vial containing 1 ml of methanol and 10 ml of scintillation medium (4 g of 2,5-diphenyloxazol (PPO) (Merck), 0.1 g of 2,2-*p*-phenylbis-(5-phenyloxazol) (POPOP) (Merck) and 100 g of naphthalene in a medium consisting of 800 ml of dioxane and 200 ml of toluene). The radioactivity of the samples was counted about one day later in a Beckman 1650 liquid scintillation counter with a counting efficiency of 89% for ^{14}C. The ether phases or fractions of them were also pipetted into scintillation vials, and the ether was evaporated to dryness. After that 10 ml of scintillation medium for non-aqueous samples (4 g of PPO and 0.1 g of POPOP in 1000 ml of toluene) was added to the vials and the radioactivity was determined as described above with a counting efficiency of 93% for ^{14}C.

2.4. *Studies on transfer of p-nitrophenyl glucuronide into the ether phase*

In order to determine the transfer of *p*-nitrophenyl glucuronide into the organic phase, 250 nCi per sample of [2,6-^{14}C]*p*-nitrophenol was used in the enzyme assay and the incubation time was 60 min in order to increase the production of radioactive glucuronide. After extraction the three ether phases were combined, evaporated to dryness and the remainder was dissolved in 100 μl of H_2O. Fifty μl of the solution was pipetted on Whatman No. 1 chromatography paper, and the chromatogram was developed in ethanol–1 M ammoniumacetate (9:1) by ascending chromatography. *p*-Nitrophenol was located with ammonia fume and *p*-nitrophenyl glucuronide with alkaline silver nitrate [11]. After detection, the spots were cut out and their radioactivity was determined in the scintillation counter using the scintillation medium for non-aqueous samples. The counting efficiency of ^{14}C from the paper was 68%.

3. Results and discussion

Diethyl ether proved to be the best extraction solvent tested. Hexane, benzene, toluene and different benzene-diethyl ether mixtures only partly extracted *p*-nitrophenol (table 1). Di-isopropyl ether was almost as efficient as diethyl ether in the separation of *p*-nitrophenol from its glucuronide. About 0.5% of radioactive *p*-nitrophenol remained in the water phase after three ether washings. The amount of *p*-nitrophenol removed in the third washing was less than the amount left in the water phase after it. Less than 0.4% of the conjugated glucuronide was found in the ether phase after extraction. The solution was buffered to a pH below the pK_a-value of *p*-nitrophenol, 7.2 [12], in

Table 1
The efficiency of the extraction of [2,6-^{14}C]*p*-nitrophenol (30 nmoles, 40 nCi) by different organic solvent systems from the reaction mixture used in UDP glucuronyltransferase determination.

Solvent	Radioactivity (%) left in the aqueous phase after three washings
Benzene	2.0 ∓ 0.04
Benzene + diethyl ether (1:1)	1.1 ∓ 0.13
Benzene + diethyl ether (1:3)	0.73 ∓ 0.06
Benzene + diethyl ether (1:5)	0.59 ∓ 0.01
Hexane	79.2 ∓ 0.43
Toluene	1.7 ∓ 0.12
Di-isopropyl ether	0.55 ∓ 0.04
Diethyl ether	0.51 ∓ 0.02

The extraction was repeated three times with 5 ml of the solvent after the addition of 0.7 ml of 3% TCA and 1.0 M Tris-maleate buffer, pH 6. Six experiments were performed in each case. The standard errors of the means are indicated.

Table 2
The hydrolysis of the reaction product by β-glucuronidase in the absence and presence of D-glucaro-1,4-lactone.

Sample	Radioactivity (cpm) in the aqueous phase
−UDPGlcUA	854 ∓ 103
+UDPGlcUA	18 489 ∓ 1076
UDPGlcUA + β-glucuronidase	2391 ∓ 275
UDPGlcUA + β-glucuronidase + D-glucarolactone	19 205 ∓ 539

The radioactivity of the aqueous phase has been given. The usual incubation with 40 nCi of [2,6-^{14}C]p-nitrophenol (30 min) was stopped by adding 0.3 ml of 1.5 M acetate buffer, pH 5. β-Glucuronidase (300 units, from bovine liver, type B 1, 1000 000 units per g, Sigma) was then added to the tubes. β-Glucuronidase was inhibited in the reference tubes by D-glucaro-1,4-lactone (5 mM) (Pfizer, Folkestone, England). After 30 min incubation the reaction was stopped with 3% TCA, extracted with ether, and the radioactivity of the aqueous phase was determined as described in the section on experimental procedure. Five experiments were performed in each group.

order to maximize the extraction efficiency of this weak acid by the organic solvent. The buffering with a Tris-maleate buffer also reduced standard deviations of the method and thus obviously stabilized the extraction system in some way.

In order to prove that the radioactivity remaining in the water phase after incubation was indeed in the glucuronide formed, the generally accepted criterion [13], based on the utilization of β-glucuronidase and on its specific inhibition by D-glucaro-1,4-lactone, was used. β-Glucuronidase treatment revealed that the increase in radioactivity in the water phase of the samples containing added UDPglucuronic acid was due to the glucuronide formed (table 2). After the addition of β-glucuronidase radioactivity in the aqueous phases of the tubes containing UDPGlcUA was at the level of the tubes containing no added UDPGlcUA. D-Glucaro-1,4-lactone completely abolished this effect of β-glucuronidase. The formation of radioactive p-nitrophenyl glucuronide was also confirmed by chromatography of the reaction mixture in ethanol–1 M ammoniumacetate (9:1) after incubation, with subsequent autoradiography.

The standard errors of the means of the present method in comparison with the older spectrophotometric methods [1] modified as [9, 10] are given in table 3. We can see that the usefulness of the radiochemical method compared with other methods increases when the reaction rate is decreased. The accuracy of determinations representing low activities is very important in kinetic studies of the enzyme. The theoretical sensitivity limit of the method depends on the specific activity of the radioactive aglycone. With the preparation commercially available today this is about 30 pmoles of the glucuronide, corresponding to 1000 cpm in the final counting. Due to this high sensitivity the method also enables the examination of tissues with low UDP glucuronyltransferase activities. With the present radiochemical method we were also able to demonstrate a p-nitrophenyl glucuronide synthesis by liver microsomes in the absence of added UDPGlcUA, as confirmed by autoradiography after paper chromatography in ethanol–1 M ammoniumacetate (9:1). This synthesis is obviously due to endogenous active D-glucuronic acid bound to the microsomal membranes.

Table 3
Standard errors of the means of the present radiochemical method and of the spectrophotometric methods based either on the measurement of the consumption of p-nitrophenol or formation of p-nitrophenyl glucuronide in different conditions.

UDPGlcUA concentration and incubation time	Method		
	Present	Spectrophotometric substrate consumption	Spectrophotometric product formation
0.5 mM, 10 min	1.32	12.5	17.5
1.5 mM, 15 min	1.28	7.08	14.7
4.0 mM, 20 min	1.14	[illegible].40	5.50

Modifications of the spectrophotometric methods with 0.1 ml of reaction mixtures were used [9, 10]. Fifteen experiments were performed in each group.

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