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In vitro cleavage of paracetamol glucuronide by human liver and kidney β-glucuronidase: determination of paracetamol by capillary electrophoresis

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Abstract

A capillary electrophoresis (CE) method was developed using paracetamol glucuronide as a novel probe for human β-glucuronidase activity. Using UV detection without prior sample clean-up procedures, fast and reliable quantitation of the released paracetamol was possible. The method showed good precision, accuracy and sensitivity with a limit of detection of 0.25 μ*M* (38 ng/ml) and a limit of quantitation of 1 μ*M* (151 ng/ml). The suitability of the method has been shown for enzyme kinetic studies using different liver and kidney homogenates, respectively. Our data clearly demonstrate that paracetamol glucuronide is cleaved by human β-glucuronidase thereby releasing paracetamol. The CE method presented is not only a valuable tool for measuring human β-glucuronidase activity, but also allows investigation of the contribution of deglucuronidation of paracetamol glucuronide to the disposition of paracetamol.

Keywords: Paracetamol glucuronide

1. Introduction

Paracetamol is a commonly used analgesic that is mainly metabolized by conjugation with glucuronic and sulphuric acid [1]. Glucuronide conjugates may accumulate during chronic therapy, particularly in patients with impaired kidney function [2,3]. A glucuronidation–deglucuronidation cycle can influence disposition and action of xenobiotics and endogenous compounds. Release of the parent compound from glucuronide conjugates has been suggested for drugs such as clofibric acid, lorazepam, and diflunisal, respectively [4–6]. One enzyme that contributes to cleavage of glucuronides is β-glucuronidase which is expressed in most tissues and body fluids [7]. Three synthetic substrates are commonly used for measuring β-glucuronidase activity: 4-nitrophenyl-β-D-glucuronide, phenolphthalein-β-glucuronide and 4-methylumbelliferyl-β-D-glucuronide (MUG) [8,9]. In contrast, paracetamol glucuronide is a pharmacologically relevant compound, that might be involved in a β-glucuronidase mediated

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glucuronidation–deglucuronidation cycle. Cleavage of paracetamol glucuronide by human β-glucuronidase has not been reported so far. However, competitive inhibition of cleavage of the synthetic substrate MUG by paracetamol glucuronide was demonstrated with human liver and kidney β-glucuronidase [10].

Investigation of paracetamol glucuronide cleavage by β-glucuronidase requires a sensitive and selective method for determination of paracetamol in which paracetamol glucuronide is effectively separated. There are different methods for determination of paracetamol described in the literature. The most commonly employed techniques are spectrophotometric quantitation [11], HPLC with UV detection [12] and immunoassays [13]. These techniques require more or less complex sample preparation procedures, whereas for capillary electrophoresis (CE) only a minimum of sample clean-up like centrifugation is required. Therefore in the last few years several CE methods have been developed for determination of paracetamol [14,15] using micellar electrokinetic capillary chromatography (MECC), which is a modification of CE combining the advantages of CE and chromatography. This technique is employed when paracetamol has to be separated effectively from plasma proteins [15]. However, these methods have not been used for separation of paracetamol glucuronide and paracetamol. For separation of glucuronides and their corresponding aglycones like morphine and its 3- and 6-glucuronide [16,17] or 7-hydroxycoumarin and its glucuronide [18], there are some CE methods described in the literature, whereas no data are available dealing with the separation of paracetamol glucuronide and paracetamol. We therefore developed a CE method for quantification of paracetamol following in vitro cleavage of paracetamol glucuronide by human β-glucuronidase.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade. Paracetamol (4-acetamidophenol) was from Fluka (Neu-Ulm, Germany), paracetamol glucuronide (4-acetamidophenyl-β-D-glucuronide), D-saccharic acid 1,4-lactone and bovine serum albumin were obtained from Sigma (Deisenhofen, Germany). Sodium tetraborate decahydrate, sodium hydroxide, sodium carbonate, sodium acetate and EDTA were supplied by Merck (Darmstadt, Germany). Triton X-100 and Tris base [tris(hydroxymethyl)aminomethane] were obtained from Serva (Heidelberg, Germany).

2.2. Preparation of human liver and kidney homogenates

Frozen human liver and kidney samples (obtained as surgical waste during partial hepatectomy or nephrectomy, respectively) were homogenized in 3 ml of 20 m*M* Tris–HCl, pH 7.4 at 4°C using an Ultra Turrax homogenizer (Bachofer, Reutlingen, Germany) for 3×30 s at full speed. Protein contents of homogenates were determined according to the method of Lowry et al. [19].

2.3. Incubation of homogenates with paracetamol glucuronide

Incubation mixtures contained 11.25 μg protein in 50 μl of assay buffer (50 m*M* sodium acetate, pH 5.0; 10 m*M* ETDA; 0.01% (w/v) bovine serum albumin; 0.1% (v/v) Triton X-100). Incubations were carried out for 2 h at 37°C in the presence of increasing amounts of paracetamol glucuronide (250 μ*M*–10 m*M*). The enzymatic reactions were stopped by adding 150 μl of 50 m*M* sodium carbonate solution and the mixture was centrifuged for 10 min at 13 000 rpm. The supernatant was analyzed by CE without further sample pretreatment. Inhibition experiments for β-glucuronidase of liver homogenate were carried out in the same way in the presence of 10 m*M* D-saccharic acid 1,4-lactone.

2.4. Standardization

Stock standard solutions of paracetamol glucuronide and paracetamol were prepared in assay buffer or in assay buffer containing heat-denatured liver homogenate (100°C, 15 min, 250 μg protein/ml) at concentrations of 25 and 10 m*M*, respectively and stored at −20°C. Working standard solutions were prepared from these stock solutions every two weeks and stored at −20°C until they were used. Cali-

bration curves showed that storage for 3 weeks or more led to partial degradation of paracetamol glucuronide resulting in increasing blanks.

Calibration samples were prepared by diluting the paracetamol stock solution in assay buffer containing heat-denatured liver homogenate (250 μg protein/ml). The final paracetamol concentrations were 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 or 100.0 μ*M*, respectively. Calibration samples were incubated in a 50 μl mixture containing 2.5 m*M* paracetamol glucuronide and 11.25 μg of protein for 2 h at 37°C, followed by addition of 150 μl sodium carbonate (50 m*M*) and a final centrifugation step. Since no sample clean-up procedure was used, no internal standard was added and paracetamol was quantified using external calibration. Calibration curves were obtained by plotting the areas of the paracetamol peak against the standard concentration.

2.5. Instrumentation and CE conditions

A Prince CE instrument from Lauerlabs (Emmen, The Netherlands) was used. The capillary was coupled to a 785A programmable UV detector (Applied Biosystems, Weiterstadt, Germany). Data acquisition was carried out using the Hyperdata Chromsoft software from Bischoff (Leonberg, Germany). CE was performed on a 75 cm×100 μm capillary (effective length 55 cm) from CS Chromatographie Service (Langerwehe, Germany). A 60 m*M* borate buffer, pH 11.0 was used for separation of paracetamol glucuronide and paracetamol. The buffer was freshly prepared every week and stored at 4°C until it was used. The buffer reservoir was changed before every CE series was started. Electrophoretic separation of paracetamol glucuronide and paracetamol was performed with a constant voltage of +18 kV resulting in a current of approximately 100 μA. Samples were injected by a pressure of 50 mbar for 0.07 min. Peak shape was improved using the sample stacking method by injecting purified water (0.07 min, 50 mbar) before and after the sample. The detector was set at 250 nm.

At the beginning of every series of analyses the capillary was rinsed with separation buffer for 10 min and equilibrated applying the separation voltage (+18 kV) for 20 min. The capillary had to be rinsed for 10 min with 1 *M* sodium hydroxide solution once a week to reduce adsorption of the analyte to the inner wall.

2.6. Assay validation

For determination of assay accuracy and variability various amounts of paracetamol were incubated in 50 μl of mixtures containing 2.5 m*M* paracetamol glucuronide and heat-denatured liver homogenate (11.25 μg protein). These mixtures were then analyzed alone or together with the incubation samples in every series of experiments as quality controls.

3. Results and discussion

The capillary electrophoresis method described here allows the determination of paracetamol following cleavage of paracetamol glucuronide by human liver and kidney β-glucuronidase. Except one centrifugation step for separation of proteins no further sample clean-up procedures are required. For this reason we did not use any internal standard and employed external standard calibration. Standardization was carried out using heat-denatured human liver homogenate instead of the native tissue homogenates. Migration times were 12.8 and 14.4 min for paracetamol glucuronide and paracetamol, respectively. Authenticity of paracetamol released by β-glucuronidase in liver and kidney homogenates was proven by several lines of evidence. First, retention times in calibration and biological samples were identical (Fig. 1). Second, no interfering peak appeared in the electropherograms at the paracetamol migration time in incubation blanks and calibration samples although paracetamol glucuronide was present as shown in Fig. 1. This fact also indicates that there was no paracetamol formation due to decomposition or nonspecific metabolism of paracetamol glucuronide. Third, cleavage of paracetamol glucuronide was completely inhibited by the specific β-glucuronidase inhibitor D-saccharic acid 1,4-lactone.

The separation buffer was changed prior to each CE series of analyses and remained stable for at least 40 samples. Larger sample series ($n>60$) resulted in migration time shifts which caused loss of separation

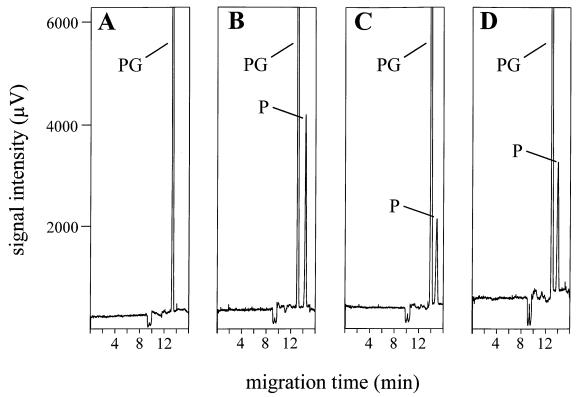

Fig. 1. Electropherograms of mixtures incubated for 2 h at 37°C in the presence of 5 m*M* paracetamol glucuronide and of 11.25 μg protein, respectively (paracetamol glucuronide: PG; paracetamol: P). A: Incubation blank containing heat-denatured liver homogenate. B: Calibration sample containing 50 μ*M* paracetamol and heat-denatured liver homogenate. C: Kidney homogenate after incubation with 5 m*M* paracetamol glucuronide. D: Liver homogenate after incubation with 5 m*M* paracetamol glucuronide.

efficacy and finally led to overlapping of paracetamol glucuronide and paracetamol peaks.

The method showed good linearity over the entire concentration range investigated (0.5–100 μ*M* paracetamol). A typical standard curve for paracetamol determination was $y=2120x-127$ ($r^2=0.9992$). There was an excellent correlation between the amount of paracetamol added and the quantitative result obtained by CE ($y=0.9979x+0.1940$, $r^2=0.9997$). The values of accuracy of the method are summarized in Table 1. Reproducibility of the method was tested by repeated analyses of samples containing heat-denatured human liver homogenate instead of native tissue homogenate spiked with different amounts of paracetamol. The paracetamol glucuronide concentration of these mixtures was 2.5 m*M*. Table 2 summarizes the values for the intra- and inter-assay variability. Reproducibility was better than 10% for the two higher concentrations (5 and 50 μ*M* paracetamol). For 1 μ*M* it increased slightly to approximately 12% and for lower concentrations the coefficient of variation was more than 35% whereas the bias was still acceptable with approximately 14%. Therefore the limit of quantification of paracetamol in our method was set at 1 μ*M* (151 ng/ml) and the limit of detection (signal-to-noise-ratio>3) was 0.25 μ*M* (38 ng/ml).

Table 1
Accuracy of the determination of paracetamol in incubation mixtures containing heat-denatured liver homogenate

Concentration added (μ*M*)	Concentratrion found (μ*M*)	Recovery (%)
0.5	0.49	98.5
1.0	1.04	104.0
2.5	2.56	102.4
5.0	4.81	96.2
10.0	9.79	97.9
25.0	26.40	105.6
50.0	50.55	101.1
100.0	99.50	99.5

Table 2
Intra- and inter-assay variability in the determination of paracetamol following incubation with heat-denatured liver homogenate

Concentration added (μ*M*)	*n*	Concentration found (μ*M*)	Bias (%)	C.V. (%)
Intra-assay variability				
0.5	6	0.43±0.16	−14.3	38.4
1.0	12	1.08±0.13	7.8	11.8
5.0	12	5.09±0.27	1.7	5.4
50.0	12	50.2±1.2	0.4	2.4
Inter-assay variability				
1.0	8	1.08±0.06	1.8	5.5
5.0	8	5.06±0.22	1.1	4.4
50.0	8	51.2±4.7	2.4	9.2

The applicability of the method described was proven by an enzyme kinetic study. Two samples of human liver and kidney homogenates, respectively, were incubated with different concentrations of paracetamol glucuronide in the range from 250 μ*M* to 10 m*M*. Preliminary experiments showed that paracetamol formation was linear for incubation time from 1 to 4 h and for protein contents from 1.125 to 22.5 μg. Therefore incubations were carried out for 2 h at 37°C in the presence of 11.25 μg of protein. The resulting electropherograms of a calibration sample, an incubation blank and two incubation samples containing 5 m*M* paracetamol glucuronide are depicted in Fig.1.

Paracetamol formation followed Michaelis–Menten kinetics for both tissues as it is shown in Fig. 2. The K_m values were similar for both types of tissue (2.2 and 2.1 for the two liver samples and 2.4 and 2.2 m*M* for the two kidney samples, respectively). These values are similar to the inhibition constant

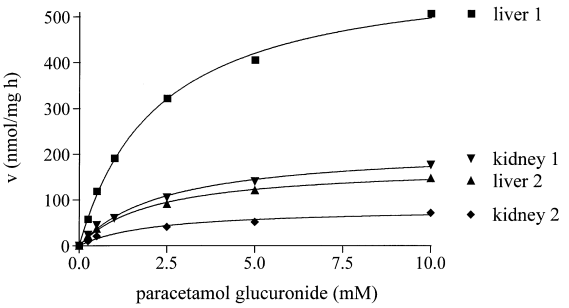

Fig. 2. Kinetics of paracetamol formation after incubation of paracetamol glucuronide with two different liver and kidney homogenates, respectively. Mixtures contained 11.25 μg protein and were incubated with different concentrations of paracetamol glucuronide at 37°C for 2 h.

(K_i) values (1.6 and 2.0 m*M* for liver and kidney, respectively) as determined by inhibition of cleavage of 4-methylumbelliferyl-β-D-glucuronide [10]. The maximum rates of paracetamol formation (V_{max}) showed pronounced variability due to the interindividual and organ specific expression of the enzyme. The individual values were 607.9 and 174.7 nmol/mg/h for the two liver samples and 214.3 and 81.4 nmol/mg/h for the two kidney samples, respectively.

Our data demonstrate that paracetamol glucuronide is cleaved by human β-glucuronidase which might have consequences for disposition of paracetamol in vivo since the enzyme could be involved in a glucuronidation–deglucuronidation cycle which modifies the amount of the active compound. Moreover our results show that paracetamol glucuronide can be used as a novel probe for β-glucuronidase activity in human tissue homogenates where the released paracetamol is measured by CE. The method is superior to conventional HPLC or spectrophotometry as it does not require sample clean-up. Only small amounts of chemicals (e.g., 1 ml of buffer solution per day) and no organic solvents are needed, therefore minimizing the costs of analysis. In summary, the speed, accuracy and precision of this method make it a valuable and cost-effective tool for the determination of human β-glucuronidase activity and for investigating the contribution of deconjugation of paracetamol glucuronide to disposition of paracetamol.

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