ELSEVIER

Journal of Chromatography B, 685 (1996) 181–184

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

High-performance liquid chromatographic quantification of 4-methylumbelliferyl-β-D-glucuronide as a probe for human β-glucuronidase activity in tissue homogenates

Bernhard Sperker, Monika Schick, Heyo K. Kroemer*

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstraße 112, Stuttgart 70376, Germany

Received 30 January 1996; revised 11 March 1996; accepted 11 March 1996

Abstract

An internally standardized HPLC method to determine the concentration of 4-methylumbelliferone liberated from 4-methylumbelliferyl-β-D-glucuronide by human β-glucuronidase was developed. The assay allows the precise and rapid measurement of specific enzyme activity in human tissue homogenates. Without prior extraction the incubation mixture can be separated using a C_8 column followed by fluorescence detection. The assay showed good accuracy and precision with a detection limit of 20 n*M* and a limit of quantification of 167 n*M*. The suitability of the method was shown in enzyme kinetic experiments with human liver homogenates.

Keywords: 4-Methylumbelliferyl-β-D-glucuronide; β-Glucuronidase; Enzymes

1. Introduction

Human β-glucuronidase (EC 3.2.1.31) is an acid hydrolase responsible for proteoglycan degradation in lysosomes. Genetic deficiency leads to lysosomal storage of undegraded glycosaminoglycans known as Sly syndrome [1]. Determination of β-glucuronidase acitivity is important for many clinically relevant questions as enzyme replacement therapy in lysosomal storage disease [2] and altered β-glucuronidase activities observed in many human diseases [3–5]. Moreover, β-glucuronidase may play a substantial role in human drug metabolism. First, glucuronide conjugates of drugs which often accumulate during chronic therapy may be cleaved by β-glucuronidase thereby releasing the active moiety [6,7]. Second, one approach to enhance selectivity of anticancer agents is to administer the glucuronidated compound as a prodrug (e.g., doxorubicin glucuronide) which is activated by high intra-tumor activity of β-glucuronidase [8]. In both instances exact determination of β-glucuronidase activity is pivotal to assess the effects of the enzyme for drug metabolism. For measuring β-glucuronidase activity three substrates are commonly used. *p*-Nitrophenyl-β-D-glucuronide and phenolphthalein-β-glucuronide are employed in spectrophotometrical assays [9,10] but the linear range of analysis is rather limited. Fluorometrical determination of 4-methylumbelliferone (MU) liberated from 4-methylumbelliferyl-β-D-glucuronide (MUG) provides a sensitive assay with a larger linear measuring range [11]. However, commonly used fluorescence photometers without any chromatographic separation cannot distinguish intrin-

*Corresponding author.

PII S0378-4347(96)00139-9

sic fluorescence of MUG from MU-fluorescence. Moreover the conventional approach does not allow for internal standardization. We therefore established a precise HPLC method with internal standardization and applied it for the quantification of β-glucuronidase activity in tissue homogenates.

2. Experimental

2.1. Chemicals

All solvents used were of HPLC quality and chemicals were of analytical grade. MU, MUG and D-saccharic acid 1,4-lactone (SL) were from Sigma (Deisenhofen, Germany). Tetrabutylammonium hydrogensulfate and 9-chloromethylanthracene (9-CMA) were from Fluka (Buchs, Switzerland).

2.2. Preparation of liver homogenates and enzymatic reaction

A 300-mg amount of frozen human liver samples (obtained as surgical waste during partial hepatectomy) 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 were determined according to the method of Lowry et al. [12].

Incubation mixtures contained 2.25 μg of protein in 50 μl of assay buffer [200 m*M* sodium acetate, pH 5; 10 m*M* EDTA; 0.01% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100]. In the presence of increasing amounts of MUG (156 μ*M*–5 m*M*) incubations were carried out at 37°C for 30 min. The enzymatic reaction was stopped by adding 150 μl of 200 m*M* sodium carbonate. Subsequently 4 μl of 5 m*M* 9-CMA in dimethyl sulfoxide were added as an internal standard and the mixture was centrifuged for 5 min at 13 000 rpm to separate from residual particles and precipitated protein.

2.3. Standardization

Stock standard solutions of MU and MUG were prepared in assay buffer at concentrations of 1 m*M* and 50 m*M*, respectively, and stored at −20°C until they were used. Working standard solutions were diluted in assay buffer from the stock solutions.

Calibration was done by adding increasing amounts of MU (0.167 μ*M*–20 μ*M*) to mixtures containing heat-denatured liver homogenate (15 min, 100°C) and calibration curves were obtained by plotting the peak-area ratios of MU and the internal standard 9-CMA against the MU concentration.

2.4. HPLC conditions

The modular liquid chromatographic system (Shimadzu, Duisburg, Germany) consisted of a LC-9A pump unit, a SIL-9A auto-injector with a 100-μl loop, a RF 530 fluorescence detector (excitation at 355 nm, emission at 460 nm) and a C-R6A integrator. The analytical column was a C_8 (Nucleosil 100, 5 μm, 125×4.6 mm I.D.) and the precolumn was a Polygosil C_{18}, 10 μm (Bischoff, Leonberg, Germany). The mobile phase consisted of methanol–10 m*M* tetrabutyl-ammonium hydrogensulfate buffer (50:50, v/v) and the flow-rate was 1.0 ml/min.

2.5. Assay validation

To determine the accuracy and precision of the assay, quality controls were created by adding different concentrations of MU to the incubation mixtures containing heat-inactivated liver homogenate and stored in aliquots at −20°C. Stability of quality controls was monitored after 24 and 48 h incubation at room temperature and 3 freeze–thaw cycles, respectively.

3. Results and discussion

Many methods for quantification of β-glucuronidase activity have been reported in the literature [9–11]. Most of them, however, have not been validated and standardized. The assay described here allows a precise and internally standardized determination of β-glucuronidase acitvity in human liver homogenates. Using 4 MUG as an enzyme substrate and HPLC analysis one can combine the sensitivity of a fluorometrical assay with the advantage of internal standardization and separation of different components in the assay mixture.

MUG, MU and 9-CMA were readily separated with retention times of 2.3, 4.5 and 20 min, respectively. Authenticity of the MU liberated by β-glucuronidase in liver homogenates was proven by several lines of evidence. Firstly we observed identical retention times in calibration samples and biological samples, secondly, there was a concentration-dependent inhibition of MUG-cleavage by the specific β-glucuronidase inhibitor SL, thirdly, measurement of blanks (liver homogenate plus 9-CMA) did not reveal any interference at the MU retention time and finally, use of heat-denatured liver homogenates for calibration did not give rise to formation of MU although MUG was present (Fig. 1).

The method showed good linearity over a concentration range of 167 n*M*–20 μ*M*. At the lowest concentration the signal-to-noise ratio was better than 10. A representative calibration curve for the determination of MU was $y=0.469x+0.0341$ ($r=0.9999$). Table 1 summarizes the data concerning the accuracy of our assay. A close correlation between the concentration added and the concentration measured by HPLC analysis ($y=0.98x+84.1$, $r=0.9995$) was observed.

Precision was determined by multiple analyses of quality controls consisting of denatured liver homogenates, containing MUG and 9-CMA spiked with known amounts of MU. Intra-assay and inter-assay variabilities are shown in Table 2. Reproducibility was between 3 and 8% when multiple samples were measured within one assay and between 6 and 10% when determined in different assays. At concentrations lower than 167 n*M* variability is smaller than 10%, whereas the deviation of the concentration found from the concentration added rises up to 24%. The limit of detection is 20 n*M* (signal-to-noise ratio=3).

Enzyme kinetic studies show the applicability of the method described. Human liver homogenates were incubated with different concentrations of MUG in the range from 156 μ*M* to 5 m*M*. The concentration of MU released from MUG was measured and the rate of MU formation was plotted against the MUG concentration (Fig. 2). The liver samples examined showed a Michaelis–Menten type kinetics with K_m values of 1.77 and 2.34, and V_{max} values of 664 n*M*/mg h and 3729 n*M*/mg h, respectively.

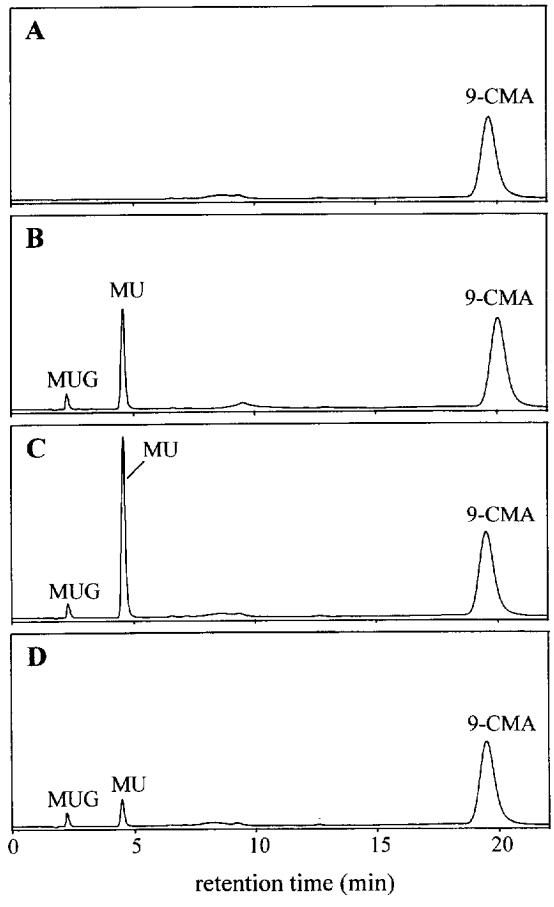

Fig. 1. HPLC chromatograms of mixtures incubated at 37°C for 30 min each containing 2.25 μg of protein. As internal standard 4 μl of 5 m*M* 9-CMA were added subsequently to a total volume of 200 μl. (A) Blank consisting of human liver homogenate. (B) Calibration sample containing 3.33 μ*M* MU and 2.5 m*M* MUG in denatured liver homogenate. (C) Liver homogenate after incubation with 2.5 m*M* MUG. (D) Liver homogenate after incubation with 2.5 m*M* MUG in the presence of 100 μ*M* SL.

These data demonstrate the suitability of our HPLC assay for the measurement of MU liberated from MUG by human liver β-glucuronidase. The K_m values observed are similar to the values reported in previous experiments [13,14]. Precision and accuracy of the method makes it a valuable tool in many clinical and pharmacological applications requiring the determination of β-glucuronidase activity in human tissues.

Table 1
Accuracy of the determination of MU in incubation mixtures containig liver homogenate

Concentration added (n*M*)	Concentration found (n*M*)	Recovery (%)
167	180	109.3
333	339	101.8
740	746	100.8
1000	1040	104.0
3333	3150	94.5
4500	4726	105.0
10 000	10 290	102.9
20 000	19 460	97.3

Table 2
Precision of the determination of MU following incubation with denatured liver homogenates

Concentration added (n*M*)	*n*	Concentration found (n*M*)	Bias (%)	C.V. (%)
Intra-assay variability				
167	5	180±15	9.3	8.3
740	5	746±23	0.81	3.1
4500	5	4726±231	5.0	4.9
20 000	5	20 622±759	3.1	3.7
Inter-assay variability				
740	7	680±68	−8.1	10
4500	7	4250±278	−5.6	6.5
20 000	7	19 490±1279	−2.6	6.6

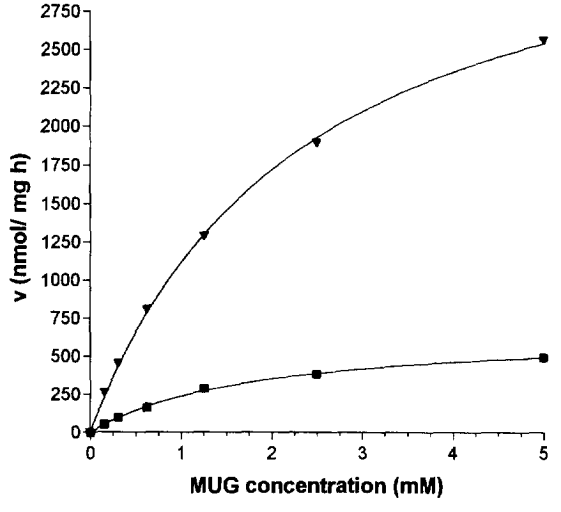

Fig. 2. Kinetics of MU formation after incubation of MUG with two different human liver homogenates. Mixtures contained 2.25 μg protein and different concentrations of MUG were incubated at 37°C for 30 min.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany), Grant No. Kr 945/4-1.

References

[1] W.S. Sly, B.A. Quinton, W.H. McAlister and D.L. Rimoin, J. Pediatr., 82 (1973) 249.
[2] C. Vogler, M. Sands, A. Higgins, B. Levy, J. Grubb, E.H. Birkenmeier and W.S. Sly, Pediatr. Res., 34 (1993) 837.
[3] H. Ohta, M. Ono, C. Sekiya and M. Namiki, Clin. Chim. Acta, 208 (1992) 9.
[4] A.K. Saha, R.H. Glew, D.P. Kotler and J.A. Omene, Clin. Chim. Acta, 199 (1991) 311.
[5] C. Camisa, A. Hessel, C. Rossana and A. Parks, Dermatologica, 177 (1988) 341.
[6] P.J. Meffin, D.M. Zilm and J.R. Veenendaal, J. Pharmacol. Exp. Ther., 227 (1983) 732.
[7] F.M. Brunelle and R.K. Verbeeck, Biochem. Pharmacol., 46 (1993) 1953.
[8] K. Bosslet, J. Czech, G. Seemann, C. Monneret and D. Hoffmann, Cell Biophys., 24/25 (1994) 51.
[9] W.H. Fishman, K. Kato, C.L. Anstiss and S. Green, Clin. Chim. Acta, 15 (1967) 435.
[10] G. Szasa, Clin. Chim. Acta, 15 (1967) 275.
[11] A.G. Rao and P. Flynn, Biotechniques, 8 (1990) 38.
[12] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[13] F.E. Brot, C.E. Bell and W.S. Sly, Biochemistry, 17 (1978) 385.
[14] M.C. Gehrmann, M. Opper, H.H. Sedlacek, K. Bosslet and J. Czech, Biochem. J., 301 (1994) 821.