Journal of Chromatography, 424 (1988) 136–140
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3944

Note

Determination of phenol sulphotransferase activity by high-performance liquid chromatography

TARJA HONKASALO and ERKKI NISSINEN*

Orion Pharmaceutica, Research Center, P.O. Box 65, 02101 Espoo (Finland)

(First received June 30th, 1987; revised manuscript received September 1st, 1987)

Phenol sulphotransferase (PST; EC 2.8.2.1) catalyses the sulphate conjugation of phenolic and catechol drugs, other phenolic xenobiotic compounds and catechol neurotransmitters in the presence of cofactor 3′-phosphoadenosine-5′-phosphosulphate (PAPS) [1]. The various organs of human and animal bodies contain at least two forms of PST [2,3]. One form of the enzyme is relatively thermolabile (TL form) and it catalyses the sulphate conjugation of micromolar concentrations of dopamine and other catechols, as well as other phenolic monoamines, and is therefore also called the M form [4]. The other form is thermostable (TS form) and catalyses the sulphate conjugation of micromolar concentrations of phenol, *p*-nitrophenol (pNP) and other simple phenols and is consequently also called the P form [5]. At millimolar concentrations pNP and phenol are also capable of serving as substrates for the TL form of the enzyme [6].

Virtually all of the circulating dopamine in humans and 70–80% of circulating norepinephrine are sulphate-conjugated [7]. Dopamine sulphate is also formed in the human central nervous system [8]. These observations raise the possibility that, in addition to its important role in drug metabolism, PST might also play a role in the termination of neurotransmitter actions of catecholamines [1].

PST activity can be determined by a large number of different techniques. These include spectrophotometry [9], fluorimetry [10] and thin-layer chromatography [11]. The most frequently used methods are radiochemical assays, which include either salt precipitation [12] or ion-exchange chromatography [13, 14]. Although these methods are sensitive, they require the use of rather expensive isotopes. The latter method also involves time-consuming extractions.

Here we describe a fast and sensitive assay, which can be used to determine

0378-4347/88/$03.50

both the TL and TS forms of PST activity in different tissues. The method is based on the separation and quantification of *p*-nitrophenylsulphate (pNPS), which is formed from pNP, by high-performance liquid chromatography (HPLC), with UV detection.

EXPERIMENTAL

Chemicals

pNP was from J.T. Baker (Deventer, The Netherlands). pNPS and PAPS were from Sigma (St. Louis, MO, U.S.A.). Adenosine-5′-triphosphate (ATP) was from Boehringer Mannheim (Mannheim, F.R.G). Dithiothreitol (DTT) and 2,4-dichloro-6-nitrophenol (DCNP) were from Aldrich-Chemie (Steinheim, F.R.G.). Methanol (HPLC grade) was from Orion Pharmaceutica (Espoo, Finland). All other reagents were of analytical grade and purchased from commercial sources.

Tissue preparation

Female rats (Han:WIST) weighing ca. 200 g were killed with carbon dioxide. Livers were quickly removed and washed with cold 0.9% sodium chloride. Tissues were kept at −80°C until they were homogenized. For the PST determination the livers were homogenized 1:5 (w/v) in ice-cold 10 m*M* sodium phosphate buffer (pH 7.2), which contained 10 m*M* DTT. The homogenate was centrifuged at 15 000 *g* for 20 min at 4°C, and the supernatant was centrifuged at 100 000 *g* for 60 min at 4°C. The final supernatant was kept in small aliquots at −20°C until assayed. Protein determinations were carried out using a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, U.S.A.).

PAPS generating system

Preparation of the PAPS was performed using the method of Van Kempen and Jansen [10] with some modifications. The buffer was 10 m*M* sodium phosphate (pH 7.2), and the final supernatant was concentrated in an Amicon Model 8050 ultrafiltration apparatus using PM 10 membrane (Amicon, Danvers, MA, U.S.A.). The final protein concentration in the incubation mixture was 70 mg.

Assay procedure

Determination of PST activity was carried out in a total volume of 250 μl, which contained the following components: 50 μl of 10 m*M* sodium phosphate buffer (pH 7.2), 50 μl of enzyme preparation and 100 μl of PAPS generating system. The reaction was started by adding 50 μl of 12.5 m*M* pNP when assaying the activity of PST TL form and 50 μl of 50 μ*M* pNP when determining the activity of PST TS form. The blank was incubated without PAPS. The mixture was incubated for 30 min at 37°C except for the study of the time course. The reaction was stopped by the addition of 25 μl of 4 *M* perchloric acid. Protein precipitate was removed by centrifugation. A 20-μl aliquot was injected into the liquid chromatograph.

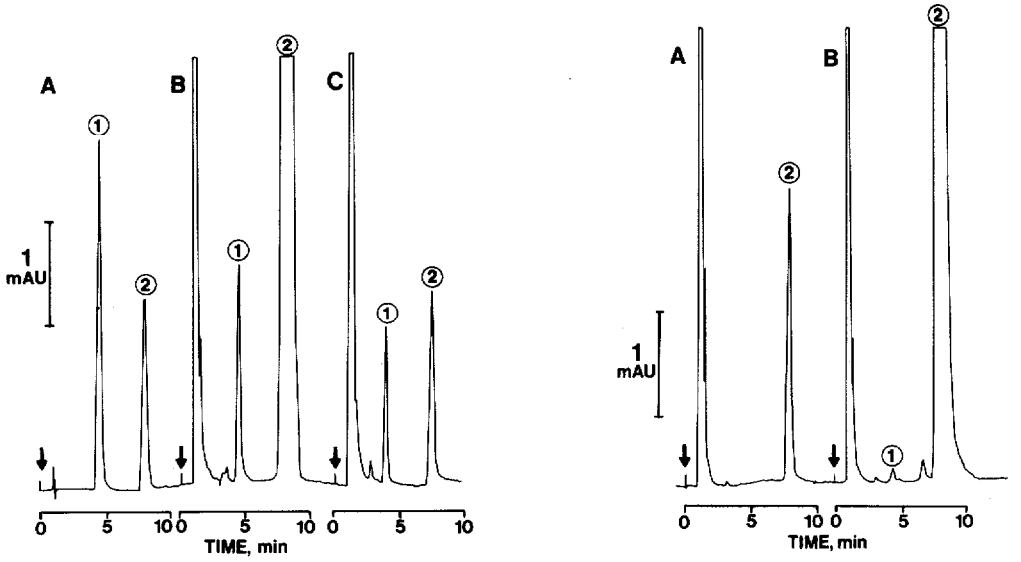

Fig. 1. Chromatograms obtained using (A) standards, (B) the TL form of rat liver PST and (C) the TS form of rat liver PST. Both the TL and TS activities were determined with 0.5 mg of protein. Chromatographic conditions: 5-μm Viosfer-ODS column (150×4 mm I.D.); mobile phase, 25% methanol in 50 m*M* sodium phosphate (pH 3.0); flow-rate, 1.5 ml/min; detection at 300 nm; sensitivity, 0.005 a.u.f.s.; injection volume, 20 μl. Peaks: 1=pNPS, 2=pNP.

Fig. 2. Chromatograms obtained using (A) the TS form of rat liver PST without PAPS preparate and (B) the TL form of rat liver PST with 5 m*M* DCNP. Other conditions are same as in Fig. 1.

Inhibition studies

For studies involving DCNP, the samples were preincubated for 20 min at 37°C in the presence of various concentrations of DCNP, but without pNP (substrate). The control samples had buffer in place of DCNP.

Chromatography

The HPLC system consisted of a Waters Model 6000 A pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne Model 7125 injector with 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.) and a 150 mm×4 mm I.D., 5-μm Viosfer ODS column (Violet, Rome, Italy) fitted with a 10-μm C_{18} guard column (RCSS, Guard PAK, Waters Assoc.). The eluted components were detected by UV absorption at 300 nm with a Kratos Spectroflow 773 absorbance detector (Kratos Analytical, Ramsey, NJ, U.S.A.). The mobile phase consisted of 50 m*M* sodium phosphate in 25% methanol. The pH was adjusted to 3.0 with sodium hydroxide. The flow-rate was 1.5 ml/min. The enzyme activity was calculated as nmol pNPS formed per min per mg protein.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained with crude rat liver homogenate. The retention time for the product pNPS is 4.8 min and for the substrate pNP

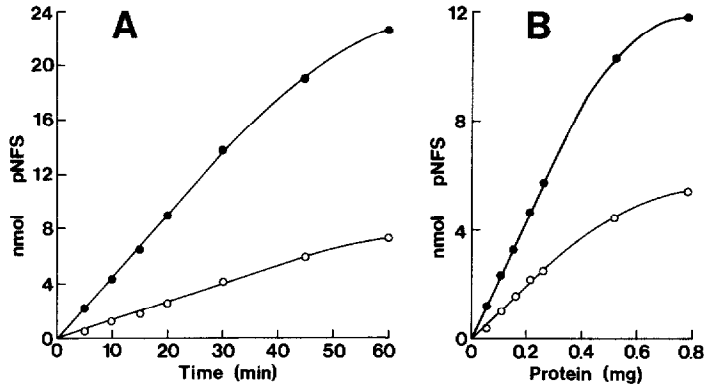

Fig. 3. (A) Time course of enzymatic pNPS formation using 0.5 mg of protein: (○) TS form; (●) TL form. (B) The linearity of the PST assay using increasing amounts of rat liver preparation: (○) TS form; (●) TL form. The reaction was stopped after 30 min.

9.0 min (Fig. 1A). Fig. 1B describes the chromatographic pattern of the PST TL form and Fig. 1C that of the PST TS form. The blank (Fig. 2A) shows that, under our experimental conditions the enzyme was totally inactive without the addition of PAPS. The presence of 5 m*M* DCNP, a specific PST inhibitor, in the incubation mixture caused a 96% inhibition in the activity of the PST TL form (Fig. 2B), which confirms that peak 1 (pNPS) is formed from pNP by the action of PST.

The calibration curve for pNPS indicated a linear relationship between the peak height and the amount of pNPS from 0.065 to 15 nmol. The detection limit at a signal-to-noise ratio of 1:2 was 15 pmol, making it possible to detect very low PST activities.

The rate of pNPS formation was linear for both forms of PST for an incubation time up to 45 min (Fig. 3A). The amount of pNPS increased linearly with protein concentration for both forms of PST up to 0.5 mg (Fig. 3B). The PST TL activity in rat liver ($n=6$) with 2.5 m*M* pNP as substrate was 1.88 ± 0.18 nmol/min per mg protein. The reproducibility of the assay using the standard procedure was 1.9%, expressed as a coefficient of variation. This result reflects the accuracy of HPLC with UV detection in the assay of enzyme activities.

The Michaelis–Menten constant (K_M) for the TL form of rat liver PST was found to be 1.15 ± 0.25 m*M* ($n=3$) and a V_{max} value of 3.55 ± 0.15 nmol/min per mg protein was calculated. The K_M value for the TS form of rat liver PST was 2.30 ± 0.30 μM ($n=2$) and a V_{max} value of 340 ± 40 pmol/min per mg protein was obtained. These values are very similar to those that have been reported for the human brain [1] and platelet [6] enzyme.

These two forms of PST have also different sensitivities to inhibition caused by DCNP. The TS form is more sensitive to DCNP inhibition than the TL form [15]. By using the described HPLC assay system, the following IC_{50} values (IC_{50} is the concentration of DCNP which causes 50% inhibition of PST activity) were obtained: 400 μM for the TL form and 7.3 μM for the TS form. These results were virtually identical with those found for human platelets [6, 16].

The PAPS generating system was comparable with the commercial PAPS as cofactor for this PST assay. A 100-μl amount of the PAPS generating system containing 70 mg of protein in the final incubation mixture produced as much pNPS as 20 μM commercial PAPS, which is the saturating amount. Also the inhibition studies gave the same results irrespective of the source of PAPS. The use of the PAPS generating system can be justified since it is easy to use and can be preserved for several months at $-20°C$.

The feasibility of the described PST assay was tested by determining the activity of the PST TL form in human platelets and jejunum, in rat brain and in guinea pig ileum. The activity of the PST TS form was determined in human jejunum and in guinea pig ileum (results not shown).

HPLC with UV detection at 280 nm has been used previously for determination of pNP conjugates in rat plasma and urine [17]. Here we describe a new method to assay the activities of the two forms of the PST enzyme by using HPLC with UV detection at 300 nm. At this wavelength pNPS still absorbs fairly well without interference of PAPS. The method is precise, sensitive and rapid, allowing the handling of ca. 40 samples per day, which makes it useful for rapid screening and inhibition studies of both forms of PST in different tissues.

REFERENCES

1 W.F. Young, Jr., H. Okazaki, E.R. Laws, Jr. and R.M. Weinshilboum, J. Neurochem., 43 (1984) 706.
2 J.A. Roth and A.J. Rivett, Biochem. Pharmacol., 31 (1982) 3017.
3 T.P. Mavs, R.K. Pearson, R.J. Anderson, L.C. Woodson, C. Reiter and R.M. Weinshilboum, Biochem. Pharmacol., 31 (1982) 849.
4 G. Rein, V. Glover and M. Sandler, in M. Sandler and E. Usdin (Editors), Phenolsulphotransferase in Mental Health Research, Macmillan, London, 1981, pp. 98–126.
5 R.M. Weinshilboum, Fed. Proc., Fed. Am. Soc. Exp. Biol., 45 (1986) 2223.
6 C. Reiter, G. Mwaluko, J. Dunnette, J. Van Loon and R.M. Weinshilsboum, Naunyn-Schmiedeberg's Arch. Pharmacol., 324 (1983) 140.
7 J.A. Rivett, B.J. Eddy and J.A. Roth, J. Neurochem., 39 (1982) 1009.
8 G.M. Tyce and D.K. Rorie, J. Neurochem., 39 (1982) 1333.
9 R.K. Banerjee and A.B. Roy, Biochim. Biophys. Acta, 151 (1986) 573.
10 G.M.J. Van Kempen and G.S.I.M. Jansen, Anal. Biochem., 46 (1972) 438.
11 R.D. Sekura, C.J. Marcus, E.S. Lyon and W.B. Jakoby, Anal. Biochem., 95 (1979) 82.
12 A. Foldes and J.L. Meek, Biochim. Biophys. Acta, 327 (1973) 365.
13 A. Baranczyk-Kuzama, R.T. Borchardt, S.C. Schasteen and C.L. Pinnick, in M. Sandler and E. Usdin (Editors), Phenolsulphotransferase in Mental Health Research, Macmillan, London, 1981, pp. 55–73.
14 R.T. Borchardt, A. Baranczyk-Kuzama and C.L. Pinnick, Anal. Biochem., 130 (1986) 334.
15 R.M. Weinshilbaum, Selected Topics Clin. Enzymol., 2 (1982) 373.
16 G. Rein, V. Glover and M. Sandler, Biochem. Pharmacol., 31 (1982) 1893.
17 G. Diamond and A. Quebbemann, J. Chromatogr., 177 (1979) 368.