SULPHATE CONJUGATION OF p-HYDROXYTRIAMTERENE BY PLATELET PHENOL SULPHOTRANSFERASE: ASSAY CONDITIONS AND CORRELATION WITH METABOLISM IN MAN

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1 Sulphate conjugation catalyzed by phenol sulphotransferase (PST) is an important pathway in the metabolism of many drugs including triamterene. Variations in PST activity in an easily obtained tissue such as the platelet might reflect individual differences in the sulphate conjugation in other organs and tissues. Human platelets contain at least two forms of PST, a thermolabile (TL) form for which dopamine is a substrate and a thermostable (TS) form for which low concentrations of p-nitrophenol serve as a substrate.

2 p-OH-triamterene, the major metabolite of triamterene, is conjugated with sulphate in vivo. p-OH-triamterene was a substrate for platelet PST with an apparent Michaelis-Menten value of 26 μM. Thermal stability studies indicated that p-OH-triamterene was a substrate for only the TS form of platelet PST.

3 When platelet homogenates from 29 individual subjects were tested, there was a significant correlation between PST activities measured with 4 μM p-nitrophenol and with p-OH-triamterene (r = 0.985, P < 0.0001) but not between activities measured with dopamine and with p-OH-triamterene (r = 0.023, P > 0.2). These results confirmed that p-OH-triamterene was a substrate for only the TS form of human platelet PST.

4 The same 29 subjects were treated with 1 mg/kg of triamterene orally. 24-h urinary excretions of triamterene, p-OH-triamterene and p-OH-triamterene sulphate averaged 15.3%, 6.3% and 78.4%, respectively, of the total of triamterene plus measured metabolites excreted. The excretion of triamterene plus the two metabolites averaged 43.1 ± 2.6% (mean ± s.e. mean) of the ingested dose. There was not a significant correlation between the proportion of p-OH-triamterene excreted as sulphate conjugate and the activities of either the TS or TL forms of platelet PST activity.

Introduction

Triamterene is a widely used diuretic. In man triamterene is metabolized to form p-OH-triamterene which is then conjugated with sulphate (Lehmann, 1965; Andrasch et al., 1971; Gundert-Remy et al., 1979). Triamterene and its metabolites have been found in the kidney stones of some patients who have taken the drug chronically (Ettinger et al., 1979; 1980; Patel, 1981). Since p-OH-triamterene is less soluble than either the parent compound or its sulphate conjugate (Werness et al., 1982), it is possible that individual variations in the metabolism of triamterene might contribute to the relative risk for the development of nephrolithiasis during therapy with the drug.

The sulphate conjugation of phenolic compounds like p-OH-triamterene is catalyzed by phenol sulphotransferase (E.C. 2.8.2.1, PST; Roy, 1981). PST activity is present in a variety of human tissues including the blood platelet (Hart et al., 1979; Anderson & Weinshilboun, 1980; Anderson et al., 1981). Platelets contain at least two independently regulated forms of PST activity with different physical properties and different substrate specificities (Rein et al., 1981; Reiter & Weinshilboun, 1981; 1982a). One form of the enzyme is relatively thermolabile (TL) and catalyzes the sulphate conjugation of α-methylcatecholamines and of dopamine and other monoamines (Reiter & Weinshilboun, 1981; 1982a; Mwaluko & Weinshilboum, 1982). The other form is thermostable (TS) and catalyzes the sulphate conjugation of micromolar concentrations of phenol and
Subjects from smokers and blood received measurement the samples subject 24 h each were conditions and 24 h later for assayed (Reiter p-nitrophenol, 1981; 1982a, 1982b; 1982c).

The experiments described here were performed to test further the hypothesis that variations in platelet PST activity might reflect variations in the sulphate conjugation of drugs. 

p-OH-triamterene was used as a model compound. Paracetamol is a substrate for both the TS and TL forms of platelet PST (Reiter & Weinshilboum, 1981; 1982a), and a significant correlation was found between the activities of both forms of platelet PST and the proportion of paracetamol excreted as the sulphate conjugate (Reiter & Weinshilboum, 1982b; 1982c).

The experiments described here were performed to test further the hypothesis that variations in platelet PST activity might reflect variations in the sulphate conjugation of drugs. p-OH-triamterene was used as a model compound. It was necessary first to determine whether p-OH-triamterene was a substrate for platelet PST, and, if so, whether it was a substrate for the TS or TL form of the enzyme. It was then possible to determine whether variations in platelet PST activity reflected individual variations in the sulphate conjugation of p-OH-triamterene in vivo.

Methods

Subjects and study procedure

Twelve women and seventeen men participated in the study. The average age of the women was 29.5 ± 7.4 years (mean ± s.d.) and the average age of the men was 30.7 ± 6.4 years. The subjects were not related, suffered from no acute or chronic illness, were nonsmokers and were taking no medications. All subjects were white. Each subject fasted overnight and had a blood sample withdrawn between 07.30-08.30 h for the measurement of platelet PST activity. He or she then received a 1 mg/kg oral dose of powdered triamterene that was taken with water. Food was permitted 2 h after ingestion of the drug. Urine from each subject was collected for 24 h, and the urine samples were assayed immediately for triamterene and p-OH-triamterene sulphate. A 20 ml aliquot of each 24 h urine sample was stored at −20°C, and was assayed 24 h later for p-OH-triamterene.

Blood samples used to establish optimal PST assay conditions were obtained from randomly selected adult blood donors at the Mayo Clinic Blood Bank. All subjects gave written informed consent. This study was approved by the Mayo Clinic Human Studies Committee.

Platelet preparation

Platelets were isolated and platelet homogenates were prepared as described elsewhere (Anderson & Weinshilboum, 1980; Anderson et al., 1981). All platelet homogenates were prepared within 1 h of the time that the blood samples were obtained. Pooled platelet homogenates from four to six individuals were used in all experiments except those in which platelet PST activities of individual subjects were determined.

PST assay

Two assay procedures were used to measure platelet PST activity. One procedure was used when dopamine and p-nitrophenol were substrates and the other was used when p-OH-triamterene was the substrate. When 60 µM dopamine and 4 µM p-nitrophenol were used as substrates, PST activity was measured by the method of Foldes & Meek (1973) as modified by Anderson & Weinshilboum (1980). The only change made in the method described previously was a 50% reduction in the volumes of all reagents to reduce the expense of radioactive isotope.

The second assay procedure was used when p-OH-triamterene was the sulphate acceptor substrate. One hundred µl of a 40-fold diluted platelet homogenate was used as the enzyme source. The final concentration of p-OH-triamterene was 70 µM. The assay was performed exactly as it was with dopamine and p-nitrophenol as substrates up to the point at which the reaction was terminated. The reaction was stopped by heating the tubes in a boiling water bath for 2 min. One ml of 15 mM ammonium bicarbonate was then added to the reaction tubes, and the contents of the tubes were transferred to 3 × 7 mm ECTEOLA Cellulose ion exchange columns that had been formed in the tips of 6 inch disposable plastic transfer pipettes. The ECTEOLA Cellulose used to make the columns had been equilibrated with 25 mM ammonium bicarbonate. Prior to sample application the columns were ‘washed’ with three 2 ml aliquots of 15 mM ammonium bicarbonate. After the samples were applied, the columns were eluted with 3 ml of 50 mM ammonium bicarbonate. This first 3 ml sample was discarded. The columns were then eluted with two 4 ml aliquots of 50 mM ammonium bicarbonate. These aliquots were collected in a 20 ml glass scintillation counting vial. Ten ml of RPI 3a70 was added to the vials, and the radioactivity was measured in a Beckman LS-7500 liquid scintillation counter.

One unit of PST activity represented the formation of 1 nmol of product per hour of incubation at 37°C. Results for both assay procedures were expressed per 10⁸ platelets, a method that has been shown to reduce the variance of the assay when compared with results expressed per mg platelet protein (Anderson et al., 1981).
**Thermal stability experiments**

Thermal stability experiments were performed exactly as previously described (Reiter & Weinsilboum, 1982a).

**Synthesis of p-OH-triamterene**

*p*-OHTriamterene was synthesized by the method of Hawes & Gorecki (1977). The identity of the compound was verified by ultraviolet and by infrared spectroscopy. In addition, behaviour of this compound during high performance liquid chromatography in two different solvent systems was identical with that of samples of authentic *p*-OHTriamterene supplied by Dr D.E. Rollins of the University of Utah, Salt Lake City, UT, and by Smith Kline and French Laboratories, Philadelphia, PA. The authenticity of both of the donated samples had been verified by mass spectroscopy.

**High performance liquid chromatography**

Urinary triamterene, *p*-OHTriamterene and *p*-OHTriamterene sulphate were measured with a modification of the high performance liquid chromatography procedure described by Werness et al. (1982).

**Kinetic analysis**

Michaelis-Menten ($K_m$) values were estimated by the method of Wilkinson (1961) with a computer program written by Cleland (1963). A Hewlett-Packard Model 9845B computer was used for these calculations.

**Materials**

[^35S]-3'phosphoadenosine - 5' - phosphosulphate ([^35S]-PAPS) (2.0–4.5 Ci/mmol) was purchased from New England Nuclear Corporation, Boston, MA. *p*-Nitrophenol, dithiothreitol (Cleland's reagent) and bovine serum albumin were purchased from Sigma Chemical Company, St Louis, MO. Dopamine- HC1 was obtained from Calbiochem, San Diego, CA. ECTEOLA Cellulose (Cellex E) was purchased from BioRad Laboratories, Richmond, CA. *p*-Toluene-sulphonic acid, dihydropryan, and 2,4,7-triamino-6-*p*-tetrahydropranylxyloxyhexylpteridine were purchased from Aldrich Chemical Company, Milwaukee, WI. Triamterene and *p*-OHTriamterene sulphate were donated by Smith Kline and French Laboratories, Philadelphia, PA.

**Results**

**Ion exchange chromatography PST assay procedure**

Many sulphate conjugated compounds are precipitated by the barium hydroxide step used to terminate the PST reaction in the assay procedure described by Foldes & Meek (1973). Precipitation of the reaction product gives the false impression that such compounds are poor substrates (Roy, 1981). To test whether *p*-OHTriamterene sulphate might be precipitated by barium, a pooled platelet homogenate was assayed for PST activity with *p*-OHTriamterene as a substrate. The reaction was terminated in separate reaction tubes either by barium precipitation as described by Foldes & Meek (1973) or by heating the samples. Adequate water was added to the heated sample to make the volume identical with that in the sample subjected to barium treatment. The *p*-OHTriamterene sulphate in both samples was then isolated by high performance liquid chromatography, and the radioactivity in the reaction product was measured. Only 16% as much radioactively labeled *p*-OHTriamterene sulphate was present in the sample precipitated with barium as in an identical sample that had not been exposed to barium. Therefore, the Foldes & Meek (1973) procedure was inappropriate for use with this substrate, and ion exchange chromatography as described by Baranzyk-Kuzma et al. (1981) and modified by Mwaluko & Weinsilboum (1982) was used to separate the radioactively labeled reaction product from radioactive PAPS in the PST assay when *p*-OHTriamterene was used as a substrate.

The elution pattern obtained from such an ion exchange column is shown in Figure 1. In this case the assay procedure was modified so that the column was eluted with four 1 ml fractions followed by a series of fractions that were 2 ml in volume. The radioactive peak of the *p*-OHTriamterene sulphate formed during the reaction was well separated from the large, late radioactive peak that represented the elution of[^35S]-PAPS (Figure 1). For routine assays only the fractions containing *p*-OHTriamterene sulphate were collected (see Methods).

**Effect of enzyme quantity and incubation time on PST activity**

When *p*-OHTriamterene was used as a substrate with a pooled platelet homogenate, there was a linear increase in product formation with increasing quantity of homogenate ranging from dilutions of 16 to 512-fold. A 40-fold dilution of homogenate was chosen for use in routine assays. Product formation also increased in a linear fashion with increasing incubation time for up to 50 min. Thirty min was chosen as the standard incubation time for use in all subsequent experiments.

**Effect of pH on PST activity**

PST activity in a pooled platelet homogenate was measured at various pH values with *p*-OH-
triamterene as substrate and with sodium acetate, potassium phosphate and Tris-HCl as buffers. Optimal activity was present with the use of a 16 mM sodium acetate buffer that resulted in a reaction pH of 7.3.

Effect of substrate concentration on PST activity

Enzyme activity was measured in a pooled platelet homogenate with nine different concentrations of p-OH-triamterene that ranged from 1.9 to 500 µM. Activity decreased at concentrations above approximately 100 µM. An apparent $K_m$ value of 26 µM was calculated from a Lineweaver-Burk plot of the results of this experiment. Data obtained with concentrations of p-OH-triamterene above 62.5 µM were not used in the calculation because of apparent substrate inhibition. The apparent inhibition found at very high p-OH-triamterene concentrations must be viewed with caution, however, since the solubility of the compound was limited at concentrations above 250 µM. In all subsequent experiments a final concentration of p-OH-triamterene of 70 µM was used since enzyme activity was optimal at that concentration.

Product identification

The product of the PST assay was identified as p-OH-triamterene sulphate by high performance liquid chromatography. Radioactivity in 'active' samples eluted as a peak with the same retention time as authentic p-OH-triamterene sulphate. This major peak accounted for 87% of the total radioactivity applied to the column and for virtually all of the radioactivity eluted after the contribution of blank samples was subtracted.

Thermal stability studies

A thermal stability experiment was performed to determine whether p-OH-triamterene was a substrate for the thermolabile (TL), the thermostable (TS), or for both forms of human platelet PST. Aliquots of a pooled platelet homogenate were preincubated for 15 min at temperatures ranging from 37 to 49°C. PST activity was measured in each aliquot with 4 µM p-nitrophenol, 60 µM dopamine and 70 µM p-OH-triamterene as substrates (Figure 2). As expected, PST activity measured with dopamine, a substrate for the TL form of the enzyme, was 50% inactivated at approximately 38.9°C, while activity measured with p-nitrophenol, a substrate for the TS form of the enzyme, was 50% inactivated at approximately 44.9°C. The thermal inactivation pattern of the enzyme activity measured with p-OH-triamterene was nearly identical with that of the TS form of activity (Figure 2). These results suggested that at a concentration of 70 µM p-OH-triamterene
was a substrate for only the TS form of platelet PST activity.

**PST activity in platelets of individual subjects**

There are wide variations among individuals in the activities of the TS and the TL forms of human platelet PST. Advantage was taken of this individual variation to test further the hypothesis that p-OH-triamterene was a substrate for the TS form of platelet PST. Platelet PST activities in blood samples from each of 29 subjects were measured with 4 \( \mu M \) p-nitrophenol, 60 \( \mu M \) dopamine and 70 \( \mu M \) p-OH-triamterene as substrates. Average platelet PST activities for these subjects are shown in Table 1. There were no significant differences between the sexes in platelet PST activities. There was not a significant correlation between PST activities measured with p-nitrophenol and activities measured with dopamine, substrates for the TS and TL forms, respectively \( (r = -0.043, \ P > 0.2, \) Figure 3a). This observation confirmed the results of previous studies which indicated that the activities of the TS and the TL forms of platelet PST were regulated independently (Reiter & Weinshilboum, 1981; 1982a; Carter et al., 1981). PST activities measured with p-OH-triamterene correlated significantly with activities measured with p-nitrophenol \( (r = 0.985, \ P < 0.0001, \) Figure 3b) but not with those measured with dopamine \( (r = 0.023, \ P > 0.2, \) Figure 3c). These results confirmed that at the concentration studied p-OH-triamterene was a substrate for only the TS form of human platelet PST activity.

**Metabolism of triamterene in vivo**

One purpose of these experiments was to determine whether individual variations in platelet PST activity might reflect variations in the sulphate conjugation of p-OH-triamterene in man. Triamterene is metabolized in vivo to form p-OH-triamterene. Therefore, the 29 subjects whose platelet PST activities are shown in Figure 3 were each given a 1 mg/kg oral dose of triamterene. A 24 h urine sample was then collected for the measurement of triamterene, p-OH-triamterene and p-OH-triamterene sulphate. The 24 h urinary excretion data are shown in Table 2. There were no statistically significant differences between men and women in the excretion of triamterene and its metabolites. The average total 'recovery' of triamterene and metabolites was 43.1 \( + 2.6\% \) (mean \( \pm \) s.e. mean).

**Table 1** PST activities in platelet samples from 29 subjects were measured with three different substrates. Enzyme activities are expressed as units per \( 10^9 \) platelets.

<table>
<thead>
<tr>
<th>Substrate (( \mu M ))</th>
<th>PST activity</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenol 4 ( \mu M )</td>
<td>0.33 ( \pm ) 0.03</td>
<td>0.13-0.68</td>
</tr>
<tr>
<td>Dopamine 60 ( \mu M )</td>
<td>0.73 ( \pm ) 0.03</td>
<td>0.52-1.21</td>
</tr>
<tr>
<td>p-OH-Triamterene 70 ( \mu M )</td>
<td>0.22 ( \pm ) 0.02</td>
<td>0.09-0.46</td>
</tr>
</tbody>
</table>
were only small individual variations in the proportion of p-OH-triamterene that was conjugated with sulphate (88.1 to 97.4%). Finally, when the proportion of sulphate conjugate in these 29 individuals was correlated with individual levels of platelet PST activity, there were no statistically significant correlations with the activities of either the TS or the TL forms of platelet PST (Figure 4).

Discussion

Sulphate conjugation catalyzed by PST plays an important role in the metabolism of many drugs including triamterene (Roy, 1981). Little is known of the regulation of sulphate conjugation in man. The observation that PST activity is present in an easily obtainable human tissue, the platelet, has raised the possibility that variations in platelet PST activity might reflect individual variations in the sulphate conjugation of drugs in man (Anderson & Weinshilboum, 1980; Anderson et al., 1981; Weinshilboum & Anderson, 1981). Studies performed with one model compound, paracetamol, have demonstrated that platelet PST activity does reflect variations in the sulphate conjugation of that drug (Reiter & Weinshilboum, 1982b; 1982c). The series of experiments described here was performed to test the hypothesis that variations in platelet PST activity might reflect variations in the level of sulphate conjugation of drugs other than paracetamol. Triamterene was the model compound used for these studies. In man triamterene is metabolized to form p-OH-triamterene, a compound that is sulphate conjugated. These two metabolites plus the parent drug account for over 95% of drug that is excreted (Lehman, 1965). The bioavailability of triamterene has been reported to be about 50% (Sörgel et al.,

![Figure 3 Correlation of PST activities in platelets from 29 subjects. Enzyme activities were measured with 4 μM p-nitrophenol, 60 μM dopamine and 70 μM p-OH-triamterene. Each value represents the mean of three determinations.](image)

Table 2 Urinary excretion of triamterene and triamterene metabolites. Excretion of each compound is expressed as a percentage of the sum of urinary triamterene, p-OH-triamterene and p-OH-triamterene sulphate (% of total excreted). p-OH-triamterene sulphate excretion is also expressed as a percentage of the sum of p-OH-triamterene and p-OH-triamterene sulphate excreted (% of total metabolites). 'Recovery' refers to the percentage of the ingested dose that was excreted as triamterene plus its metabolites.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean ± s.e. mean (n = 29)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary excretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( % of total excreted)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triamterene</td>
<td>15.3 ± 0.8</td>
<td>7.9–27.1</td>
</tr>
<tr>
<td>p-OH-triamterene</td>
<td>6.3 ± 0.4</td>
<td>2.2–10.2</td>
</tr>
<tr>
<td>p-OH-triamterene sulphate</td>
<td>78.4 ± 0.9</td>
<td>65.8–86.8</td>
</tr>
<tr>
<td>p-OH-triamterene sulphate ( % of total metabolites)</td>
<td>92.5 ± 0.5</td>
<td>88.1–97.4</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>43.1 ± 2.6</td>
<td>10.2–67.5</td>
</tr>
</tbody>
</table>
SULPHATE CONJUGATION OF p-HYDROXYTRIAMTERENE

Figure 4 Correlation of platelet PST activities in 29 subjects with the urinary excretion of p-OH-triamterene sulphate expressed as a percentage of the sum of p-OH-triamterene plus p-OH-triamterene sulphate excreted. See text for details.

1982), a figure close to the 43.1% value that might be estimated from our urinary excretion data (Table 2). Plasma levels of triamterene after a therapeutic dose of 150 mg have been reported to be approximately 100 ng/ml (Gundert-Remy et al., 1979).

The results of these experiments have demonstrated that p-OH-triamterene is a substrate for platelet PST, and, at the concentration studied, is a substrate for only the TS form of the enzyme. In contrast to results obtained with paracetamol, there was little individual variation in the percentage of p-OH-triamterene that was converted to the sulphate conjugate among the 29 subjects studied, and there was not a significant correlation between this percentage and either TS or TL platelet PST activities.

There are several possible explanations for the difference between results obtained with triamterene and those obtained with paracetamol. Obviously, many factors other than the level of PST activity may be involved in the regulation of sulphate conjugation. For example, the availability of sulphate plays an important role in regulating the proportion of paracetamol that is sulphate conjugated after the ingestion of toxic doses of that drug (Slattery & Levy, 1979). However, sulphate availability is probably much less significant after the ingestion of therapeutic doses of paracetamol (Lin & Levy, 1982). The relatively low doses of triamterene used in this study, and the fact that almost all of the p-OH-triamterene formed was sulphate conjugated, makes it unlikely that variations in the availability of sulphate can explain differences between the results of these experiments and similar studies performed with paracetamol (Reiter & Weinshilboum, 1982b; 1982c). The most likely explanation for the difference is that, unlike paracetamol which is itself a substrate for PST, triamterene must first be converted to p-OH-triamterene. If the hydroxylation step were rate limiting there might be only small individual vari-

ations in the proportion of p-OH-triamterene that was sulphate conjugated, and there might not be a significant correlation between that proportion and platelet PST activity. The results of these experiments raise the possibility that hydroxylation is the rate-limiting step in the metabolism of triamterene in man.

Even though this study did not demonstrate a significant correlation between platelet PST activity and the sulphate conjugation of p-OH-triamterene in vivo, these results did help to clarify the characteristics of an ‘ideal’ compound for use in future studies of the regulation of sulphate conjugation in man. First, such a compound should be safe for use in man. Second, sulphate conjugation should be a major metabolic pathway for the compound. Third, unlike triamterene, the compound itself should be a PST substrate without a requirement for prior metabolism. Finally, it would be ideal if the compound were a substrate for only one form of PST. Although many compounds have been identified that are substrates for only the TL form of PST, all substrates for the TS form of the enzyme that have been carefully studied are also TL substrates. However, the affinities of the two forms of PST for such ‘dual’ substrates vary greatly (Reiter & Weinshilboum, 1981; 1982a; Reiter et al., 1982). Whether p-OH-triamterene represents the first ‘pure’ TS substrate remains an open question because of the relative insolubility of the compound. The poor solubility of p-OH-triamterene makes it impossible to determine whether, like phenol and p-nitrophenol, it might also serve as a substrate for the TL form of the enzyme at millimolar concentrations.

The experiments described here have answered some questions about the role of sulphate conjugation in the metabolism of triamterene in man, but other questions remain unanswered. For example, these studies were performed with only a single dose of triamterene. It remains to be determined whether
similar results would be found in patients who have taken the drug chronically or in individuals who develop renal stones that contain triamterene and its metabolites. Finally, these experiments represent only one facet of a broader attempt to understand the biological basis of individual variations in human drug metabolism. It remains to be determined whether variations in PST activity might be responsible for important individual differences in the sulphate conjugation of a large number of drugs other than paracetamol.

References


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