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Limitation to the use of the urinary S-/R-mephenytoin ratio in pharmacogenetic studies

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The reproducibility of the S-/R-mephenytoin ratio was examined in urines stored at −20° C over 2 years. Large changes in this ratio were observed in some urine samples stored for only a few months under these conditions. The changes observed in the S-/R-mephenytoin ratio are attributed to the decomposition of an acid labile metabolite of S-mephenytoin which is eliminated in the urine. The instability of this metabolite makes it desirable to process urine shortly after its collection in order to avoid inaccurate phenotype assignments based upon the urinary S-/R-mephenytoin ratio. If rapid processing of urines is impractical, additional methods are described for preventing improper phenotype assignment of subjects.

Keywords S-/R-mephenytoin pharmacogenetics cytochrome P-450MP polymorphic metabolism analytical artifact

Introduction

The urinary ratio of S-/R-mephenytoin is widely used for characterizing phenotypic expression of the cytochrome P-450MP isoenzyme (CYP2C gene product) (Drohse *et al.*, 1989; Guttendorf *et al.*, 1990; Nakamura *et al.*, 1985; Sanz *et al.*, 1989). This method is based upon the marked difference in the disposition of S-mephenytoin between individuals who do and do not express this isoenzyme. In individuals with a deficiency in P-450MP activity, there is a diminished formation of (S)-4-hydroxy mephenytoin and an increased urinary recovery of S-mephenytoin (Kupfer *et al.*, 1984; Meier *et al.*, 1985; Wedlund *et al.*, 1984, 1985). Associated with the formation of 4-hydroxy-mephenytoin is the concurrent formation of another metabolite, an acid labile metabolite of mephenytoin (Wedlund *et al.*, 1987). This product is excreted in the urine and can be converted to S-mephenytoin by strong acid, thereby altering the urinary S-/R-mephenytoin ratio.

We observed a pronounced intrasubject variability in the urinary S-/R-mephenytoin ratio (Guttendorf *et al.*, 1990), raising concern that the acid labile metabolite may also be unstable under less acidic conditions. The purpose of this study was to determine the reproducibility of the S-/R-mephenytoin ratio under normal urine handling conditions. Thus, the urinary S-/R-mephenytoin ratio was measured daily in urine left at room temperature for 1 week and in urine stored at –20° C for 1, 3, 6 or 24 months.

Methods

Subjects

The studies were performed using urines collected from 166 subjects (154 men and 12 women, aged 18 to 81 years) who ingested 100 mg of racemic mephenytoin. Written informed consent was obtained from each subject and approval for the study was obtained from the University of Kentucky Investigational Review Board. The experimental protocol has been described previously (Guttendorf *et al.*, 1990).

Samples

Stability studies were performed by comparing the urinary S-/R-mephenytoin ratio obtained initially (within 1–2 weeks of collection) with the value after storage of urine for 1 month ($n = 50$), 3 months ($n = 30$) and 6 months ($n = 12$). For samples stored for 2 years the initial S-/R-ratio was determined within 3 months of collection and again 2 years later ($n = 116$). Aliquots of urine were stored at –20°C until extracted and analyzed by capillary gas chromatography (Wedlund *et al.*, 1984). The extraction procedure employed was modified to include 0.01 N NaOH and 0.01 N HCl washes of the initial dichloromethane urine extract in order to diminish the number of extraneous chromatographic peaks. Within and between run variance in the S-/R-

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ratio was less than 7% upon repeated examination of several urines over a 2 week period.

A measure of the maximum potential change in the S-/R-mephenytoin ratio was obtained by shaking 0.5 ml aliquots from some of these samples with concentrated (12 M) HCl (0.1 ml) for 10 min at room temperature. In addition, stability studies were carried out with freshly collected urine samples at room temperature for 1 week. Urine pH was monitored with a Beckman 3560 digital pH meter.

Data analysis

The difference between the initial urinary S-/R-mephenytoin ratio and the S-/R- ratio after storage at –20° C was examined for significance ($P < 0.05$) by the paired *t*-test. The maximum potential change in the S-/R-ratio was determined by dividing the difference in the S-/R-ratio in the presence and absence of acid by the initial S-/R-ratio (i.e. $[(S\text{-}/R\text{-})_{acid}\text{-}(S\text{-}/R\text{-})_{initial}]/(S\text{-}/R\text{-})_{initial}$). Correlations between observed changes in the S-/R-ratio and the maximum potential change in this ratio were evaluated by linear regression analysis.

Results and discussion

The S-/R-mephenytoin ratio in urines from efficient metabolizers increases significantly after storage of urines for 1, 3, 6 or 24 months at –20° C. The mean percentage increases in the S-/R-mephenytoin ratio after 1, 3, 6 or 24 months of storage were 6.9% (range 0–104%), 20% (range 0–139%), 177% (range 0–2,600%) and 216% (range 0–1,100%), respectively. Although the urinary S-/R-mephenytoin ratio in urines from efficient metabolizers increased markedly over 2 years, this ratio showed little change in the urines from 16 poor metabolizers after 2 years of storage (Figure 1). The urine pH varied between pH 5–7, but there was no evidence of any relationship between urine pH and the percent change in the urinary S-/R- mephenytoin ratio with storage.

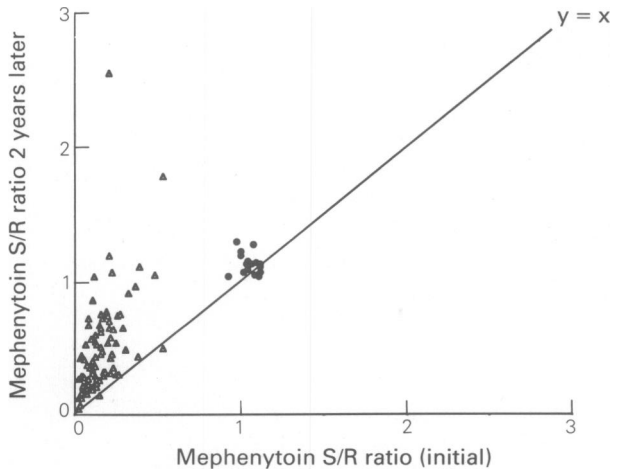

Figure 1 Relationship between the initial urinary S-/R-mephenytoin ratio and the urinary S-/R-ratio in the same urine 2 years later in efficient (▲ EM, $n = 100$) and poor (● PM, $n = 16$) metabolizers of mephenytoin.

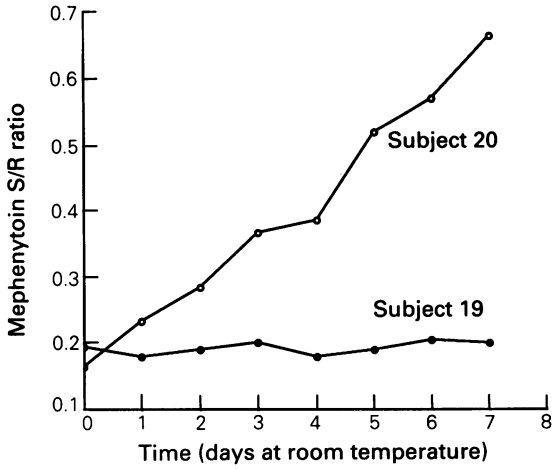

Figure 2 Urinary S-/R-mephenytoin ratio for two efficient metabolizer subjects *vs* the length of time urine remained at room temperature. Despite a marked difference in the effect of storage at room temperature, both urines demonstrated comparable changes in the S-/R-ratio (○, 45 fold *vs* ●, 42 fold) on treatment with concentrated acid. The pH values of the urines were, 6.55 (subject 19) and 5.04 (subject 20).

A strong correlation ($r = 0.81$) was observed between the change in the S-/R-mephenytoin ratio in urine samples from efficient metabolizers after storage for 3 months at –20° C and the maximum potential change in the S-/R-ratio obtained by treating urine with concentrated HCl (data not shown). The most marked change in the S-/R-ratio with storage occurred in those urines which displayed the most pronounced change in the ratio when urine aliquots were treated with acid. Surprisingly, the S-/R-mephenytoin ratio appeared to display much more stability in some urines from rapid metabolizers than in others (Figure 1). On further examination, a striking interindividual difference was noticed in the stability of this ratio when urines were kept at room temperature (Figure 2). The urine from the two subjects whose data are shown in Figure 2 exhibited a similar maximum potential change in the S-/R-mephenytoin ratio on treating their urine with strong acid. However, the urine from one subject showed little change in the S-/R-ratio over a 7 day period, while in the other urine the S-/R-mephenytoin ratio increased several hundred percent. One possible explanation for this observation may lie with the extent to which the acid labile metabolite is conjugated in various subjects and in the relative stability of the conjugated and unconjugated form of this metabolite. The stability in the S-/R-ratio following acid treatment was also examined in 50 separate urine samples. A comparison of these ratios from freshly treated urine and after storage of urine for 1 year showed that they were not significantly different (slope = 0.979; $r = 0.957$) (data not shown). Although the urinary S-/R-mephenytoin ratio is a useful index for phenotyping, some caution may be necessary when applying it for routine screening. Ideally, the S-/R-ratio should be measured in urine within at least 1 month of collection. In 7% of the individuals, changes in the urinary S-/R-ratio after even 6 months of storage can be large enough to produce incorrect phenotype assignments. An alternative solution to the instability of the acid labile metabolite is to extract urine samples

immediately with dichloromethane and to save the organic extract for later analysis. The polarity of the acid labile metabolite precludes its extraction into dichloromethane and most other organic solvents. In urines which have been stored for extended periods of time, it is possible to recheck the phenotype assignment by treating these samples with concentrated (12 N) HCl (0.1 ml). Even in old urine samples, individuals expressing the P-450MP isoenzyme still exhibit a marked change (several fold) in this ratio following acid treatment of the urine. The true poor metabolizer, however, produces so little of this metabolite that even acid treatment of the urine has only a minimal influence on the ratio. In all the urine samples we have analyzed from poor metabolizers of mephenytoin, the S-/R-ratio was never increased above 1.2 on treatment of the urine sample with 12 N HCl.

It would be prudent for researchers using the urinary S-/R-mephenytoin ratio to examine its stability under typical conditions in their laboratories. In addition, one should double check the accuracy of the poor metabolizer phenotype assignment based upon the urinary S-/R-ratio by treating aliquots from those urines with strong acid. Otherwise, it is possible that the frequency of the poor metabolizer phenotype will be overestimated.

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