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Note

Quantitation of *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine in human urine by high-performance liquid chromatography

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Approximately 90% of a carbamazepine-10,11-epoxide oral dose is eliminated as *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (*trans*-dihydrodiol) and *trans*-dihydrodiol conjugates in human urine [1,2]. Due to the nearly complete urinary recovery of these terminal metabolic products of carbamazepine-10,11-epoxide, their measurement in urine provides a useful means for assessing the effect of anticonvulsant therapy on the carbamazepine-epoxide metabolitic pathway [3,4]. Investigations of this type, however, require confidence in the analytical conditions and methods employed for quantitation of urinary *trans*-dihydrodiol levels. Previous studies of this metabolite have provided little more than a description of the assay conditions used [5–7]. Thus, before initiating work on *trans*-dihydrodiol urinary elimination, the sensitivity, reproducibility, and selectivity of a new assay for urinary *trans*-dihydrodiol was examined and tested with patient urine. The stability of the *trans*-dihydrodiol in urine was also investigated along with the incubation conditions for hydrolysis of its conjugated form.

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EXPERIMENTAL

Chemicals

The *trans*-dihydrodiol, 9-hydroxymethyl-10-carbamoyl acridane, carbamazepine, and carbamazepine-10,11-epoxide were gifts from Ciba-Geigy (Basel, Switzerland). Primidone was kindly supplied by Ayerst Labs. (New York, NY, U.S.A.). The *p*-hydroxymephenytoin was a generous gift from Dr. Grant Wilkinson (Vanderbilt University, Nashville, TN, U.S.A.). Phenobarbital, *p*-hydroxyphenobarbital, diphenylhydantoin, and *p*-hydroxydiphenylhydantoin were purchased from Sigma (St. Louis, MO, U.S.A.). Phensuximide was a gift from Dr. Robert Yokel (University of Kentucky, Lexington, KY, U.S.A.). Phenacetin was a gift from Dr. Joe Bogardus (Bristol Labs., Syracuse, NY, U.S.A.). Acetonitrile, ethyl acetate, methanol (all HPLC grade), and glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). The β-glucuronidase (lyophylized powder from limpets, Type L-II) was purchased from Sigma. Sodium acetate was obtained from Mallinckrodt (Paris, KY, U.S.A.). All chemicals and reagents were used without further purification.

Standards and reagents

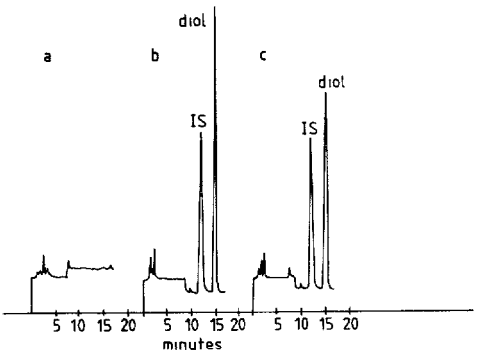
Standards for the *trans*-dihydrodiol, ranging in concentration from 1.25 to 41.6 μg/ml, were prepared by serial dilution of a 208 μg/ml methanol stock solution. *p*-Hydroxymephenytoin (24 μg/ml) served as the internal standard. A 0.5 *M* solution of sodium acetate buffer (adjusted to pH 4.5 with glacial acetic acid) was used to prepare enzyme solutions for hydrolysis of urine samples.

Analytical procedure

Internal standard (0.1 ml) was added to 15-ml PTFE-lined screw-capped tubes and the methanol evaporated to dryness under nitrogen at 37°C. To each tube, 0.1 ml of a ten-fold diluted urine sample was added followed by 0.5 ml of acetate buffer containing 300 U of β-glucuronidase. The samples were incubated for 4 h at 37°C. At the end of the incubation, the samples were extracted with 5 ml of ethyl acetate. After mixing and centrifugation, the organic phase was transferred to a 5-ml Reacti-vial® and evaporated to dryness under nitrogen at 37°C. The samples were reconstituted in 0.1 ml mobile phase and a 20-μl aliquot was chromatographed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 6000 pump, a Beckman 340 Organizer injector (20-μl), a guard column (50×4.6 mm, 30–38 μm ODS), a Supelco C_8 column (150×4.6 mm, 5 μm), a Waters 441 absorbance detector (wavelength 214 nm), and a Hewlett-Packard integrator (3390 A). Separation of the solutes was accomplished at ambient temperature using a mobile phase of acetonitrile–water (15:85, v/v) and a flow-rate of 1.1 ml/min. The stability of the *trans*-dihydrodiol was examined over a 70-day period in individually frozen urine aliquots and in a bulk urine sample which was thawed prior to removal of each aliquot for analysis.

RESULTS

Chromatograms of the extracts from the blank, spiked, and patient urine sample are shown in Fig. 1. These chromatograms indicate an absence of endogenous



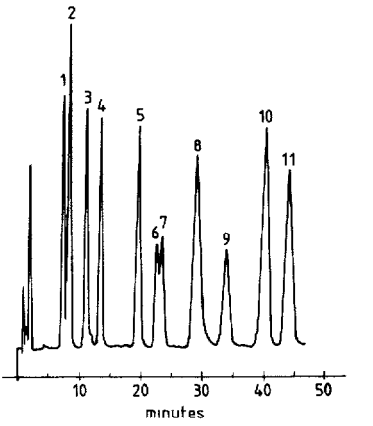


Fig. 1. Chromatograms of a blank (a), spiked (b), and patient urine sample (c). The retention times are 11.64 min for *p*-hydroxymephenytoin (internal standard, IS, 24 μg/ml) and 14.48 min for the *trans*-dihydrodiol (diol) corresponding to 15.6 μg/ml in b and 12.1 μg/ml in c.

Fig. 2. Elution profile of some anticonvulsant drugs and their metabolites. Peaks: 1 = *p*-hydroxyphenobarbital; 2 = primidone; 3 = *p*-hydroxymephenytoin; 4 = carbamazepine-10,11-*trans*-dihydrodiol; 5 = phenacetin; 6 = phenobarbital; 7 = phensuximide; 8 = *p*-hydroxydiphenylhydantoin; 9 = carbamazepine-10,11-epoxide; 10 = mephenytoin; 11 = 9-hydroxymethyl-10-carbamoylacridane.

interfering substances in urine and baseline resolution of the internal standard and *trans*-dihydrodiol metabolite. The potential for interference from other anticonvulsants was also examined. As shown in Fig. 2, none of the substances tested was found to co-elute with the internal standard or the *trans*-dihydrodiol.

The reproducibility of this assay was assessed by calculating the mean concentration for each individual point of the standard curve over a three-month time period. The coefficient of variation about each point on the curve was less than 7% and linearity was maintained ($r = 0.9998$) over a 30-fold concentration range.

The intra- and inter-day variation in *trans*-dihydrodiol concentrations from spiked urine samples are presented in Table I. The coefficient of variation was less than 5% for all but the lowest inter-day concentration examined.

The time required for the hydrolysis of urinary conjugated *trans*-dihydrodiol was investigated by incubating patient urine with β-glucuronidase at 37 °C from 0 to 4 h. The *trans*-dihydrodiol urinary concentration observed as a function of incubation time is shown in Fig. 3. Under the conditions employed, the hydrolysis of the urinary *trans*-dihydrodiol conjugate was complete in 30 min. Comparison of the *trans*-dihydrodiol recovery before and after hydrolysis indicates that approximately 22% of this urinary metabolite was excreted in the conjugated form.

The stability of the *trans*-dihydrodiol metabolite in urine stored at −20 °C was assessed over a 70-day period by repeated assay of a urine sample collected from a patient receiving carbamazepine. No trend toward decreasing *trans*-dihydrodiol levels with time could be detected in either individual urine aliquots or the bulk urine sample stored at −20 °C. Variations observed in the *trans*-dihydrodiol concentrations during this time period were within the limits of assay error (data not shown).

TABLE I

INTRA- AND INTER-DAY REPRODUCIBILITY IN *trans*-DIHYDRODIOL CONCENTRATIONS

Spiked concentration (μg/ml)	*n*	Measured concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Intra-day			
1.25	11	1.45 ± 0.03	1.86
5.20	11	5.23 ± 0.21	3.96
15.60	11	15.65 ± 0.28	1.77
20.80	11	20.56 ± 0.42	2.06
41.60	11	39.33 ± 0.83	2.15
Inter-day			
1.25	10	1.17 ± 0.19	15.82
2.60	9	2.52 ± 0.12	4.72
15.60	9	15.31 ± 0.30	1.99
24.96	10	24.83 ± 0.59	2.38
41.60	10	39.85 ± 0.77	1.92

DISCUSSION

The large quantity of *trans*-dihydrodiol metabolite eliminated in the urine from patients receiving carbamazepine required urine samples used in this assay to be diluted ten-fold prior to enzyme hydrolysis and extraction. Dilution of urine to this extent minimized interference from endogenous substances and improved the assay selectivity. The sensitivity of the assay, defined as peak height for the

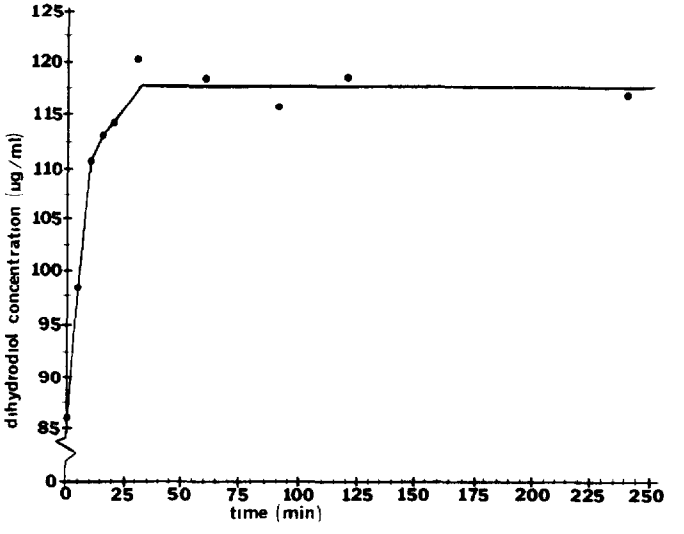


Fig. 3. The concentration of *trans*-dihydrodiol versus the duration of incubation with β-glucuronidase.

trans-dihydrodiol equal to twice the background noise, was approximately 0.5 μg/ml. This sensitivity limit is well below the concentration typically encountered for this metabolite in urine from patients receiving carbamazepine [2–4]. The excellent separation of the *trans*-dihydrodiol and its internal standard (*p*-hydroxymephenytoin) from the other anticonvulsant drugs and their metabolites which might be encountered in patient urine indiciated little potential for assay interference due to anticonvulsant polytherapy. The elution profile of 2-hydroxy and other metabolites of carbamazepine, however, could not be determined in this assay due to the non-availability of pure reference compounds. Although the internal standard selected for this assay is itself a major metabolite of an anticonvulsant drug, this should not create a problem. Mephenytoin, although approved as an anticonvulsant, is rarely used in the United States and is no longer prescribed by the neurologist at the University of Kentucky due to its potentially severe adverse effects.

The assay showed good linearity and reproducibility for the *trans*-dihydrodiol over a 30-fold concentration range with a coefficient of variation of less than 5% for all but the lowest *trans*-dihydrodiol concentrations examined. The reproducibility of this assay indicated that subtle changes in *trans*-dihydrodiol elimination should be detectable with this assay method.

An investigation of the time-dependent hydrolysis of conjugated *trans*-dihydrodiol suggested cleavage of the urinary conjugate(s) was complete in 30 min. This rate is considerably more rapid than the overnight or 24-h incubation conditions employed for hydrolysis of *trans*-dihydrodiol conjugates by other investigators [1,8]. In spite of the shorter incubation time, the estimated recovery of *trans*-dihydrodiol eliminated as conjugated metabolite (22%) in this study was similar to the 10–30% reported by others [1,3,4,8]. One explanation for the shorter hydrolysis time may be the β-glucuronidase employed in these incubations. The Type L-II β-glucuronidase used in the present study has been reported to be more efficient in cleaving conjugated metabolites than other forms of β-glucuronidase [9].

Although investigation of the stability of the *trans*-dihydrodiol in urine is essential for establishing acceptable urine handling procedures, no previous study has reported on the stability of this metabolite in patient urine. This work indicates the *trans*-dihydrodiol is stable in urine over a 70-day period at −20°C. Furthermore, repeated freezing and thawing of urine for removal of aliquots during this time did not appear to affect the *trans*-dihydrodiol stability.

In conclusion, the methodology developed for analysis of urinary concentrations of *trans*-dihydrodiol and its conjugated products in patient urine is reproducible, selective, and sensitive. Examination of the incubation time required for the hydrolysis of conjugated *trans*-dihydrodiol indicated incubations could be completed in less than 1 h rather than the 12–24 h reported previously. Finally, stability studies of the *trans*-dihydrodiol suggests urine containing this metabolite need not be assayed immediately, but can be kept for at least two months if stored at −20°C.

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