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QUANTITATION OF 3'-HYDROXYPENTOBARBITAL IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PAUL L. CARY* and BRIAN E. PAPE

Toxicology and Therapeutic Drug Monitoring Laboratory, Department of Pathology, Health Sciences Center, University of Missouri, Columbia, MO 65212 (U.S.A.)

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SUMMARY

A quantitative method for the determination of 3'-hydroxypentobarbital (3'HP) in serum has been developed using reversed-phase high-performance liquid chromatography. A sensitivity limit of 0.5 μg/ml in 100 μl serum was obtained by detection at 215 nm. Serum concentrations of 3'HP were measured in patients receiving high-dose pentobarbital therapy and pentobarbital:3'HP concentrations are discussed.

INTRODUCTION

Pentobarbital is a short-acting barbiturate used for a variety of sedative and hypnotic purposes [1]. Studies indicate that 3'-hydroxypentobarbital (3'HP) is the major metabolite in man [2, 3]; two additional metabolites are N-hydroxypentobarbital and 3'-carboxypentobarbital [4, 5].

Recently, high-dose pentobarbital therapy (HDPT) has demonstrated beneficial effects in the treatment of some of the consequences of severe brain insult [6—13]. Clinical HDPT usually results in steady-state serum concentrations of 20—50 μg/ml pentobarbital, inducing barbiturate coma and requiring artificial ventilation and continuous physiologic monitoring [14]. These consequences of HDPT, as well as the interpatient variability of pentobarbital pharmacokinetics [15], necessitate the availability of therapeutic drug monitoring procedures.

Gas chromatography (GC), high-performance liquid chromatography (HPLC), and ultraviolet spectroscopy (UV) have been used to quantitate pentobarbital [16—18]. More recently, this laboratory modified a homogeneous enzyme immunoassay to allow pentobarbital quantitation in 50 μl of serum [19, 20]. Although EMIT® serum barbiturate reagents have

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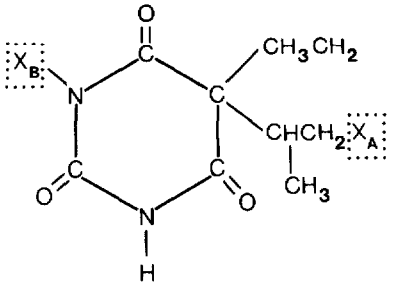


COMPOUND	X_A	X_B
PENTOBARBITAL	CH_3CH_2	H
3′-HYDROXYPENTOBARBITAL	$CH_3CH(OH)$	H
N-HYDROXYPENTOBARBITAL	CH_3CH_2	OH
3′-CARBOXYPENTOBARBITAL	CH_2COOH	H

been modified to quantitate pentobarbital previously, cross-reactivity towards other barbiturates or metabolites were not reported [21, 22]. Therefore, as part of the methodologic evaluation, the quantitation of 3′HP was necessary in order to investigate the potential analytical interference (bias) due to immunoassay antibody—enzyme cross-reactivity towards 3′HP. Additionally, the quantitation of 3′HP and pentobarbital in patients receiving HDPT allowed comparison between low dose/high dose drug:metabolite concentration ratios for pentobarbital as compared with other barbiturate analogues [23—25].

Hydroxylated barbiturate metabolites have been quantitated by radioimmunoassay (RIA), gas chromatography—mass spectrometry (GC—MS), thin-layer chromatography (TLC), and gas chromatography (GC) [23, 24, 26—29]. While all of these methods are considered to be selective, disadvantages have been recognized: RIA requires antibody production and the use of radioisotopes; GC—MS requires expensive instrumentation; TLC requires a large specimen volume; and GC requires the methylation of barbiturate compounds, which has been subject to criticism because of incomplete derivatization and shortened GC column life. HPLC has been used to quantitate other barbiturates and their metabolites and has proven to be sensitive, selective, and rapid [30—32]. This report describes a reversed-phase HPLC assay for the selective quantitation of 3′HP at concentrations greater than 0.5 μg/ml in 100 μl of plasma.

EXPERIMENTAL

Instrumentation

Analyses were carried out with a Perkin-Elmer Series 3 high-performance liquid chromatograph equipped with a Whatman Partisil PXS-10/25 ODS-2 column (250 mm × 4.6 mm I.D., 10 μm particle size) and guard column (45 mm × 5.0 mm I.D.) packed with CO:Pell ODS (Whatman, Clifton, NJ, U.S.A.), and a Model LC-55 variable-wavelength detector (Perkin-Elmer, Norwalk, CT,

U.S.A.) at 215 nm. Chromatographic conditions were as follows: mobile phase, tetrahydrofuran—water (5:95); flow-rate, 2.5 ml/min; column temperature, 50°C.

A Beckman Microfuge B centrifuge (Beckman, Palo Alto, CA, U.S.A.) was used to centrifuge serum extracts.

Reagents

Analytical grade 3'-hydroxypentobarbital was verified by GC—MS and TLC. 3'HP can be synthesized to obtain analytical material [33]. A 10 μg/ml stock standard solution of 3'HP was prepared in drug-free human serum. Serum standards (2, 4, 6, and 10 μg/ml) were prepared by diluting a stock solution of 3'HP with drug-free serum.

Tetrahydrofuran (UV) was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ethyl acetate, pesticide grade, was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A., Cat. No. E-191.). Hydrochloric acid used was ACS grade. Water was deionized and passed through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

Procedure

To 100 μl of standard or patient sample, 50 μl of 3 *N* hydrochloric acid and 1.0 ml of ethyl acetate were combined in a 1.5-ml polypropylene microcentrifuge tube, and vortexed for 30 sec. The extraction tubes were centrifuged for 10 min at 11,000 *g*; 0.8 ml of organic layer was transferred to a glass conical tube and the organic layer was evaporated under nitrogen at 35°C. The residue was reconsituted with 40 μl of methanol—water (50:50) and 30 μl were quantitatively injected into the chromatograph.

Pentobarbital was quantitated by HPLC using a modified procedure reported by Salvadori et al. [17]. Measurements were made at 239 nm and chromatographic conditions were as follows: mobile phase methanol—water (45:55); flow-rate 2.5 ml/min; column temperature 50°C.

RESULTS AND DISCUSSION

Chromatographic conditions allowed the separation of endogenous coextractables from 3'HP (Fig. 1). The HPLC pentobarbital method, using a more polar mobile phase, resulted in the apparent elution of 3'HP in the void volume of the column. HPLC analysis also suggested the presence of an additional metabolite (P1) in all samples containing 3'HP. This peak was not present in drug-free serum or in serum spiked with 3'HP. Assuming that 3'HP and P1 have the same analytical recovery and molar absorptivity at 215 nm, peak area data for P1 suggested that the concentration of P1 and 3'HP were grossly equivalent. P1 was not identified.

Analytical recovery of 1—10 μg/ml 3'HP added to serum was 85 ± 7%. The analysis of drug-free patient sera indicated that nonselective detector response (due to HPLC system fluctuation or endogenous coextractables) was equivalent to less than 0.025 μg/ml of 3'HP. However, the distortion of 3'HP peak shape prohibited accurate integration at concentrations less than 0.5 μg/ml (equivalent to 30 ng of 3'HP injected on-column).

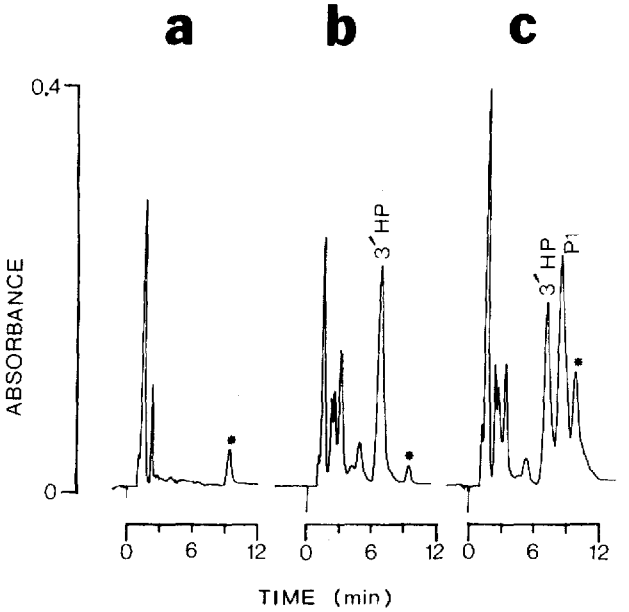


Fig. 1. HPLC chromatograms illustrating: (a) blank serum extract, (b) drug-free serum spiked with 5 μg/ml of 3'-hydroxypentobarbital (3'HP), (c) patient serum extract containing 3.5 μg/ml of 3'HP and unidentified metabolite P1. * designates serum interference.

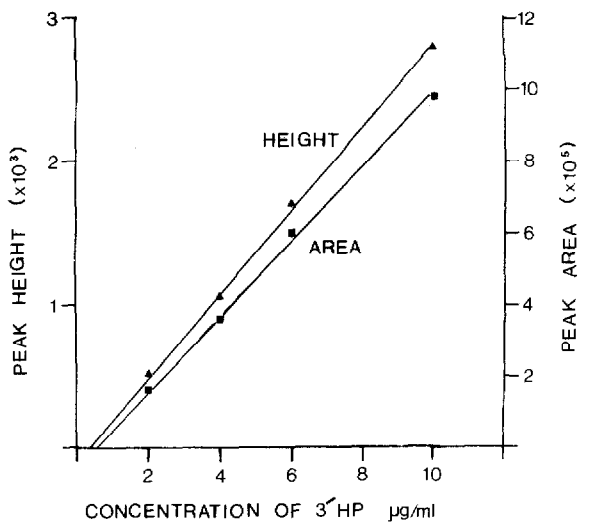


Fig. 2. Calibration curve for 3'-hydroxypentobarbital quantitation using peak area (■) and peak height (▲) calculations.

A standard curve obtained by linear regression of concentration versus peak height or peak area was used to quantitate 3'HP from 0.5—10 μg/ml (Fig. 2). Between-day variation was examined by analysis of variance of slopes and intercepts from four standard curves (Table I). The slopes for these standard curves had coefficients of variation (C.V.) of 15.4% and 10.6% for calculations based on peak area and peak height, respectively. Such variance is not unusual and

TABLE I

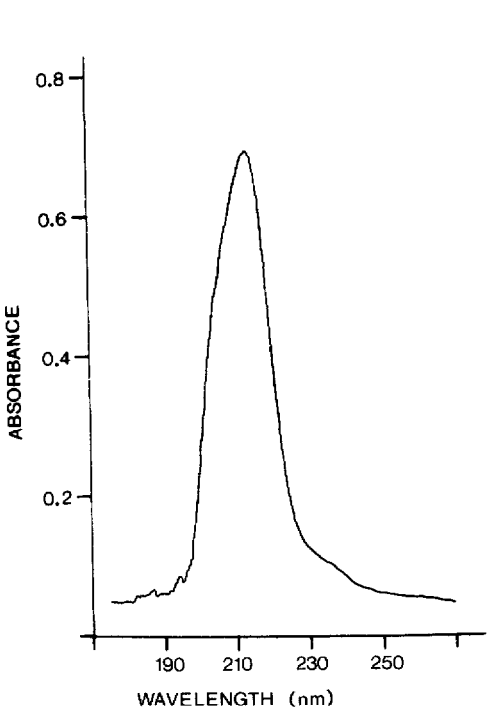
ANALYSIS OF VARIANCE ON SLOPES AND INTERCEPTS FOR THE 3'HP STANDARD CURVES

Analysis	Slope				Intercept	
	r	Value	C.V.	S.E.	Value	S.E.
Peak area						
1	0.999	1.034	3.86	0.039	−0.388	0.249
2	0.998	0.963	7.83	0.075	−0.145	0.412
3	0.996	0.920	16.57	0.152	0.390	0.835
4	0.998	0.708	13.06	0.092	1.693	0.507
Peak height						
1	0.999	0.299	0.54	0.002	−0.086	0.010
2	0.999	0.255	4.23	0.010	0.035	0.059
3	0.998	0.243	11.08	0.026	0.153	0.147
4	0.999	0.239	7.28	0.017	0.267	0.095

appears to be related to changes in column performance due to reversible deactivation of the C_{18} column associated with solvent bonding or accumulation of impurities from samples and/or mobile phase [34, 35]. Statistical analysis of the variance of each standard curve, using the general linear test approach, indicated equal regression parameters for slope and intercept, with $p < 0.05$ [36].

The UV absorption of 3'HP in the HPLC mobile phase was determined (Fig. 3). The λ_{max} at 217 nm is due to $\pi \rightarrow \pi^*$ transition of C=C in the barbiturate ring. Analysis of 100-μl sample volumes required a high degree of sensitivity from our method. The relatively low molar absorptivity of unionized 3'HP in neutral (or acidic) mobile phase at 254 nm ($\eta \rightarrow \pi^*$ transition) did not allow detection below 10 μg/ml of 3'HP. Alternatively, the use of alkaline buffers (> pH 7.6) to increase the spectrophotometric selectivity and sensitivity of barbiturates will slowly dissolve silica columns causing loss in efficiency [34]. Numerous reported procedures for the analysis of barbiturates using reversed-phase HPLC have utilized wavelengths < 220 nm when neutral mobile phases have been employed for chromatographic separation [37—39]. Differences in absorbance from 254 nm to 215 nm indicated a 75-fold theoretical increase in spectroscopic sensitivity for 3'HP. While a corresponding decrease in analytical selectivity was experienced, the procedure was designed to minimize interference from biological coextractable constituents. No interference from other commonly prescribed barbiturates was noted; however, the HPLC behavior of other barbiturate metabolites was not investigated.

3'HP was quantitated in sera from 26 patients receiving HDPT (Fig. 4). Pentobarbital concentrations, determined by separate HPLC analysis, ranged from 3—36 μg/ml (mean = 19 μg/ml); while 3'HP was < 4 μg/ml (mean = 1.4 μg/ml). Even in patients (n = 8) treated for between seven and ten days, the highest 3'HP concentration recorded was 3.6 μg/ml. Regression analysis of pentobarbital versus 3'HP suggested no significant relationship between the concentration of drug and metabolite (Fig. 5). However, samples were randomly



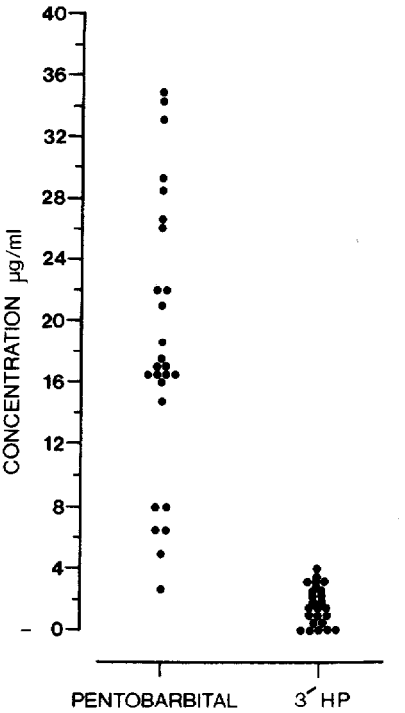


Fig. 3. UV spectrum of 3′-hydroxypentobarbital in tetrahydrofuran—water (5:95).

Fig. 4. Serum concentrations of pentobarbital and 3′-hydroxypentobarbital in patients receiving high-dose pentobarbital therapy. Analysis by HPLC. n = 26.

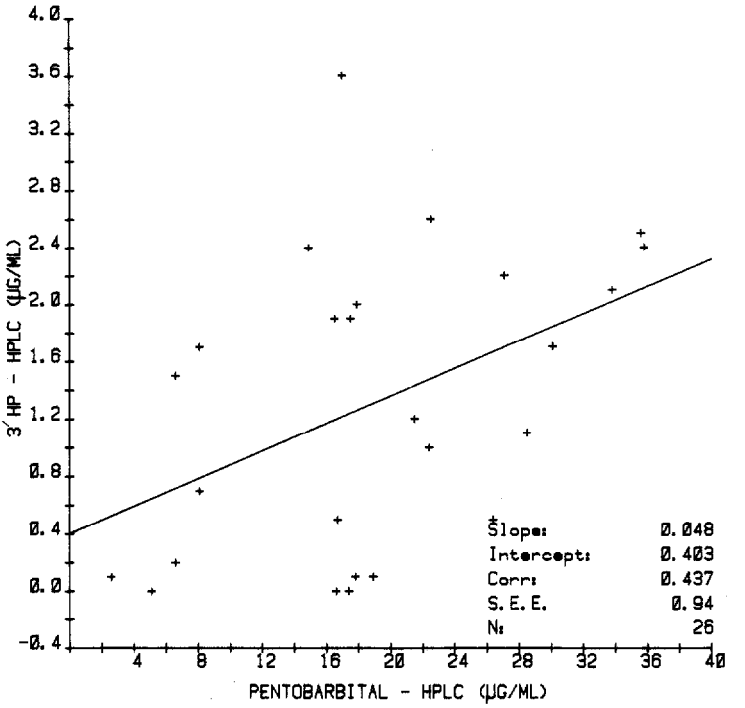


Fig. 5. Concentrations of pentobarbital and 3′-hydroxypentobarbital in high-dose pentobarbital patients. Analysis by HPLC.

acquired and were not selected for patients presumed to be at steady-state.

While data on blood concentrations of hydroxylated pentobarbital are limited, Robinson and McDowall [25] reported concentrations of hydroxypentobarbital from four pentobarbital overdoses at $\leqslant 4$ μg/ml. Additionally, their results for other, high-dose, short-acting barbiturates indicate drug/hydroxylated metabolite ratios much greater than one with poor correlation, which is consistent with our findings. Other studies involving low dose barbiturate administration also indicate that serum barbiturate:hydroxylated metabolite concentration ratios are > 1.0 and are variable [23, 24].

Although 3'HP is considered biologically inactive [40] its quantitation in serum may prove helpful in the investigation of pentobarbital biotransformation. Additionally, the ability to quantitate 3'HP and other hydroxylated barbiturates is useful in assessing the metabolite cross-reactivity of non-specific barbiturate procedures.

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