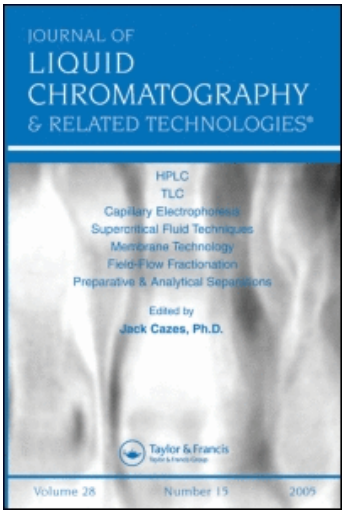

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENACEMIDE IN BIOLOGICAL FLUIDS

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ABSTRACT

A sensitive and reliable high performance liquid chromatographic procedure has been developed for the quantitation of phenacemide in plasma or urine. After simple extraction of the drug with ethylacetate from alkalinized samples and evaporation to dryness, the reconstituted extract was chromatographed using a C_8 reversed phase analytical column with UV detection at 254 nm. Regression analyses for the calibration plots obtained on 3 different days for the drug concentrations ranging 1-15 mcg/ml indicated excellent linearity ($r > 0.999$) and reproducibility (CV<

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4%, $p > 0.01$). The mean recovery of spiked phenacemide in plasma and urine from the lower limit of quantitation (1 mcg/ml) to 15 mcg/ml was 97.9 and 96.3%, respectively and their respective CV was 3.53 and 2.58%. The method was applied to monitor the plasma vs. time profile of the drug following a single bolus IV dose of 12 mg/kg in a dog.

INTRODUCTION

Phenacemide, a straight chain analog of 5-phenylhydantoin is an anticonvulsant drug used for the control of severe epilepsy, particularly mixed forms of complex psychomotor seizures (1,2). Usual daily dose in adults varies widely from 1 to 5 g depending upon the patient's response to the drug. Its clinical value and use are, however, often limited by its potential of serious toxicity (2). Phenacemide is well absorbed after oral dose, and is known to undergo extensive metabolism in the liver (3).

Although this drug has been in clinical use for many years, information about its pharmacokinetic behavior in man is scarce, partly, due to the deficiency of sensitive and specific analytical methods to quantitate the drug in plasma. The present method which appears to be the first HPLC procedure for the assay of phenacemide in biological samples is simple and reliable, and has high sensitivity and specificity. It should be useful for pharmacokinetic and bioavailability studies of phenacemide in man.

EXPERIMENTAL

Materials - Phenacemide (4) and Oxamniquine (5) were used without further purification. Acetonitrile (6), ethylacetate (6) and water were HPLC grade. All other chemicals were of U.S.P. or ACS quality, and were used as received.

Apparatus - The liquid chromatograph equipped with a single wavelength U.V. detector (7) at 254 nm and fixed-volume injector (8) with a 20-ul sample loop was isocratically operated at ambient temperature. A stainless steel reversed-phase octyl column (15 cm x 4.6 mm i.d.) containing 5 um Ultrasphere packing (9) was used with a guard column. Chromatograms were recorded on a strip chart recorder (10) at a speed of 0.5 cm/min.

Mobile Phase - The eluting medium consisting of 0.05 M acetate buffer and acetonitrile (50% V/V) was prepared and degassed by bubbling helium gas for 5 min prior to use. The final pH of the mobile phase was adjusted to pH 4.2 by glacial acetic acid. Column equilibration with the eluting solvent was established by pumping (11) the mobile phase at a rate of 0.2 ml/min for overnight. The flow rate was set at 1.0 ml/min during analysis.

Stock Solutions - An appropriate quantity of phenacemide and oxamniquine was accurately weighed and dissolved separately in a 50 ml volumetric flask to prepare a 100 mcg/ml solution in acetonitrile. The standard solutions of phenacemide in the concentrations between 1 and 15 mcg/ml and containing 2.5 mcg/ml

of oxamniquine as a calibration marker were prepared by diluting the appropriate quantities of stock solutions with the mobile phase. These solutions were used to construct standard calibration plots and to compare the recovery of the drug from spiked plasma or urine samples.

Extraction Procedure - One half ml of dog plasma (dosed or spiked) in a 12-ml glass centrifuge tube was rendered alkaline with 0.1 ml of 2 N NaOH, and was mixed thoroughly. After addition of 2.5 ml ethylacetate, the mixture was vortexed at high speed for 2 min and centrifuged at 3000 rpm for 5 min. Two mls of the organic layer were transferred to a 2-ml volumetric tube and evaporated to dryness under a nitrogen gas stream at ambient temperature. The residue was reconstituted in 200 ul of elution solvent containing 0.5 mcg (2.5 mcg/ml) of oxamniquine. The sample was then shaken on a vortex mixer for 30 sec., and 20 ul of the resulting solution was injected into the column for analysis. The extraction and analysis of the drug from spiked urine samples were the same as described for plasma assay.

Calibration plots - Rconstituted extracts of spiked plasma and urine standard samples were chromatographed, and calibration plots were obtained by plotting the peak height ratios (phenacemide/oxamniquine) versus the concentration of the drug. An aliquot (0.5 ml) of blank dog plasma or human urine in a 12-ml centrifuge tube was spiked with different amounts of the phenacemide stock solution to prepare seven standard solutions in concentrations ranging 1-15 mcg/ml.

Quantitation - After subjecting unknown plasma or urine samples to the described extraction and chromatographic procedures, the amounts of phenacemide were determined by comparing the peak height ratios of phenacemide to oxamniquine obtained from unknown samples with the calibration plots prepared from spiked standard samples.

Recovery Studies - The extraction efficiency of phenacemide from dog plasma or human urine was determined by comparing the peak height ratios of the drug to the external standard obtained after direct injection of the solutions containing known quantities of phenacemide (1,3,5,8,10,12 and 15 mcg/ml) dissolved in the mobile phase with those obtained after extracting the drug from spiked plasma samples containing the equivalent amounts of the drug. At each of the seven phenacemide concentrations used three to six replicate samples were measured.

Animal Study - To demonstrate the applicability of the assay to the quantitation of phenacemide in plasma, a single bolus intravenous dose of 12 mg/kg in 2 ml of Tween 80/alcohol solution (50/50 V/V%) was administered into the cephalic vein of a mongrel dog weighing 30 kg. Blood samples (5 ml) were withdrawn from the jugular vein using heparinized vacutainer tubes at the intervals of 0, 5, 18, 32, 47, 77 127 min. and 3, 5, 7, 10 and 12 hrs after dose. Blood samples were immediately processed and the plasma (2 ml) was stored in screw-capped plastic vials at -4°C until assayed within a week.

RESULTS AND DISCUSSION

Chromatograms obtained at the lower limit of sensitivity for drug free plasma or urine extracts showed no interferring peaks at the retention times of phenacemide and the calibration marker, oxamniquine. Fig. 1 shows typical chromatograms for the samples prepared from blank dog plasma (A), plasma spiked with 3 mcg/ml of phenacemide (B), and the sample containing the drug and oxamniquine (C). Using the chromatographic conditions described phenacemide and oxamniquine were well separated and their retention times were 2.2 and 6.8 min, respectively. Both peaks were sharp and symmetrical, thus facilitating the accurate measurement of the peak height ratios. No interference by the metabolites or other normal constituents of plasma was recorded.

Standard plots obtained for plasma and urine samples were both highly linear in the concentration range of 1.0-15.0 mcg/ml. Linear regression analyses of the standard calibration plots for dog plasma and human urine samples were, respectively, $y = 0.069 x - 0.010$ and $y = 0.070 x - 0.012$ where y and x are the peak height ratio and phenacemide concentration, respectively. The small negative intercepts indicate that the blank plasma has negligible interferences for the drug by its metabolites. The correlation coefficients of both lines were higher than 0.999.

The day-to-day reproducibility of the assay for plasma samples was evaluated by comparing the least-squares linear regression analyses of the three standard plots obtained from

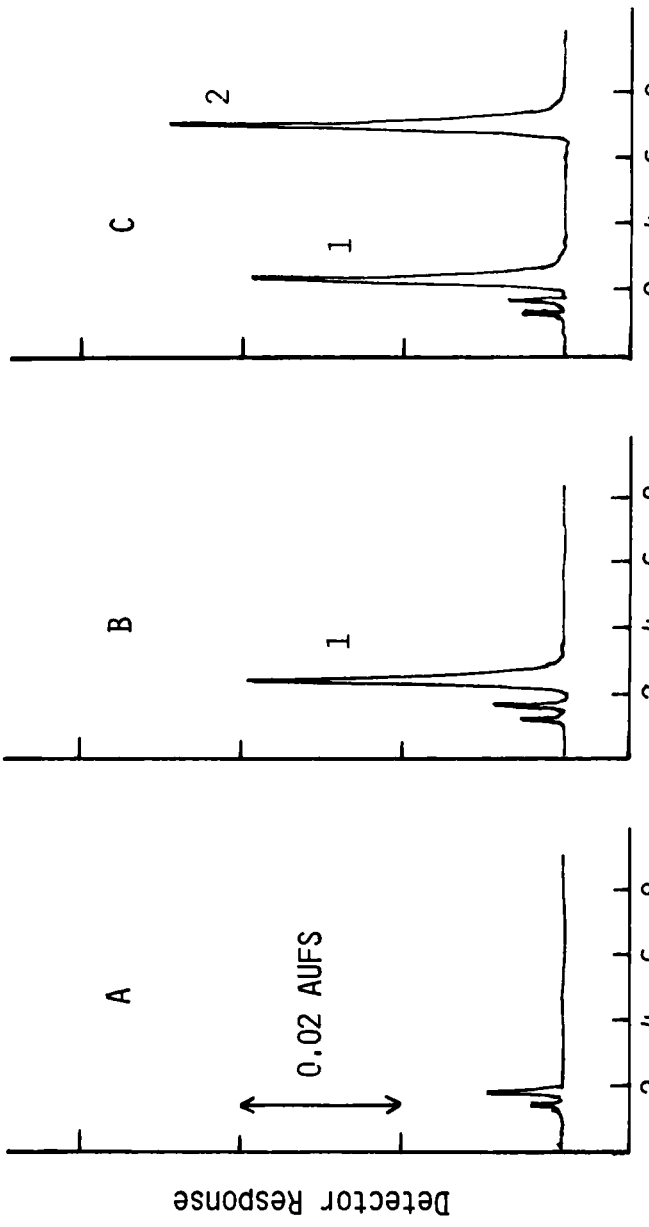

Fig. 1. Chromatograms of blank dog plasma (A); dog plasma containing phenacemide (3 mcg/ml) (B) and dog plasma extract containing phenacemide and the external standard.
Key: 1-Phenacemide, 2-Calibration Marker

TABLE 1

Reproducibility Data for the Standard Plots in Dog Plasma

Standard Plot[a]	Slope[b]	Intercept[b]	Correlation[b] Coefficient
1	0.0699	0.011	0.9995
2	0.0701	0.010	0.9993
3	0.0697	0.010	0.9994

a - obtained in 3 different days
b - the mean of 3-6 determinations at each drug concentration

spiked dog plasma standards at three different days over three week period. The results of this evaluation are summarized in Table 1. The average correlation coefficient was higher than 0.999 and the coefficient of variation of the slopes of the three lines was less than 4%. Analysis of variance of the data showed no detectable difference in the slopes of the three standard plots ($F=2.9, p>0.01$). The results thus confirmed excellent linearity of the calibration lines and high reproducibility of the assay. With little variation in the slopes of the standard plots among multiple determinations, the method should be accurate and precise within the assay day as well as between assay days. The calibration data obtained using human urine samples were in close agreement with the results of plasma data.

The recovery and precision of the assay were assessed by comparing the peak height ratios (phenacemide/oxamniquine) obtained from spiked dog plasma samples of different phenacemide

TABLE II

Standard Calibration and Recovery Data of Phenacemide from Spiked Dog Plasma

Phenacemide Added(mcg/ml)	n[a]	Mean Recovery mcg/ml	Standard Deviation	Recovery[b] %	CV[c] %
1	6	0.97	0.011	97.0	1.1
3	5	2.99	0.183	99.6	6.1
5	5	5.0	0.238	100.0	4.8
8	5	7.8	0.262	97.5	3.3
10	4	9.5	0.250	95.0	2.5
12	3	11.8	0.432	98.7	3.6
15	4	14.6	0.809	97.6	3.4

a - Number of replicate samples
b - Average recovery = 97.9%
c - Average CV = 3.53 %

concentrations (1-15 mcg/ml) to the peak height ratios for the samples containing the equivalent amounts of the drug and external standard directly dissolved in the mobile phase. Three to six replicate samples were measured at each drug concentration and the results are shown in Table II. The average recovery of the drug was 97.9% and its coefficient variation was 3.53%. The results obtained indicated that the concentration of the drug in the samples between 1.0 and 15 mcg/ml had no noticeable effect on recovery.

Table III shows the standard calibration and recovery of phenacemide from spiked human urine samples between the concentrations of 1-15 mcg/ml. The average recovery of the drug was 96.3% and its coefficient of variation was 2.58%.

TABLE III

Standard Calibration and Recovery Data of Phenacemide from Urine Samples

Phenacemide Added(mcg/ml)	n[a]	Mean Recovery mcg/ml	Standard Deviation	Recovery %	CV %
1	5	0.93	0.036	93.3	3.9
3	5	2.81	0.066	93.7	2.1
5	5	4.74	0.059	94.9	2.4
8	5	8.12	0.235	101.6	2.9
10	5	9.72	0.013	97.2	1.2
12	5	11.63	0.290	96.9	2.5
15	5	14.5	0.449	96.6	3.1

a - Number of replicate samples
b - Average recovery = 96.3%
c - Average CV = 2.58%

The sensitivity of the assay defined as the minimum amount drug concentration corresponding to two times signal to noise ratio was found to be approximately 0.2 mcg per ml of plasma or urine. The level of sensitivity is relatively low, but the assay was found to be adequate for monitoring the plasma level vs. time profile of phenacemide in the dog after a bolus intravenous administration of a 12 mg/kg dose which is less than an average therapeutic dose in man.

Higher sensitivity of the method could be obtained by the use of either a larger sample loop or a smaller dilution volume during sample reconstitution.

The plasma level versus time profile after a bolus IV dose (12 mg/kg) drug in a dog is shown in Fig. 2. It shows that the

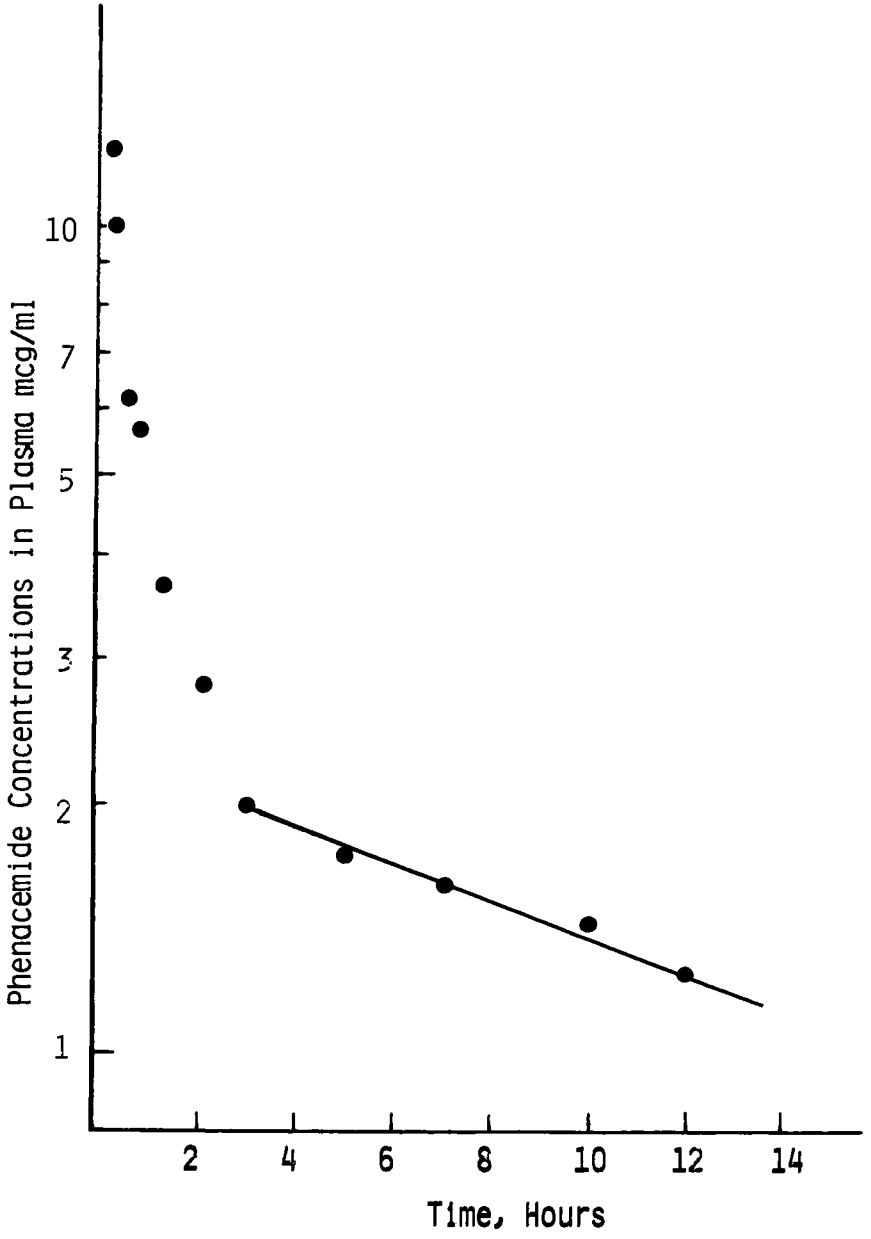

Fig. 2. Plasma concentration of phenacemide in a dog after bolus IV dose of 12 mg/kg.

disposition of phenacemide in this animal follows the two-compartment body model as represented by:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where C is the plasma phenacemide concentration; A and B are the intercepts on the concentration axis; and α and β are the first-order hybrid rate constants for the rapid and slow disposition phases, respectively. The apparent half-life for α phase was found to be 26 min and the half-life for the terminal elimination phase was 13 hours. From 0-12 hrs post-administration, the plasma levels of phenacemide in the dog were approximately in the range of 1-40 mcg/ml. The volume of distribution at steady state (Vd_{ss}) was 55 L indicating that the drug was bound to tissue components.

In conclusion, the new reversed phase HPLC method developed in this study is simple, sensitive and reliable, and is suitable for the quantitation of phenacemide in biological fluids.

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