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DETERMINATION OF LOW LEVELS OF 5,5-DIPHENYLHYDANTOIN IN SERUM BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitative measurement of 5,5-diphenylhydantoin (DPH) in serum. The determination involves simple extraction and gas–liquid chromatographic analysis of the underivatized drug using dehydroisoandrosterone as an internal standard. Levels of DPH in the range of 0.50–8.00 μg/ml can be measured with a maximum possible error of ± 0.11 μg/ml. This assay has been utilized in a pharmacokinetic investigation of DPH elimination in healthy subjects who had ingested a 400-mg dose of sodium DPH.

INTRODUCTION

A pharmacokinetic investigation of drug elimination capacity in man was being carried out in this laboratory[1] and it was decided to include 5,5-diphenylhydantoin (DPH) as one of the drugs to be studied. Subsequently, a sensitive and specific assay for DPH was required.

Most of the analytical methods for the measurement of DPH plasma or serum levels have been developed for routine clinical application[2–10] with only a few of the procedures displaying sufficient sensitivity, accuracy and precision at subtherapeutic levels to be potentially useful as a research tool for our purposes[11–15]. Of these latter techniques, the colorimetric procedure involving nitration of DPH followed by diazotization[11] and the gas–liquid chromatographic (GLC) methods using either silylation[12] or "on-column" methylation[13] of DPH could be subject to error due to incomplete derivatization of the drug or derivative decomposition prior to or during instrumental analysis. The radioimmunoassay technique tends to give high DPH plasma values since the antibodies used in the assay cannot discriminate between DPH and the major metabolite 5-*p*-hydroxyphenyl-5-phenylhydantoin[15].

Recently, it has been demonstrated in a quantitative GLC assay for amobarbital that the use of unconventionally coarse diatomaceous earth solid support (60–80 mesh AW-DMCS Chromosorb W) coated with methyl silicone liquid phase (SE-30) yielded little if any irreversible adsorption of the drug, good sample resolution and

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excellent sensitivity as compared to results obtained with 80–100 mesh support material[16]. Using this stationary phase, a GLC procedure was developed for the determination of DPH in serum without the need for derivative formation.

The use of an internal standard for a GLC microdetermination is virtually essential, but the selection of a suitable compound is sometimes difficult. For this GLC procedure, the best standard among several compounds tried was Δ^5-androsten-3-β-ol-17-one (dehydroisoandrosterone; DIAS), which was clearly separated from serum endogeneous substances and had a retention time slightly longer than that for DPH.

This GLC assay was used to investigate the pharmacokinetics of DPH elimination in healthy volunteers who had ingested 400 mg of sodium DPH.

MATERIALS AND METHODS

Gas–liquid chromatography

An F & M Model 402 biomedical gas chromatograph equipped with a flame ionization detector was used. The column was a glass U-shaped tube (120 cm × 4 mm I.D.) packed with 3% SE-30 on 60–80 mesh AW-DMCS Chromosorb W (Chromatographic Specialties, Brockville, Canada) and it was conditioned at 270° for 16 h with a slow flow of helium carrier gas. The operating conditions were as follows: column temperature, 215°; detector temperature, 280°; injection port temperature, 250°; air flow-rate, 360 ml/min; helium carrier gas flow-rate, 80 ml/min; hydrogen flow-rate, 37 ml/min. The detector sensitivity was set at range 10 and the attenuation was either × 8 or × 16, depending on the sample.

Reagents and materials

5,5-Diphenylhydantoin (DPH) and dehydroisoandrosterone (DIAS) were supplied by Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and Calbiochem (Los Angeles, Calif., U.S.A.), respectively. [4-^{14}C]DPH was obtained from New England Nuclear (Boston, Mass., U.S.A.). All solvents used were ACS quality and were supplied by Fisher Scientific Co. (Pittsburgh, Pa., U.S.A.). Reacti-Vials, 0.3 ml, were obtained from Pierce Chemical Co. (Rockford, Ill., U.S.A.). Butyl-PBD scintillation fluid came from Amersham/Searle Corp., (Arlington Heights, Ill., U.S.A.). The phosphate buffer (pH 6.65) was prepared by adding 133 ml of 0.5 *M* potassium dihydrogen phosphate to 89.2 ml of 0.5 *M* disodium hydrogen phosphate and then diluting to a volume of 1 litre[17].

Standard curves

The standard solutions were prepared by dissolving known amounts of DPH in chloroform–methanol (1:1) solutions containing 2.0 mg/ml DIAS. The concentration range of the DPH standards was 0.30 to 3.00 mg/ml. Prior to injection of the standards, the active sites of the column were saturated with 10 μg DPH. A 1.0-μl aliquot of each standard was injected into the GLC column and the DPH/DIAS peak height ratio was determined and plotted against the amount of DPH applied to the column. A typical standard curve is shown in Fig. 1. The retention time values of DPH and DIAS were 2.4 and 3.8 min, respectively.

Extraction and analysis procedure

A 0.10-ml aliquot of 0.10 mg/ml DIAS solution (chloroform) was added to a 15-ml centrifuge tube and was concentrated with a stream of nitrogen. Then, 2.0 ml of a serum sample were added, followed by 1 ml of phosphate buffer (pH 6.65). The contents of the tube were mixed thoroughly and were then extracted with three 4-ml amounts of diethyl ether using a Vortex mixer each time. The organic layers were combined and placed in a 15-ml centrifuge tube equipped with a ground-glass top and stopper. The ethereal extract was washed with 1 ml of 0.01 *N* hydrochloric acid by shaking the tube contents for 1 min, followed by centrifugation at 700 *g* for 10 min. The organic layer was transferred to a dry 15-ml centrifuge tube and was concentrated. The residue was transferred to a 0.3-ml Reacti-Vial using three 0.1-ml volumes of chloroform–methanol (1 :1), concentrating after each transfer. All samples were stored under vacuum until analyzed, at which time 10.0 μl of chloroform–methanol (1:1) were added to the Reacti-Vial and then a 2.0-μl aliquot was injected into the GLC column. After two serum sample determinations, the column temperature was raised to 250° and held there for 30 min to allow the elution of high-boiling substances extracted from serum. The amount of DPH in each sample was determined by measuring the DPH and DIAS peak heights, calculating the DPH/DIAS peak height ratio and then interpolation on the standard curve. A new standard curve was determined after the analysis of every two serum sample injections.

Extraction efficiency

The efficiency of the diethyl ether extraction step was determined by adding 10,000 dpm of [4-^{14}C]DPH to the centrifuge tube prior to the addition of the serum sample, followed by the counting of a 0.10-ml aliquot of the combined ethereal extract in the liquid scintillation counter using 5 ml of methanol and 15 ml of butyl-PBD in toluene (7 g/l) as the scintillation fluid. The extraction was found to be 99.4 $\pm$ 2.1% (S.E. for eight determinations) efficient regardless of the amount of DPH.

Elimination of DPH in a healthy subject

A healthy male, who had avoided other medication prior to the study, ingested 400 mg of sodium DPH in the form of four 100-mg Dilantin™ (Parke, Davis and Co., Ann Arbor, Mich., U.S.A.) capsules in the evening. Pre-drug serum and eight serum samples over a two-day period were collected and kept frozen at —20° until analyzed.

RESULTS AND DISCUSSION

A few procedures have been reported in the literature that seem to be sufficiently specific and sensitive for the quantitative measurement of DPH serum levels in a pharmacokinetic investigation[11–15]. However, most of these assays involve drug derivatization[11–13] or oxidation[14] that lengthens analysis time and may be subject to error. The analytical method described herein for the determination of DPH serum levels involves simple extraction of the drug followed by GLC analysis of the parent compound and it seems to be as specific and sensitive as those chromatographic procedures requiring methylation[13] or silylation[12] prior to measurement.

In order to obtain the sensitivity and accuracy required for the pharmacokinetic

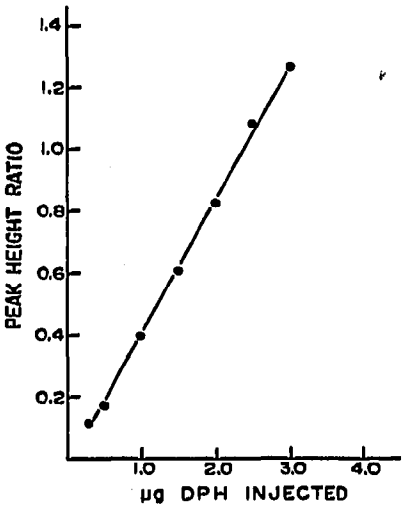

Fig. 1. Standard curve of 5,5-diphenylhydantoin. Peak height ratio (DPH/DIAS) *versus* amount of DPH injected.

study, the coarser solid support 60–80 mesh Chromosorb W coated with 3% SE-30 was used and it yielded less irreversible adsorption and subsequent peak tailing of DPH than seen with 80–100 mesh support material. It was also found that SE-30 yields better separation of DPH from serum endogenous materials than OV-17.

An internal standard was used in the GLC procedure to minimize errors in extraction and instrumental analysis. Dehydroisoandrosterone (DIAS) was selected for this purpose as it is thermally stable and well resolved under the GLC conditions, yielding a sharp symmetrical peak sufficiently separated from DPH and endogenous compounds. DIAS was initially chosen since its acetate derivative had been used previously as an internal standard in a GLC assay for DPH[4]. In Fig. 2, it can be seen

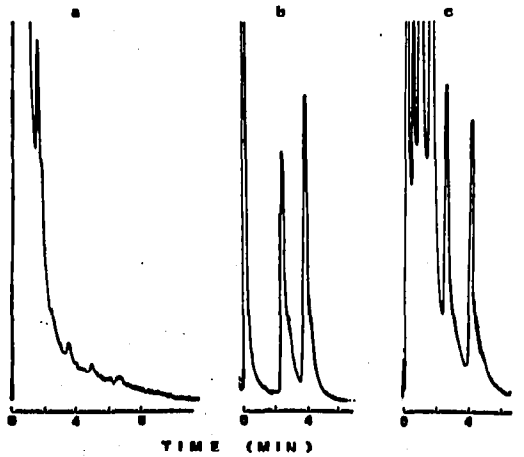

Fig. 2. Chromatograms from DPH analysis. (a) Blank serum extract. (b) A standard: DPH, 2.5 μg, 2.4 min; DIAS, 2.0 μg, 3.8 min. (c) A serum sample extract: DPH, 6.0 μg/ml, 2.5 min; DIAS, 5.0 μg/ml, 4.0 min.

that there are virtually no substances in the serum extract that interfere with either the DPH or DIAS signals.

When standards were injected into the GLC column and the peak height ratios of DPH to DIAS were plotted against the amount of DPH analyzed, linearity was obtained between 0.30 and 3.00 μg, as illustrated in Fig. 1.

The accuracy of the whole method and the precision of the GLC measurement were determined in the following way. Different amounts of DPH were added to 2.0-ml aliquots of normal human serum yielding the following concentrations: 0.75, 2.50, and 6.25 μg/ml. Each sample was then extracted according to the described procedure and duplicate GLC measurements were performed on each extract using the peak height ratio method. The results are presented in Table I and they show that

TABLE I

RECOVERY OF DPH FROM SERUM

Amount added (μg/ml)	*Amount found by GLC (μg/ml)*	*Mean ± S.E. (μg/ml)*
0.75	0.75 0.75	0.75 ± 0.00
0.75	0.70 0.58	0.64 ± 0.06
2.50	2.62 2.60	2.61 ± 0.01
6.25	6.00 6.28	6.14 ± 0.14

the maximum error of the entire extraction and analysis procedure is 0.11 μg/ml regardless of the amount of DPH in the sample. The maximum variability in the precision of the GLC measurement is 0.14 μg/ml. As described in Materials and methods, it has been shown by using a ^{14}C-labelled compound that DPH is extracted from serum

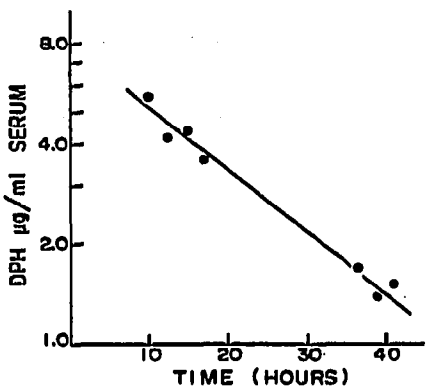

Fig. 3. Serum DPH levels in a healthy adult male following an oral dose of 400 mg of sodium DPH.

to the extent of 99.4 ± 2.1 % (S.E.) regardless of the amount of drug. It would appear that both the extraction and instrumentation errors contribute to the limitation in the accuracy of the method. From this study, it is evident that subtherapeutic levels of DPH can be quantitated with a maximum possible error of ± 0.11 μg/ml when duplicate GLC measurements are made.

This analytical procedure was developed as a research tool for a pharmacokinetic investigation of DPH elimination in man in which several healthy adult males each ingested 400 mg of sodium DPH[18]. The results of one particular study are presented in Fig. 3 and it is quite apparent that DPH serum levels can be easily followed for approximately two days in spite of the single dose.

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