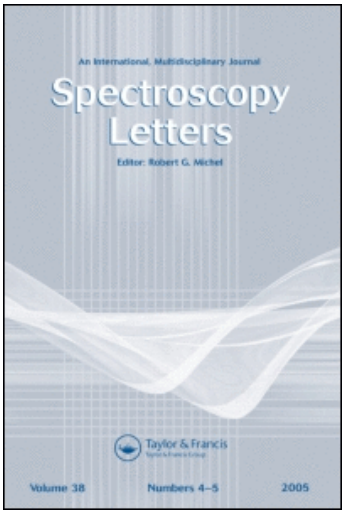

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Determination of Phenytoin, Phenobarbitone, and Methyl Phenobarbitone in Combination

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DETERMINATION OF PHENYTOIN, PHENOBARBITONE AND METHYL PHENOBARBITONE IN COMBINATION

KEY WORDS: Phenytoin, Phenobarbitone, Methylphenobarbitone, Densitometry, ^{1}H-NMR, Spectrophotometry

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ABSTRACT

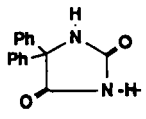
This work is concerned with the determination of a mixture of phenytoin, phenobarbitone and methylphenobarbitone by two different techniques. The first one depends on the simultaneous quantitative densitometric evaluation of thin layer chromatograms of the three drugs. The second is the application of quantitative ^{1}H-NMR spectrometry for the simultaneous determination of phenobarbitone and methyl-phenobarbitone in the presence of phenytoin, while phenytoin was determined in this mixture by modifying the benzophenone spectrophotometric procedures.[1-4]

The proposed procedures were proved using laboratory prepared mixtures of the three drugs and were successfully applied for their analysis in tablet form.

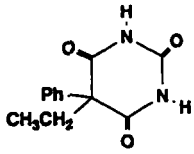
The validity of the proposed procedures was further assessed by applying the standard addition technique.

INTRODUCTION

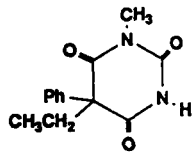
Phenytoin, phenobarbitone and methylphenobarbitone are formulated together in tablets known as Comital-L for the control of chronic epilepsy.[5]



Phenytoin



Phenobarbitone



Methylphenobarbitone

Phenytoin was determined by several methods including titrimetry[6], spectrophotometry[7], colorimetry[8], ^{1}H-NMR spectrometry[9], potentiometry[10], flow injection analysis[11], TLC [12], G.C.[13], and H.P.L.C.[14]. Phenobarbitone was determined by titrimetry[15], spectrophotometry[16], IR. spectrometry[17], TLC[18], G.C.[19], and H.P.L.C.[20], flow injection analysis[21], potentiometry[22], and polarography[23]. Methyl phenobarbitone was analysed by H.P.L.C.[24], spectrophotometry[25], and potentiometry[26].

In anticonvulsant therapy, mixtures of the three drugs are administered together. Phenytoin and phenobarbitone in a mixture have been determined by a variety of procedures including titrimetry[27-30], spectrophotometry [31-34], gas chromatography[35], H.P.L.C.[36]. Mixture of phenobarbitone and methylphenobarbitone was determined by the use of their infra-red spectra[37], gas chromatography[38,39], spectrophotometry and polarography[40].

Due to the structure similarity of the three drugs, any analytical procedure for their simultaneous determination is not an easy target, and accordingly, very few methods have been reported for the determination of this mixture in tablet form. These include H.P.L.C.[41,42], and UV spectrophotometry using the A method for the determination of methylphenobarbitone and the modified Vierordt's method for phenytoin and phenobarbitone[43]. The three drugs were also analysed by the least square's method[43].

This work deals with the determination of the three drugs in tablet form by two different techniques without previous separation.

EXPERIMENTAL

Apparatus

- Shimadzu-Dual wavelength flying spot CS-9000 densitometer.
- Precoated T.L.C. plates, silica gel 60 F_{254} 20X20 cm., 0.25 mm. thickness (Alugram).
- Hamilton syringes 50 ul. and 10 ul.
- Joel FX 90 Q- Fourier- Transform NMR spectrometer.
- Beckman DU-7 spectrophotometer

Pure samples

Phenytoin (Katwijk Chemie B.V. Holland), phenobarbitone (Medexport USSR) and methylphenobarbitone (Siegfried Switzerland) were kindly supplied by Alexandria Pharmaceutical Company. Their percentages purity were found to be 100.00, 99.98 and 100.07 respectively according to the B.P. methods 1993.[44]

Market samples

Comital-L tablets batch No. 3408002, 3408010, 4408001, 4408003 and 4408010. Each tablet claimed to contain 50 mg. of each drug manufactured by Alexandria Pharmaceutical Company under license from Bayer.

Reagents

The reagents and solvents used were of analytical and spectroscopic grades respectively. Ethanol 95% (Merck), Chloroform (Prolabo), Acetone (Prolabo), Dimethyl-d_6-sulfoxide (Sigma), Maleic acid (B.D.H.), n-heptane (Merck) and alkaline permanganate reagent (1% $KMnO_4$ in 7 N NaOH).

Prepared mixtures

Weigh accurately different amounts of phenytoin, phenobarbitone and methyl-phenobarbitone as follows:

Sample No.	Phenytoin (gm.)	Phenobarbitone (gm.)	Me. phenobarbitone (gm.)
1 (1:1:1)	0.50	0.50	0.50
2 (1:1:2)	0.50	0.50	1.00
3 (2:2:1)	1.00	1.00	0.50
4 (2:1:1)	1.00	0.50	0.50

Mix the contents of each sample thoroughly

1- Densitometric Method

- Drug standard solution for linearity:

For each drug, a 1mg.ml^{-1} ethanolic solution was prepared

- Prepared mixtures:

Weigh accurately 150 mg, 200 mg., 250 mg. and 200 mg. of samples 1,2,3, and 4 respectively in a series of beakers, add 30 ml. ethanol. Keep in a water bath at 60°C for 10 minutes with constant magnetic stirring, transfer quantitatively to 50 ml. volumetric flasks and complete to volume with ethanol.

- Procedures:

- Construction of calibration curves:

Apply separately 5, 10, 15, 20, and 25 ul. of the standard solutions of phenytoin and phenobarbitone and 5, 7, 10, 12, 15 and 20 ul. of the standard solution of

methylphenobarbitone to a thin layer chromatographic plate (20X20 cm.) using 10 and 50 ul. Hamilton syringes. Spots are spaced 2 cm. apart from each other and 2 cm. from the bottom edge of the plate. Develop the plate in a chromatographic tank, previously saturated for at least one hour with the developing mobile phase, chloroform-acetone (43.75 : 6.25 V/V), by ascending chromatography through a distance of 16 cm. at room temperature and scan the three drugs at 210 nm.

Record the area under the peak, construct the calibration curves and compute the corresponding regression equations.

- *Assay of prepared mixtures:*

Apply 10 ul. of the prepared mixtures to a silica gel plate and proceed as under construction of calibration curves. Record the area under the peak at 210 nm. for the three drugs. Calculate the concentration of the three drugs from the regression equations (1), (2) and (3) for phenytoin, phenobarbitone and methylphenobarbitone respectively.

Results obtained are shown in table (1)

- *Assay of Comital-L tablets:*

Weigh accurately and powder 10 tablets. Weigh an amount of the powder equivalent to one tablet (claimed to contain 50 mg. of each of phenytoin, phenobarbitone and methylphenobarbitone) in a beaker, add 30 ml. ethanol and keep in a water bath for 10 minutes at 60°C with continuous magnetic stirring. Filter into a 50 ml. volumetric flask and complete to volume with ethanol. Apply 10 ul. of the prepared mixtures to a silica gel plate and proceed as under construction of calibration curves, starting from " spots were spaced ...."

Results obtained are shown in table (2)

Table (1): Determination of Phenytoin, Phenobarbitone and Methylphenobarbitone in the laboratory prepared mixtures by the proposed methods

Sample No.	Proposed densitometric method Recovery ± S.D.			Proposed NMR method Recovery ± S.D.		Spectrophotometric method Recovery ± S.D.
	Phenytoin	Phenobarbitone	Me.Phenobarbitone	Phenobarbitone	Me.Phenobarbitone	Phenytoin
1	99.99 ± 0.56	99.43 ± 0.20	99.95 ± 0.73	100.50 ± 0.34	100.47 ± 0.43	100.03 ± 0.73
2	100.61 ± 0.32	99.94 ± 0.41	100.28 ± 0.56	99.64 ± 0.21	100.18 ± 0.56	100.02 ± 0.65
3	100.26 ± 0.61	99.75 ± 0.36	99.25 ± 0.91	99.75 ± 0.36	99.35 ± 0.39	100.03 ± 0.49
4	100.30 ± 0.31	100.48 ± 0.63	99.75 ± 0.49	100.10 ± 0.19	99.75 ± 0.28	99.68 ± 0.28

Table (2): Statistical analysis of the results obtained by the proposed methods and a compendial[43] method for the analysis of Comital-L tablets

Batch No.		Phenytoin			Phenobarbitone			Methylphenobarbitone		
		Densitometric method	Spectrophotometric method	Compendial method	Densitometric method	NMR method	Compendial method	Densitometric method	NMR method	Compendial method
3408002	Mean ± S.D. t F	100.55 ± 0.57 2.223 (2.447) 3.01 (9.28)	100.25 ± 0.51 1.655 (2.571) 3.77 (9.55)	99.28 ± 0.99	100.18 ± 0.32 1.232 (2.447) 1.81 (9.28)	100.27 ± 0.33 1.399 (2.571) 1.70 (19.16)	99.85 ± 0.43	102.13 ± 0.84 0.364 (2.447) 4.20 (9.28)	101.75 ± 0.30 1.948 (2.571) 1.87 (19.16)	102.30 ± 0.41
3408010	Mean ± S.D. t F	100.63 ± 0.91 1.677 (2.447) 5.45 (9.28)	100.50 ± 0.58 1.929 (2.571) 2.21 (9.55)	99.80 ± 0.39	100.72 ± 0.41 2.169 (2.447) 1.55 (9.28)	100.29 ± 0.31 0.831 (2.571) 2.71 (19.16)	100.01 ± 0.51	99.11 ± 1.12 1.835 (2.447) 6.67 (9.28)	99.28 ± 0.95 1.772 (2.571) 4.88 (19.16)	100.21 ± 0.43
4408001	Mean ± S.D. t F	100.67 ± 0.28 2.230 (2.447) 5.09 (9.28)	100.56 ± 0.75 1.269 (2.571) 1.42 (9.55)	99.90 ± 0.63	100.18 ± 0.29 1.703 (2.447) 4.73 (9.28)	100.15 ± 0.31 1.468 (2.571) 4.14 (19.16)	99.59 ± 0.63	100.75 ± 0.22 0.566 (2.447) 6.24 (9.28)	100.32 ± 0.17 0.761 (2.571) 10.83 (19.16)	100.58 ± 0.56
4408003	Mean ± S.D. t F	100.43 ± 0.52 0.505 (2.447) 1.33 (9.28)	100.26 ± 0.39 0.075 (2.571) 2.37 (19.16)	100.23 ± 0.60	99.88 ± 0.52 1.724 (2.447) 2.43 (9.28)	99.55 ± 0.40 0.967 (2.571) 4.10 (19.16)	99.05 ± 0.81	102.65 ± 0.15 1.579 (2.447) 6.91 (9.28)	101.10 ± 0.97 2.335 (2.571) 6.19 (19.16)	102.32 ± 0.39
4408010	Mean ± S.D. t F	101.87 ± 0.53 1.70 (2.447) 3.36 (9.28)	101.67 ± 0.42 1.373 (2.571) 5.35 (19.16)	100.93 ± 0.97	100.44 ± 0.63 2.172 (2.447) 1.96 (9.28)	100.41 ± 0.48 2.295 (2.571) 1.13 (9.55)	99.60 ± 0.45	100.50 ± 1.11 2.293 (2.447) 1.42 (9.28)	99.67 ± 0.41 1.854 (2.571) 5.02 (19.16)	98.84 ± 0.93

The Figures in parenthesis are the corresponding theoretical values at p=0.01

2- NMR spectrometry for the simultaneous determination of phenobarbitone and methylphenobarbitone in the presence of phenytoin

- Drug standard solutions for linearity:

Weigh accurately from 10-40 mg. of phenobarbitone and methylphenobarbitone separately, add 10 mg. of maleic acid (internal standard) and dissolve in 1 ml. DMSO in a series of NMR tubes.

- Prepared mixtures:

Weigh accurately 30, 40, 50 mg. and 40 mg. of samples 1, 2, 3, and 4 respectively in a series of NMR tubes, add 10 mg. of maleic acid and dissolve in 1 ml. DMSO.

- Procedures:

- Construction of calibration curves:

Record the NMR spectra of the prepared standard solutions and refer all peak positions to T.M.S. at 0.00. Measure the integrals of the sharp singlets at 3.36 ppm. and 6.58 ppm. for methylphenobarbitone and maleic acid respectively and the triplet at 0.92 ppm. for phenobarbitone. Construct the calibration curves relating the amount of the drug in mg. to I_m/I_s where I is the integral of the signal (mm.), the subscripts m and s refer to the drug and the internal standard respectively.

- Assay of prepared mixtures:

To calculate the weight of methylphenobarbitone (W_m), measure the integrals of the sharp singlets at 3.36 ppm. and 6.58 ppm. for methylphenobarbitone and maleic acid respectively, then substitute in the equation:

$$W_m = (H_s M_m / H_m M_s)\, I_m/I_s \,.\, W_s \quad = \quad (2 \times 246.24 / 3 \times 116.04)\, I_m/I_s \,.\, W_s \quad (4)$$

where I is the integral of the signal (mm.), H is the number of protons corresponding to the signal, M is the molecular weight (mg.). The subscripts m and s stand for methylphenobarbitone and maleic acid respectively.

To calculate the weight of phenobarbitone (W_p), measure the integral of the triplet signal at 0.92 ppm. then subtract the integral of the peak at 3.35 ppm. from the former. Calculate the weight of phenobarbitone from the equation:

$$W_p = (H_s M_p / H_p M_s)(I_p - I_m / I_s).\ W_s = (2 \times 232.24 / 3 \times 116.04)(I_p - I_m)/I_s .\ W_s \quad (5)$$

The subscript p stands for phenobarbitone. Results obtained are shown in table (1).

- Assay of Comital-L tablets:

Proceed exactly as under the densitometric method up to "filter into a 50 ml. volumetric flask and complete to volume with ethanol".

Transfer accurately 10 ml. of the prepared solution to a beaker, then evaporate the alcohol, add 10 mg. maleic acid then transfer the contents of the beaker accurately with 1 ml. DMSO to an analytical NMR tube and record the NMR spectrum. Proceed as under assay of prepared mixtures starting from " to calculate the weight of methylphenobarbitone.....". Results obtained are shown in table (2).

3- Spectrophotometric Method for the Determination of Phenytoin in the Presence of Phenobarbitone and Methylphenobarbitone

- Standard solution for linearity:

0.1 $mg.ml^{-1}$ phenytoin in 0.1 N NaOH

- Prepared mixtures:

Weigh accurately 12 mg., 16 mg., 20 mg., and 16 mg. of samples 1, 2, 3, and 4 respectively in a series of 100-ml. volumetric flasks, dissolve and complete to volume with 0.1 N NaOH. Transfer accurately 0.5 ml. from each prepared mixture to a glass stoppered test tube and complete to 1 ml. with 0.1 N NaOH.

- ***Procedure:***

- *Construction of calibration curves:*

Transfer accurately measures volumes (0.1, 0.2, 0.4 ..... 1 ml.) of phenytoin stock solution (0.1 mg.ml-1) into a series of glass stoppered test tubes, complete to 1 ml. with 0.1 N NaOH. Add 20 ml. of freshly prepared alkaline permanganate solution and keep in a water bath for 15 minutes at 90°C. Cool then add 5 ml. n-heptane and shake for 10 minutes. Separate the n-heptane layer and measure the absorbance at 247 nm. against a blank exposed to the same conditions as the experiment.

Construct the calibration curve and compute the regression equation. (6)

- *Assay of prepared mixtures:*

To the prepared mixtures add 20 ml. alkaline permanganate solution and keep in a water bath for 15 minutes at 90°C. Complete as detailed under construction of calibration curves starting from "Cool, then add .....". Results obtained are shown in table (1).

- *Assay of phenytoin in comital-L tablets:*

Weigh accurately and powder 10 tablets. Weigh an amount of the powder equivalent to one tablet in a 50-ml. volumetric flask, complete to volume with 0.1 N NaOH and shake well. Filter and take accurately 10 ml. of the filtrate in a 100-ml. measuring flask and complete to volume with 0.1 N NaOH. Proceed as detailed under construction of calibration curves starting from "Transfer accurately measured volumes .."

Results obtained are shown in table (2).

RESULTS AND DISCUSSION

Several procedures were published on the analysis of phenytoin, phenobarbitone and methylphenobarbitone in a single form. Only very few literature have been reported on the analysis of these drugs in a mixture form because of their structural similarity and in

turn the complication of the problem of their interference. This difficulty in the analysis of the mixed drugs attracted our attention to develop simple and applicable methods for the analysis of each drug in the presence of the other two drugs.

- Densitometric Method:

This work is concerned with the application of a densitometric technique for the simultaneous determination of three drugs in tablet form. The proposed method is based on the difference of their R_f values, phenytoin ($R_f = 0.34$), phenobarbitone ($R_f = 0.43$), and methylphenobarbitone ($R_f = 0.75$). Different developing systems were tried among which complete separation of the three drugs was achieved using chloroform-acetone (43.75 : 6.25 V/V).

The separated spots of the three drugs can be scanned on the same plate at 210 nm. simultaneously. A linear correlation was obtained between the peak area and the concentration in the range of 5-20 ug./spot for methylphenobarbitone and 5-25 ug./spot for both phenytoin and phenobarbitone respectively. The regression equations were computed and found to be:

$$Y = 1.7338\ X + 0.467 \text{ with a correlation coefficient } 0.9995 \quad (1)$$

$$Y = 1.656\ X - 0.21 \text{ with a correlation coefficient } 0.9999 \quad (2)$$

$$Y = 1.4974\ X + 0.0538 \text{ with a correlation coefficient } 0.9999 \quad (3)$$

for phenytoin, phenobarbitone and methylphenobarbitone respectively, where Y is the area under the peak and X is the concentration of the drug in ug./spot.

It was possible by applying the proposed densitometric procedure to determine the concentration of the three drugs in the prepared mixtures with mean accuracies 100.29 ± 0.25, 99.90 ± 0.44 and 99.81 ± 0.37 for phenytoin, phenobarbitone and methylphenobarbitone respectively.

The proposed method has been applied to the assay of the three drugs in tablet form, and the validity of the technique was further assessed by applying the standard addition technique. (Table 3).

- NMR Method:

The solubility of the three drugs and the internal standard maleic acid in DMSO and the absence of resonance signals of the solvent in the regions of interest support the choice of DMSO as a solvent for the NMR procedure.

Figure (1) shows the ^1H NMR spectra of the three drugs in tablet form. All signals were measured with reference to TMS at 0.00 ppm. The sharp singlet at 3.36 ppm. which corresponds to the three protons of the N-methyl group in methylphenobarbitone was used for it's quantitative determination by comparing it's integral to that of the sharp singlet at 6.58 ppm. of the methylene group of maleic acid. The triplet signal at 0.92 ppm. which corresponds to the $-CH_3$ in the ethyl group in both phenobarbitone and methylphenobarbitone was chosen for the determination of phenobarbitone after subtracting the integration at 3.36 ppm. corresponding to methylphenobarbitone from its integration and comparing the integration to that of the methylene group of maleic acid. It was possible by the use of equations (4) and (5) to determine phenobarbitone and methylphenobarbitone simultaneously without interference from phenytoin in laboratory prepared mixtures with mean accuracies 99.99 ± 0.34 and 99.94 ± 0.49 for phenobarbitone and methylphenobarbitone respectively. The proposed method has been applied to the assay of phenobarbitone and methylphenobarbitone in the presence of phenytoin in their tablet form without any interference. The validity of this technique was further assessed by the standard addition technique (table 3).

Table (3): Application of the standard addition technique to the analysis of Comital-L tablets by the proposed methods

	Densitometric method						***NMR method***				***Spectrophotometric method***	
	Phenytoin		**Phenobarbitone**		**Me.phenobarbitone**		**Phenobarbitone**		**Me.phenobarbitone**		**Phenytoin**	
	Added ug/ml	**Recovery %**	**Added ug/ml**	**Recovery %**	**Added ug/ml**	**Recovery %**	**Added ug/ml**	**Recovery %**	**Added ug/ml**	**Recovery %**	**Added ug/ml**	**Recovery %**
Batch No 3408010	2.50	100.00	2.50	100.08	2.50	10.40	5	99.94	5	100.20	2	100.00
	3.75	99.58	3.75	99.70	3.75	99.70	10	100.07	10	100.44	4	99.67
	5.00	99.80	5.00	99.60	5.00	100.50	20	100.74	20	99.75	8	99.75
	12.50	100.16	12.50	100.16	12.50	100.10	30	100.37	30	100.45	12	100.08
Average % ± S.D.		99.89 ± 0.26		99.89 ± 0.28		100.18 ± 0.36		100.28 ± 0.36		100.21 ± 0.33		99.88 ± 0.19

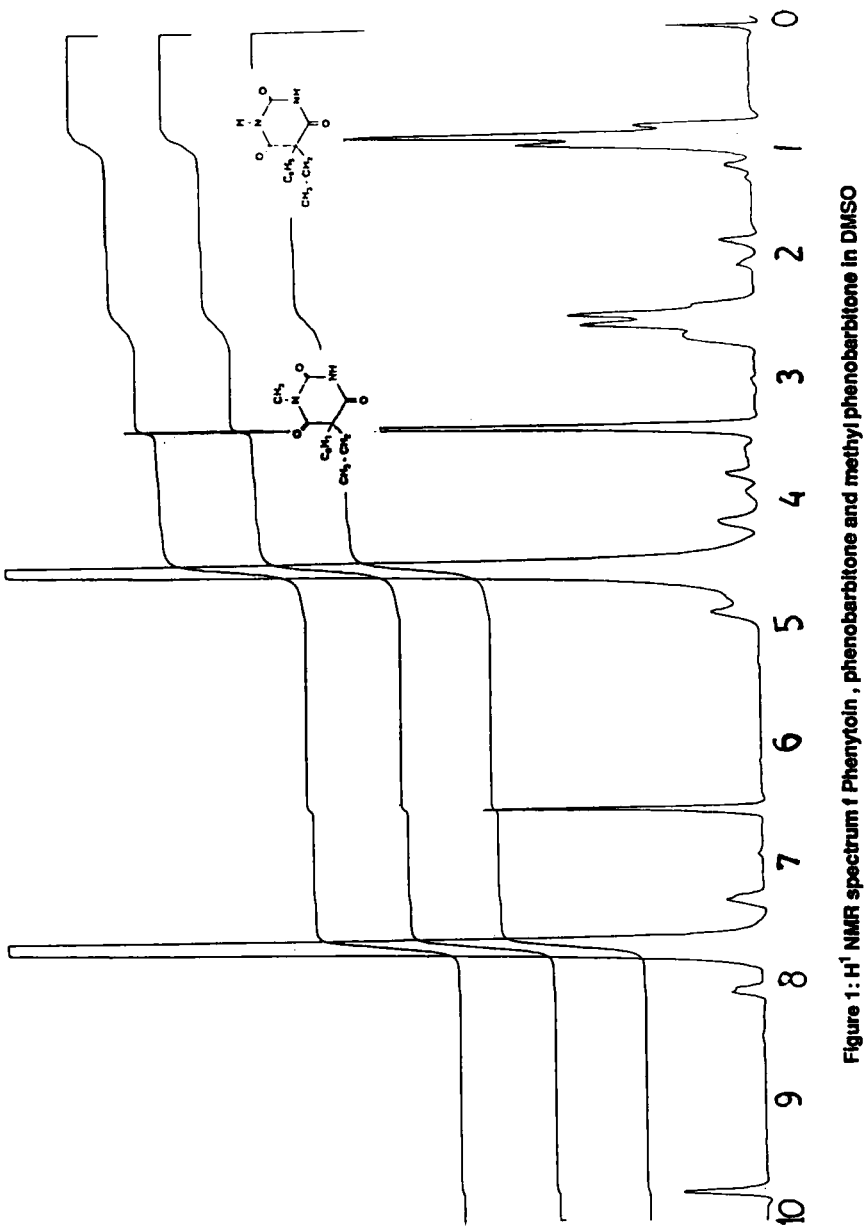


Figure 1: H^1 NMR spectrum f Phenytoin , phenobarbitone and methyl phenobarbitone in DMSO

Table (4): Statistical analysis of the results of the proposed methods and the official method for phenytoin, phenobarbitone and methylphenobarbitone separately in their pure powdered form.

	Phenytoin			Phenobarbitone			Methylphenobarbitone		
	Densitometric method	NMR method	Official method	Densitometric method	NMR method	Official method	Densitometric method	NMR method	Official method
Mean ± S.D.	99.95±0.99	100.07±0.41	100.00±0.60	100.02±0.77	99.65±0.36	99.98±0.51	99.98±0.51	99.47±0.68	100.07±0.2
n	5	8	4	5	5	4	6	5	8
Variance	0.98	0.168	0.360	0.593	0.130	0.260	0.260	0.384	4
Student's t	0.088 (2.365)	0.393 (2.228)		0.089 (2.365)	1.141 (2.365)		0.493 (2.306) 3.33 (9.01)	1.778 (2.365)	0.078
F	2.72 (5.19)	2.14 (4.35)		2.28 (6.59)	2.00 (6.59)			4.92 (9.12)	

The Figures in parenthesis are the corresponding theoretical values at p=0.01

- Spectrophotometric Method For Phenytoin:

Phenytoin has an undefined absorption spectrum but upon oxidation it is converted to benzophenone which has a well defined peak at 247 nm. Several procedures are based on this idea i.e. the quantitative determination of phenytoin through the determination of it's benzophenone oxidation product under different experimental conditions. The optimum conditions for the production of maximum absorbance at 247 nm. were studied. It was found that the used of 20 ml. alkaline $KMNO_4$ reagent, heating at 90°C for 15 minutes, extracting with 5 ml. n-heptane and shaking for 10 minutes were the optimum conditions to obtain reproducible and quantitative results.

This work was adopted for the determination of phenytoin in the presence of phenobarbitone and methylphenobarbitone either in their laboratory prepared mixtures or in their pharmaceutical formulation (Comital-L tablets) without interference. A linear correlation was obtained between the absorbance at 247 nm. and the concentration range (2-20 ug./ml.) for phenytoin from which the linear regression equation was found to be $Y = 0.07047 X - 0.00197$ (6) with a correlation coefficient 0.9999, where Y is the absorbance at 247 nm. and X is the concentration of the drug in ug./ml.

It was found that 95% of phenytoin is converted to benzophenone under the previously mentioned conditions.

Table (4) shows statistical comparison of the results obtained by the proposed methods and the official BP 1993 method (44) for the pure drugs separately, while table (2) shows statistical comparison of the results obtained by the proposed methods and those obtained by applying the reference method (43) for the tablet form. Results of the statistical analysis show no significant difference between the proposed methods and the reference methods, so the proposed methods were equally precise and accurate as the reference methods.

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