Aceton verwenden) unter Rückfluß erhitzt. Bei der Herstellung von **5a** und **5b** wurden noch jeweils 5 Tropfen etwa 40proz. HI zugesetzt. Anschließend wurde heiß filtriert und abgekühlt. Die sich abscheidenden Kondensationsprodukte wurden teilweise erst nach längerem Stehen und Anreiben kristallin. Sie wurden abgesaugt, mit C_2H_5OH gewaschen und umkristallisiert.

Die Daten zu den aufgeführten Verbindungen sind in Tab. 2 enthalten. Die Elementaranalysen (alle Elemente außer 0) ergaben stimmende Werte innerhalb der üblichen Fehlergrenzen.

Die Strukturen folgender Verbindungen wurden durch die ^{1}H-NMR-Spektren gesichert: **2a** bis **2c**, **3a** bis **3d**, **5a** bis **5c**.

Wir danken der Abteilung Chemische Analyse für die Ausführung der Elementaranalysen und die Anfertigung der IR-Spektren.

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GLC Analysis Following Oxidation of Phenytoin
An Inexpensive Method for Routine Determination of the Drug in Plasma

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Phenytoin (5,5-diphenyl-2,4-imidazolidinedione) is readily oxidized to benzophenone. Using spiro(fluorene-9,4-imidazolidine)-2′,5′-dione or 5-(4′-methoxyphenyl)-5-phenyl-2,4-imidazolidinedione as internal markers the oxidation product can be analyzed by GLC, providing an analytical method for plasma phenytoin which removes the need for preliminary extraction of the drug.

GC-Analyse des Oxidationsprodukts von Phenytoin

Eine einfache Methode für die routinemäßige Bestimmung im Plasma

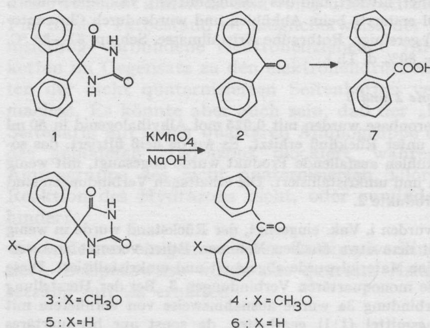
Phenytoin (5,5-Diphenylimidazolidine-2,4-dion) läßt sich leicht zu Benzophenon oxidieren. Mit Spiro(fluoren-9,4′-imidazolidin)-2′,5′-dion oder 5-(4-Methoxyphenyl)-5-phenyl-2,4-imidazolidindion als innerem Standard können die Oxidationsprodukte gc analysiert werden, so daß Phenytoin im Plasma ohne vorherige Extraktion bestimmt werden kann.

1. Introduction

Drugs with a narrow therapeutic range and a degree of intersubject variability call for careful monitoring of blood levels in their routine use. It is important that cheap and efficient analytical methods are available for their determination. A drug which falls into the category outlined above is phenytoin [22, 31]. Variation in bioavailability [27] of phenytoin formulations combined with the non-linear elimination kinetics of the drug [13, 33] increase the need for the plasma level monitoring. Although no means for phenytoin measurement was available before 1956 [22], in the last fifteen years a great number of methods have appeared in the literature. The first reliable method [40], which was subsequently refined [3, 20, 26, 30, 40—44], was spectrophotometric and depends on the assay of benzophenone, produced by permanganate oxidation of phenytoin in the sample. Later on, GLC [2, 4, 10, 18, 19, 21, 23, 24, 28, 35], TLC [39], enzyme immunoassay [8, 16, 25, 38], radioimmunoassay [5], mass fragmentographic [32] and HPLC [1, 6, 7, 14, 17, 32, 36] methods were developed for the determination of drug. All such methods tend to be subject to error because of (a) difficulties in derivatisation of the drug, (b) incomplete derivatisation, (c) decomposition of the derivative, (d) the multiple extraction steps needed to clean up the sample. In addition, for routine testing of the drug, a balance has to be struck between (e) the time taken per sample, (f) the size of sample needed, (g) the cost, and (h) sophistication of the necessary instrumentation, (i) the cost, and (j) the availability of the chemical reagents, (k) the need for experienced personnel.

The present report describes a combination of the oxidation technique of analysis of phenytoin with that of GLC. The usual oxidation of the drug (**5**) to benzophenone (**6**) was carried out in the presence of spiro(fluorene-9,4′-imidazolidine)-2′,5′-dione (**1**) or 5-4-methoxyphenyl)-5-phenyl-2,4-imidazolidinedione (**3**) used as internal standards, which were similarly oxidized to 9-fluorenone (**2**) and 4-methoxybenzophenone (**4**) respectively (Scheme 1). The conditions used may cause further partial oxidation of **2** to **7** [9, 29] but if conditions are standardized this can be accounted for in the calibration.

Scheme



The method developed has the characteristics of a routine assay procedure (e) to (j) above where GLC apparatus is available. It requires only 0.1—0.2 ml plasma (f). Derivatisation (a) is well-ensured in the oxidation technique [3, 20, 26, 30, 34, 40—44] in contrast with derivatisation for chromatographic purpose [28, 37]. It is cheap to effect (i) and (j) and does not involve prior extraction (d). In view of the simplicity of the procedure (k) the time taken to obtain a quantitative result from a sample (ca. 30 min) can be considered reasonable.

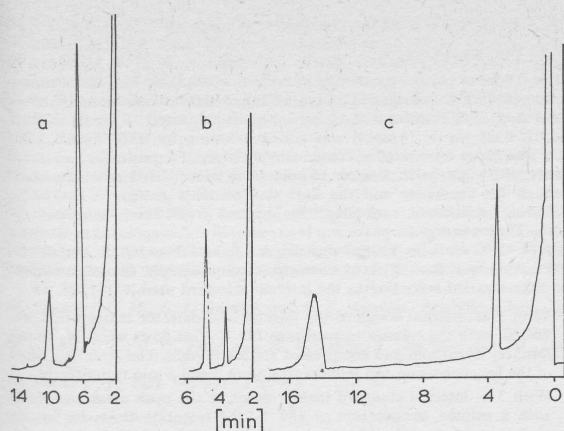


Fig. 1. GLC chromatograms of the extract following oxidation of phenytoin. a: Using spiro(fluorene-9,4'-imidazolidine)-2',5'-dione as marker added before oxidation, b: using 9-fluorenone as a merker added after oxidation, c: using 5-(4-methoxyphenyl)-5-phenyl-2,4-imidazolidinedione as a marker added before oxidation. Column conditions as in text

2. Results and Discussion

Chromatograms of sample containing therapeutic levels of phenytoin in plasma following oxidation are shown in Fig. 1.

In Fig. 1 (a) the internal standard of spiro(fluorene-9,4'-imidazolidine)-2',5'-dione (**1**) was added to the plasma sample prior to oxidation and was itself oxidized to 9-fluorenone. In Fig. 1 (b) the internal standard, 9-fluorenone (**2**) was added during extraction of benzophenone after oxidation. In both cases a plot of the peak height ratio (benzophenone/9-fluorenone) versus phenytoin concentration was linear between 1 and 40 μg/ml. However, the slope of the straight line obtained using the spiro compound as internal standard was greater than that which was derived using 9-fluorenone directly. For example, comparing the calibration curves obtained using the internal standards (a) **1** at the oxidation stage and (b) **2** at the extraction stage under the conditions specified above, the yield of 9-fluorenone (**2**) for the former case was found approximately 15% less than the theoretically expected value.

In addition, the slope obtained using the spiro compound as internal standard was itself variable and dependent on the reaction conditions during oxidation. The more vigorous the conditions (time of heating, amount of permanganate, concentration of alkali) the steeper was the slope. Consequently, the differences noted cannot be caused by recovery problems. These variations can be attributed to the partial further oxidation of 9-fluorenone to 2-phenyl benzoic acid (**7**) which remains in the aqueous alkaline phase during the extraction and does not interfere with the analysis. Surprisingly, using standard conditions of oxidation with the spiro compound as internal standard, the part-conversion of the predominant oxidation product **2** to **7** is constant, leading to linearity of the calibration plots. Using 9-fluorenone (**2**) as an internal standard in the extraction stage always produced a straight line with a constant slope described by the regression equation $y = 0.123 + 0.050\,x$ ($R = 0.99$), where y is the peak height ration (benzophenone/9-fluorenone) and x is the concentration of phenytoin in μg/ml. The intercept on the peak height ratio axis is linked with the slightly wider peak of 9-fluorenone compare to benzophenone. For data obtained from running replicate samples in the range of 2.5—20 μg/ml the precision varied from ± 6.1 to 9.1% (coefficient of variation, CV), Table.

Chromatograms of phenytoin from oxidized plasma samples to which **3** was added as internal standard are shown in Fig. 1 (c). The conversion of **3** to **4** was quantitative and gave linear plots of the ratio of peak areas or peak heights against phenytoin concentration. Thus, the linear regression line $y' = 0.014 + 0.057\,x'$ ($R = 0.99$) was obtained under isothermal conditions at 180 °C, where y' is the ratio of peak areas benzophenone/(4-methoxybenzophenone) and x' is the concentration of phenytoin in μg/ml. Data for the variation of precision shown in the Table, reveal a coefficient of variation ±1.5 to 5.1% for the range of concentrations 2.5—20 μg/ml. The results in the Table indicate that the minimum concentrations that can be measured accurately by this procedure using the internal standard **3** is considerably lower than 2.5 μg/ml. This value for phenytoin is well below the therapeutic level. When a programmed column temperature was used the plot gave a linear regression line $y' = 0.018 + 0.092\,x''$ ($R = 0.99$) where y'' is the ratio of peak heights benzophenone/4-methoxybenzophenone and x'' is the concentration of phenytoin in μg/ml.

Table Mean Recoveries of Phenytoin from Human Plasma Spiked with the Drug, as Measured by GLC of the Oxidized Sample Using either 9-Fluorenone (**2**) or 5-(4-methoxyphenyl)-5-phenyl-2,4-imidazolidinedione (**3**) as Internal Standards

Aded μg/ml	Measured* [μg/ml] ± C. V. [%]** Internal standard **2**	internal standard **3**
2.5	2.20 (9.1)	2.65 (5.1)
5.0	5.04 (6.9)	5.01 (4.9)
10.0	10.09 (7.5)	9.93 (3.2)
20.0	20.14 (6.1)	20.41 (1.5)

*Mean of four replicate samples; ** coefficient of variation (s/y · 100)

A recovery study was done to show whether some of the oxidation products **4** and **6** were being lost during oxidation or extraction. Recovery was essentially complete since **4** and **6** were 100% recovered, as calculated by relative average peak heights of plasma samples as compared to aqueous samples. In addition, regression analysis of data from water samples, using the same range of drug concentrations, resulted in identical equations to those quoted above.

Blank samples of plasma using either **1**, **2** or **3** as internal standards gave no interfering peaks nor peaks corresponding to **6** or, using suitable internal standards, **2** or **4**. However, it is worth noting that the commercially available p-methoxybenzophenone (**4**) may contain benzophenone as an impurity. Use of unpurified **4** for the synthesis of **3** [43] then inevitably leads to the simultaneous formation of phenytoin as an impurity of the internal standard. In this case analyses of blank samples (aqueous or plasma) show a benzophenone peak corresponding to an approximately 2% impurity in the internal standard. Extensive recrystallisation of p-methoxybenzophenone eventually gave a benzophenone-free material which was used as the starting point for the synthesis of **3**.

A series of experiments was carried out as above in the presence of the primary metabolite of phenytoin, 5-(p-hydroxy)-5-phenyl-2,4-imidazolidinedione. No interfering peak was noted, since its oxidation product, benzoic acid [34], is not extracted from the aqueous alkaline phase. Analyses were also carried out in the presence of methoin, which did not interfere.

The rabbit data are graphically presented in Fig. 2. Plasma phenytoin levels were readily monitored throughout the absorption phase. 9-fluorenone (3 μg) was used as internal standard

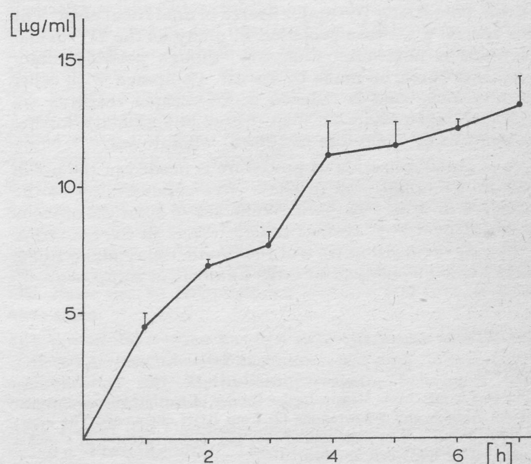


Fig. 2. Plasma phenytoin levels with standards deviations following oral administration to 2 rabbits of 70 mg phenytoin as suspension

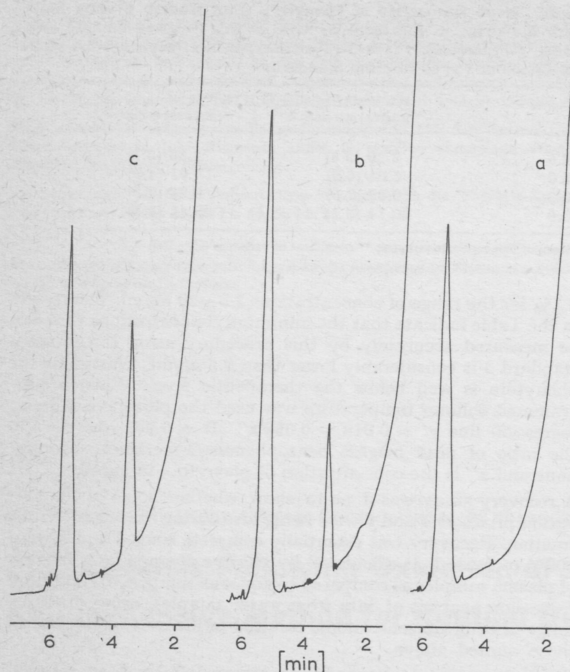


Fig. 3. Typical chromatograms of benzophenone and 9-fluorenone seen after oxidation of 0.2 ml rabbit plasma. a: Blank, b: 1 h after oral phenytoin administration, c: 5 h after oral phenytoin administration. The first peak is benzophenone while the second is 9-fluorenone

at the extraction stage. Chromatograms of benzophenone and 9-fluorenone in rabbit plasma are shown in Fig. 3. This preliminary rabbit study was done to show the utility of the procedure in following plasma phenytoin concentrations. It was later adopted for a study focusing on the absorption characteristics of drug in presence of proteins.

Although plasma samples were oxidized without any previous treatment, clear chromatograms were obtained (Figs. 1 and 3). This is attributed to two factors. Firstly, the strongly alkaline aqueous phase prevents the extraction of many of the oxidized plasma constituents such as acids, keto-acids and phenols.

Secondly, the low column temperatures used prevent interference from any high boiling point oxidation products of plasma which may be extracted in the dichloromethane.

The technique outlined above simplifies the UV and GLC methods for determination of phenytoin in several ways. Most importantly, it eliminates the extraction step for the isolation of the drug, potentially the major source of analytical error. The need for special glassware proposed in many of the UV oxidation methods is not found necessary, though possibly minor improvements could be made by its use. Compared with other GLC methods the cost is reduced, since cheaper reagents are used. The method provides a high degree of specificity for the routine analysis of phenytoin in clinical laboratories.

A degree of standardisation of procedure is needed in the use of the oxidation method, and in these circumstances any of the standards 1/2 or 3 may be used. While use of 3 has the obvious advantage of guarding against drastic errors in the oxidation stage, the longer retention time of the product may make 1 or 2 more attractive for analysis of large numbers of samples.

3. Experimental

3.1. Materials

Phenytoin was a gift from Messrs Parke Davies, 4-methoxybenzophenone (6) was from Aldrich and 9-fluorenone (2) from BDH chemicals. The internal standards 3 and 1 were synthesized [11, 12]. The structures of 1 and 3 were confirmed by IR, NMR and MS.

GLC was carried out using a Perkin Elmer F33 instrument with two FID detectors. The columns were 2 m long by 3 mm i. d. glass packed with 3% OV-17 (phenyl methyl silicone oil) on 80—100 mesh chromosorb W-HP.

3.2. Methods

3.2.1. Plasma Samples

0.1 or 0.2 ml of plasma containing phenytoin were placed in a 50 ml round-bottomed flask to which was added 0.1 ml of the internal standard used, either 1 or 3. The internal standard solutions contained 16 μg/ml in 1.5% NaOH. 8 ml NaOH (5 mol/l) was added following by 0.4 g $KMnO_4$. The flask was fitted with a teflon coated stirring bar and a condenser and boiled gently with vigorous stirring for 15 min. 15 ml of cold H_2O was then added through the condenser and the flask and contents cooled to 10—20 °C. Following addition of 4 ml CH_2Cl the mixture was stirred vigorously for 5 min. The lower organic phase was then removed and evaporated in a water bath at 41 °C until the volume was reduced to less than 100 μl. 1 μl of this concentrate was then injected onto the chromatograph. Chromatographic conditions varied according to the internal standard used.

- With 1 as internal standard the injector and detector temperature was 225 °C, with the column temperature 185 °C. Gas flows were: N_2 60 ml/min, H_2 50 ml/min and compressed air 250 ml/min. The retention times of the benzophenone and 9-fluorenone were 3 and 5 min respectively.
- With 3 as internal standard measurement of the peak areas was made with a column temperature of 180 °C, the retention times for benzophenone and 4-methoxybenzophenone being 3.5 and 14.5 min respectively. When peak height ratios were to be determined a programmed column temperature was used with an inital temperature of 180 °C for 6 min followed by a rise to 220 °C at 20°/min. All other conditions were as above. The retention times were 3.5 and 9.5 min for benzophenone and p-methoxybenzophenone respectively.
- Using 9-fluorenone (2) as internal standard the oxidation of phenytoin was carried out as above but with 3 μg of the internal standard added at the extraction stage as a solution in CH_2Cl_2.

The column was cleaned daily by holding a temperature of 250 °C for 2 h to allow elution of high boiling contaminants from plasma.

3.2.2. Aqueous Samples

The procedure used was as for the plasma samples, but, with less oxidizable material present, the amount of $KMnO_4$ was reduced to 0.2 g.

3.2.3. In vivo Study

Two white male New Zealand rabbits of about 2.5 kg were used. Each animal was starved for 48 h. Using a wide syringe and plastic tubing they were then treated orally with a suspension of 70 mg phenytoin powder in 10 ml of H_2O followed by two portions of 10 ml of H_2O. The rinses were used to ensure the complete transfer of drug into the stomach of animal. Blood samples were collected in heparinized vials from the rabbit at 1, 2, 3, 4, 5, 6 and 7 h postadministration by an incision of the marginal ear vein. The samples were centrifuged at 3500 rpm for 20 min and the plasma was stored frozen until assayed.

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Beiträge zur gaschromatografischen Bestimmung von Atropin, Theophyllin, Phenobarbital und Aminophenazon in Tabletten

P. MAJLÁT

Eine aus zwei Arbeitsgängen bestehende gc Methode wurde für die Trennung und quantitative Bestimmung von Atropin, Theophyllin, Phenobarbital und Aminophenazon in Tabletten entwickelt. Durch einfache Extraktionsverfahren werden zwei Fraktionen erhalten. Atropin wird mit BSA silanisiert und unter Verwendung des inneren Standards Homatropin chromatografiert. Theophyllin, Phenobarbital und Aminophenazon werden mit Trimethylphenylammoniumacetat behandelt und neben dem inneren Standard Amobarbital bestimmt. Die Trennungen erfolgen auf 3% OV 1 bei 160 °C und 210 °C. Das Verfahren ist noch anwendbar, wenn Atropin in geringen Mengen, wie z. B. 0,07%, bezogen auf die Gesamtmenge der Proben vorliegt. Diese Verbindungen können in verschiedenen Arzneiformen bestimmt werden.

Contribution of the GLC Assay of Atropine, Theophylline, Phenobarbital and Aminophenazone in Tablets

A GLC method consisting of two runs has been developed for the quantitative determination of atropine, theophylline, phenobarbital and aminophenazone in tablets. By using simple extraction procedures two fractions are obtained. Atropine is silylated with BSA and homatropine is applied as the internal standard. Theophylline, phenobarbital and aminophenazone are treated with trimethylphenylammonium acetate and amobarbital is used as the internal standard. Separations are carried out on 3% OV 1 at 160 °C and 210 °C. The method is applicable for determining atropine even when its amount is 0.07% of that of the sample. The procedure presented here provides possibilities for the assay of these compounds in various dosage forms.

1. Einleitung

Als Antiasthmatica sind zahlreiche Arzneipräparate mit Atropin, Theophyllin, Phenobarbital und Aminophenazon im Arzneimittelverkehr. Die Erfassung der einzelnen Komponenten wird durch die Anwesenheit weiterer Wirkstoffe erschwert. Die quantitative Bestimmung des Atropins, das in wesentlich geringerer Konzentration auftritt (z. B. 1:1500), ist besonders problematisch.

Zur Lösung derartiger Aufgaben bietet sich die GC an.

2. Ergebnisse und Diskussion

Um die erwähnten Wirkstoffe zu trennen, wurde eine Reihe stationärer Phasen untersucht. Wegen der verschiedenen Flüchtigkeit dieser Verbindungen war es unmöglich, ihre Trennung für quantitative Zwecke bei isothermen Bedingungen durchzuführen. Ferner konnten nicht alle Komponenten in einem Schritt aus der Tablettenmasse isoliert werden. Es wurde daher ein aus zwei Arbeitsgängen bestehendes, isothermes Verfahren auf OV 1 ausgearbeitet.

Eine genaue direkte gc Analyse des Phenobarbitals ist infolge seines polaren Charakters nicht durchzuführen. Sein Peak zeigt einerseits starke Schwanzbildung, andererseits sind die Analysenresultate wegen der irreversibilen Adsorption nicht reproduzierbar. Es ist deshalb die Methylierung des Phenobarbital-Moleküls erforderlich. *Mráz* und Mitarb. [8] empfahlen statt der früher verwendeten quartären Ammoniumbasen [4—6, 9, 10] die Anwendung des neutralen Trimethylphenylammoniumacetats, mit dem Phenobarbital ein 1,3-Dimethylderivat quantitativ ohne Nebenprodukte bildet.

Auch die direkte gc Bestimmung des Theophyllins ergab einen breiten, nicht gut auswertbaren Peak mit Schwanzbildung. Zur besseren quantitativen Erfassung kann man Theophyllin ebenfalls alkylieren [2, 13]. Wie bei Phenobarbital erwies sich auch bei Theophyllin Trimethylphenylammoniumacetat als geeignet. Der Peak des so entstehenden Coffeins ließ sich quantitativ auswerten.

Die Wirkstoffe werden aus der Tablettenmasse mit Chloroform isoliert und anschließend zusammen mit dem inneren Standard Amobarbital mit Methylierungsreagenz behandelt. Aminophenazon bleibt dabei unverändert.

Abb. 1 zeigt das Gaschromatogramm der methylierten Verbindungen Phenobarbital, Theophyllin und Amobarbital sowie des unveränderten Aminophenazons.

Die gc Bestimmung des Atropins erfolgt teils auf silylierten Trägern ohne vorhergehende Derivatisierung [1, 3, 12, 14, 16],