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GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHY OF BARBITURATES AFTER FLASH-HEATER DERIVATIZATION WITH DIMETHYLFORMAMIDE DIMETHYLACETAL

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SUMMARY

Glass capillary column gas chromatography has been used for quantitative and qualitative analyses of barbiturates. The barbiturates are first converted into the corresponding acetal derivatives by flash-heater derivatization, using dimethylformamide dimethylacetal as the derivatization reagent. The influence of the injection port temperature, the column temperature, the solvent used to dissolve the substances and the reagent dilution were studied in order to obtain the optimal response with good reproducibility. Calibration graphs in the concentration range 5–50 μg/ml were evaluated and the relative standard deviations for the quantitative analyses were calculated. High sensitivity was obtained when extracts from plasma containing phenobarbital in the low therapeutic concentration range were analysed using the described derivatization technique.

INTRODUCTION

Glass capillary gas chromatography (GC) is a high-resolution separation method for qualitative and quantitative analyses of complex mixtures containing components with a wide range of volatilities, polarities and concentrations. In trace analyses of species containing functional polar groups, disturbances due to irreversible adsorption or decomposition may occur. To avoid these problems, polar substances are derivatized and carefully deactivated columns are used. Recently, glass capillary GC was used for the identification and quantitation of trace amounts of barbiturates after pre-column alkylation[1]. Derivatization can also be carried out in the flash heater of the gas chromatograph. This flash-heater derivatization technique has recently been applied to glass capillary columns. The reaction occurs in the heated injection port by simultaneous injection of the sample and the reagent[2,3]. The method is applicable to heat-stable drugs that react rapidly with the derivatization reagent to form a single product. Reagents suitable for forming trimethylsilyl derivatives with narcotic drugs[2] and with aromatic carboxylic acids of pharmaceutical

interest[3] have been investigated. The method could be used for quantitative analyses in the nanogram range.

Since their introduction as derivatization reagents, N,N-dimethylformamide (DMF) dialkylacetals have been applied to a wide range of compounds of pharmaceutical interest[4]. With barbiturates the reaction appears to be quantitative and reproducible, giving the corresponding acetals with good chromatographic properties on packed columns[5]. In this study, the flash-heater derivatization technique has been applied to barbiturates using DMF dimethylacetal as reagent. Several parameters are important if optimal response, accurate results and good reproducibility are to be obtained. A concentration range of 5–50 μg/ml was carefully investigated. For the analyses of plasma samples containing barbiturates, this concentration range covers low therapeutic levels and can, if necessary, be extended to higher concentrations. It was evident that the injection technique, the injection port temperature and the initial column temperature had to be controlled carefully. The results were also influenced by the solvents used to dissolve the barbiturates and the reagent dilution. When these parameters were controlled, linear calibration graphs were obtained and reproducible analyses could be carried out. Plasma samples containing phenobarbital in the low therapeutic concentration range were analysed using the described derivatization technique with good sensitivity.

MATERIALS AND METHODS

Reagents

Barbital, allobarbital, aprobarbital, amobarbital, pentobarbital, hexobarbital and phenobarbital were of pharmacopoeial grade, supplied by Norsk Medisinaldepot (Oslo, Norway). Heptadecane was obtained from Koch-Light (Colnbrook, Great Britain) and DMF dimethylacetal in 10-ml vials was purchased from Pierce (Rockford, Ill., U.S.A.). The reagent was used either undiluted or diluted with pyridine (1:4).

Stock standard solutions of the barbiturates contained 10 mg/ml of ethyl acetate and 1 mg/ml of hexobarbital and phenobarbital in DMF and dioxan.

Analytical-reagent grade ethyl acetate, DMF, dioxan and pyridine were obtained from E. Merck (Darmstadt, G.F.R.).

Gas chromatography

A Fractovap 2300 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame-ionization detector and a capillary column splitless injector was used. The glass capillary columns (20 × 0.35 mm I.D.) (H. and J. Jaeggi, Trogen, Switzerland) were wall-coated with OV-1 (quantitative analyses) or SE-30 (qualitative analyses). The injection port temperature was 200° and the samples were injected at an oven temperature of 70° or 150°. The temperature was programmed at 5°/min up to 220°. Nitrogen was used as the carrier gas at an inlet pressure of 0.4 kp/cm^2, which gave a flow-rate of 1.4 ml/min through the column. The splitting ratio for the injector was 1:40 for the identification tests. Samples for the quantitative analyses were injected splitless. The splitter was closed before the injection and re-opened 30 sec after the injection. The sensitivity setting varied from 10 × 2 to 10 × 8 and a Spectra Physics Autolab Minigrator was connected to the gas chromatograph for peak-area measurements.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS was carried out using a MM-70-70F mass spectrometer (VG-Micromass, Altrincham, Great Britain) combined with a Pye gas chromatograph (Pye Unicam, Cambridge, Great Britain).

Choice of solvent and initial column temperature for the derivatization of phenobarbital

Three different solvents were used: ethyl acetate, dioxan and DMF. The concentrations of phenobarbital and hexobarbital (internal standard) were 50 and 20 μg/ml, respectively, in each of the test solutions. The derivatization reaction was studied by injecting 1 μl of the test solution together with 2 μl of DMF dimethylacetal and the initial column temperature was varied from 70° to 150°. The peak-area ratios (phenobarbital derivative to hexobarbital derivative) were calculated.

Dilution of the derivatization reagent

A 1-μl volume of test solution containing 50 ng of phenobarbital and 50 ng of hexobarbital (internal standard) in DMF was injected together with 2 μl of derivatization reagent from different dilutions in pyridine (undiluted, 1:2, 1:4, 1:6, 1:8 and 1:12). The peak-area ratios were calculated.

Influence of the reagent volume

A 1-μl volume of test solution containing 50 ng of phenobarbital and 20 ng of heptadecane (internal standard) in DMF was studied by injecting 1, 2, 3 and 4 μl of DMF dimethylacetal dilution (1:4). The peak-area ratios (phenobarbital derivative to heptadecane) were calculated.

Calibration graphs and reproducibility tests

A 1-μl volume of the test solution containing 5–50 μg/ml of ethyl acetate solutions of allobarbital and aprobarbital and 20 μg/ml of amobarbital (internal standard) was injected together with 2 μl of DMF dimethylacetal (undiluted). The initial column temperature was 70°.

A calibration graph for phenobarbital dissolved in DMF was constructed for the same concentration range using hexobarbital (20 μg/ml) as internal standard. The derivatization reagent was diluted (1:4) and the initial column temperature was 150°. The peak-area ratios (barbiturate derivative to internal standard derivative) were plotted against barbiturate concentration. Five assays on each solution were carried out. For the reproducibility test, solutions containing 10 or 50 μg/ml of the barbiturate and 20 μg/ml of internal standard were analysed as described for the different barbiturates. The mean and the relative standard deviations (RSD) of ten assays were calculated.

RESULTS AND DISCUSSION

Derivatization studies

The separation of the acetal derivatives of a mixture of the barbiturates is shown in Fig. 1. This shows that the derivatization method combined with a glass capillary column of high separation efficiency is suitable for the identification of barbiturates. Flash methylation of barbiturates has earlier been described using reagents

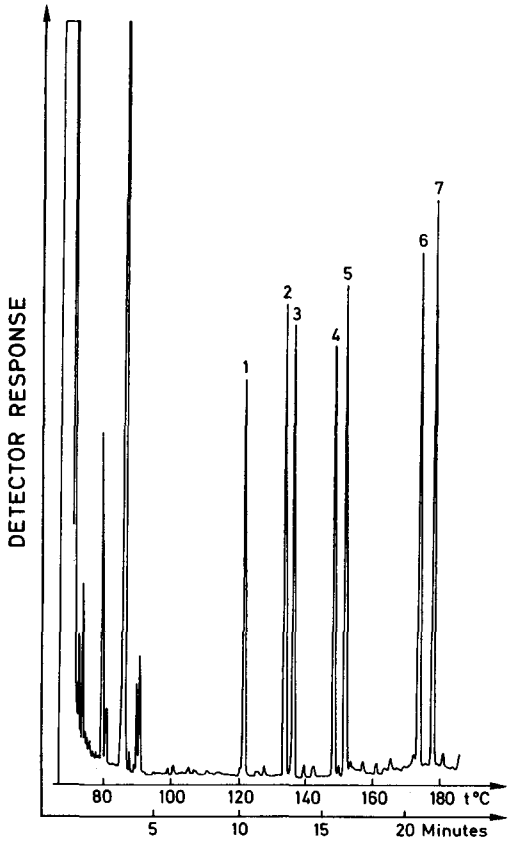


Fig. 1. Chromatogram obtained after flash-heater derivatization by injecting 2 μl of DMF dimethylacetal together with 1 μl of an ethyl acetate solution containing the following components: 1 = barbital, 2 = allobarbital, 3 = aprobarbital, 4 = amobarbital, 5 = pentobarbital, 6 = hexobarbital, 7 = phenobarbital.

such as trimethylphenylammonium hydroxide and trimethylanilinium hydroxide[6,7]. There seems to be overwhelming evidence to indicate that phenyl-substituted barbiturates undergo decomposition when chromatographed using this technique[8]. With trimethylphenylammonium hydroxide two products were formed, the methylated barbiturate and a methylated degradation product often referred to as the "early barbiturate peak". Phenobarbital, when subjected to flash alkylation in trimethylanilinium hydroxide solution, decomposes to give two derivatives in addition to the expected N,N-dimethylphenobarbital. It should be noted that these alkylating agents are strongly basic and that barbiturates are unstable in alkaline media.

The advantages of DMF dimethylacetal as the derivatization reagent lie in the rapid reaction and its relative mildness. Unlike on-column methylation, only one product was formed in our investigation. The choice of the injection port temperature also proved to be important. The temperature has to be high enough to allow evaporation and quantitative derivatization of the samples. However, impurity peaks are generated on injection of the reagent into the gas chromatograph. The

amount of these impurity peaks is reduced by lowering the flash-heater temperature. Figs. 2 and 3 show chromatograms where 2 μl of DMF dimethylacetal had been injected at injection port temperatures of 200° and 275°, respectively. Effective derivatization in this study was obtained when the injection port temperature was set at 200°.

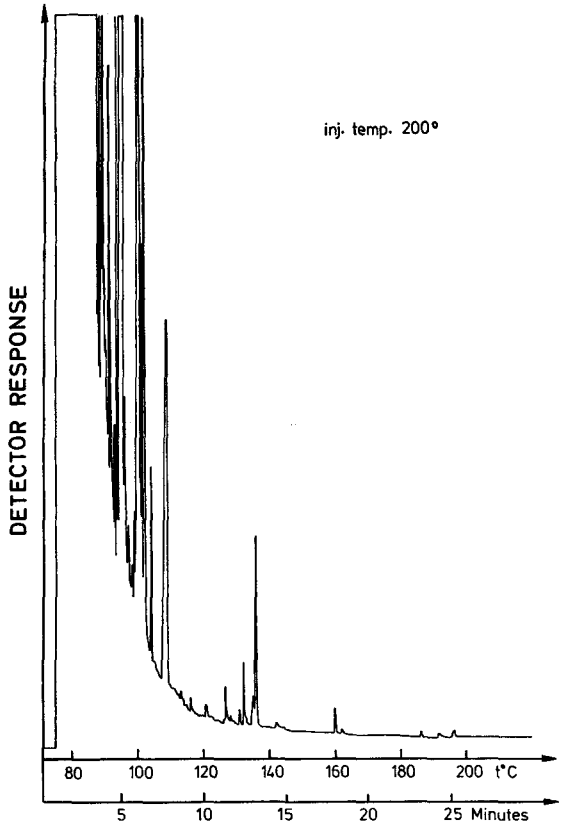


Fig. 2. Chromatogram obtained after injection of 2 μl of DMF dimethylacetal at an injection port temperature of 200°.

Sampling technique

As reported earlier, the quantitative results may be dependent of the sampling technique[3,9]. In this work, reproducible results were obtained when the sample and the reagent solutions were injected over a period of 5 sec measured by a stop-watch.

GC–MS identification of derivatives

The identity of the barbiturate derivatives was checked by GC–MS. The reaction of DMF dimethylacetal with barbiturates to form the corresponding acetal derivatives has been studied earlier[5]. Our investigation gave mass spectra with

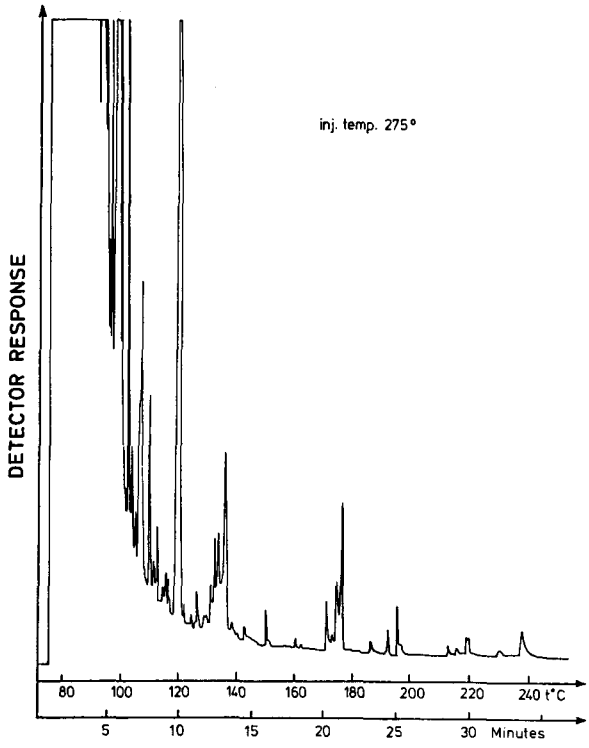
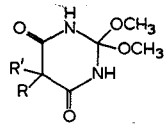


Fig. 3. Chromatogram obtained after injection of 2 μl of DMF dimethylacetal at an injection port temperature of 275°.

typical acetal fragmentation identical with the previously published data. It was concluded that two methoxy groups were introduced:



Temperature and solvent effects

The results in Table I illustrate the effect of the solvent used and the relationship of its boiling point to the initial column temperature. The derivatization of high-boiling barbiturates was most dependent on these parameters, and therefore phenobarbital and hexobarbital (internal standard) were selected as test substances. Only with a high-boiling solvent such as DMF could satisfactory results be obtained. In order to obtain a good solvent effect and to minimize the analysis time, it has earlier been recommended that the initial column temperature should be at least 15° (30–40° if possible) below the boiling point of the solvent[9]. The best sensitivity and RSD were obtained when the initial column temperature was 150°, only 3° below the boiling point of DMF. The peaks could only occasionally be detected when the temperature was programmed from 100°.

TABLE I

EFFECT OF SOLVENT AND INITIAL COLUMN TEMPERATURE ON THE FLASH-HEATER DERIVATIZATION OF PHENOBARBITAL

Solvent	*Boiling point (°C)*	*Initial column temperature (°C)*	*Average phenobarbital peak width (mm)*	*Peak-area ratio, phenobarbital to hexobarbital ($\bar{x}$)*	*RSD (%)*
Ethyl acetate	77	70		1.76	30
		120	3.0	1.97	5.3
		150	2.7	2.72	18.5
Dioxan	101	100	Peaks too small for detection		
		120	3.0	2.40	9.0
		150	2.4	2.82	4.0
Dimethyl formamide	153	100	The peaks could only occasionally be detected		
		120	3.1	1.74	41
		150	2.3	2.64	2.0

No change in the retention time was observed with the different solvents. The lower column efficiencies at lower initial column temperatures can be accounted for by the fact that the peak widths were increased (Table I).

Surprisingly good results were obtained for samples dissolved in dioxan and injected at an initial column temperature higher than the boiling point of the solvent. Selection of the solvent and careful control of the initial column temperature are essential when the flash-heater derivatization technique is applied to high-boiling barbiturates.

Fig. 4 shows a chromatogram where 50 ng of phenobarbital and 50 ng of hexobarbital dissolved in DMF were injected together with DMF dimethylacetal dilution (1:4) at an initial column temperature of 150°.

Dilution of derivatization reagent

The main problem with DMF dimethylacetal is the impurities generated, which can interfere with peaks of interest. To minimize the effect of the impurities, the reagent was diluted with pyridine and the different dilutions were tested for the derivatization of phenobarbital and hexobarbital. Impurities from the undiluted reagent interfered with these compounds when the temperature was programmed from 150°. Fig. 5 shows the peak-area ratios (phenobarbital derivative to hexobarbital derivative) after derivatization with the different reagent dilutions. The investigation showed that the 1:4 and 1:6 dilutions could be used for quantitative derivatization. The peak-area ratios decreased when a higher reagent dilution was used. The 1:4 dilution was selected for the quantitative analyses of phenobarbital and no interfering peaks could be detected in the concentration range investigated. It was obvious that the solvent used to dilute DMF dimethylacetal had to be carefully selected. When DMF was used as the solvent for the reagent dilution, broad interfering peaks were obtained.

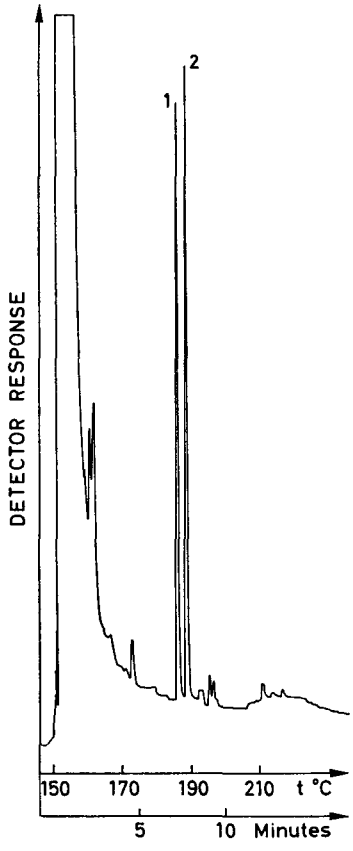


Fig. 4. Chromatogram obtained after flash-heater derivatization by injecting 1 μl of DMF dimethylacetal dilution (1:4) together with 1 μl of a solution containing 50 ng of hexobarbital (peak 1) and 50 ng of phenobarbital (peak 2).

Influence of volume of DMF dimethylacetal

The influence of the reagent volume is shown in Fig. 6. It is apparent that at least 2 μl of reagent must be injected in order to obtain a complete reaction. No significant difference was obtained when 2, 3 or 4 μl of reagent was injected. Interfering peaks were observed with 4 μl of reagent. Heptadecane was used as the internal standard in this experiment in order to study the derivatization against an underivatized standard. However, it is well known that an appropriate choice of internal standard is necessary to obtain good precision and accuracy when quantitative analyses of barbiturates are performed. As expected, a low precision was obtained with heptadecane as the internal standard, as shown by the vertical errors bars for each point on the curve in Fig. 6. This was not the case in the proposed quantitative method when an appropriate barbiturate was used as the internal standard.

Calibration graphs

Calibration graphs in the concentration range 5–50 μg/ml were constructed

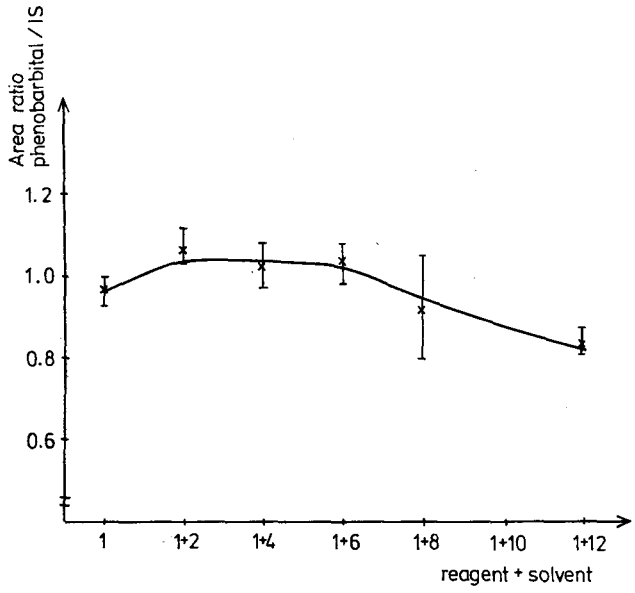


Fig. 5. Peak-area ratio of phenobarbital derivative to internal standard (IS) (hexobarbital) derivative plotted against DMF dimethylacetal dilutions used for derivatization. Concentrations of phenobarbital and hexobarbital: 50 μg/ml.

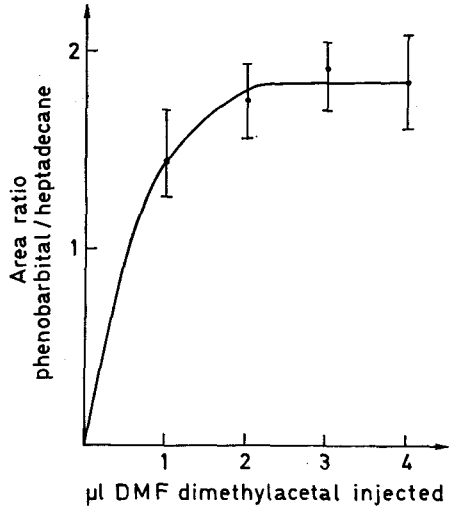


Fig. 6. Peak-area ratio of phenobarbital derivative to heptadecane plotted against volume of DMF dimethylacetal dilution (1:4) injected for 1 μl of solution containing 50 ng of phenobarbital and 20 ng of heptadecane (internal standard).

in order to check the linearity of the derivatization method. To obtain optimal results for low- and high-boiling barbiturates, different solvents and initial column temperatures were selected. The volatility of the different barbiturates made an appropriate choice of internal standard necessary. Amobarbital was used as the

internal standard and ethyl acetate as the solvent for allobarbital and aprobarbital (low-boiling barbiturates). The initial column temperature was 70° and undiluted derivatization reagent could be used with no interfering peaks. The peaks were not separated from the solvent front using higher initial column temperatures and ethyl acetate was then a suitable solvent.

An acceptable calibration graph for phenobarbital could only be obtained when DMF was used as the solvent and the temperature was programmed from 150°. The high-boiling hexobarbital was suitable as the internal standard; a short analysis time for phenobarbital was then obtained. As mentioned earlier in this paper, derivatization reagent diluted with pyridine (1:4) had to be used.

Table II gives the data for the different calibration graphs after calculating the linear regression lines and the correlation coefficients. The data from the reproducibility tests are also given in Table II. Linear calibration graphs were obtained for the different barbiturates with acceptable reproducibility.

TABLE II

DATA FROM CALIBRATION GRAPHS AND REPRODUCIBILITY TESTS AFTER BARBITURATE DERIVATIZATION

Barbiturate	*Solvent*	*Initial column temperature (°C)*	*Internal standard*	*Calibration graph equation over range 5–50 μg/ml*	*Correlation coefficient*	*RSD (%)*	
						10 μg/ml	*50 μg/ml*
Allobarbital	Ethyl acetate	70	Amobarbital	$y = 0.055x - 0.074$	0.998	2.7	2.5
Aprobarbital	Ethyl acetate	70	Amobarbital	$y = 0.056x - 0.039$	0.998	6.5	2.4
Phenobarbital	Dimethyl formamide	150	Hexobarbital	$y = 0.053x - 0.030$	0.999	3.4	2.0
Phenobarbital	Ethyl acetate	70	Amobarbital	$y = 0.033x + 0.278$	0.834	35.6	30.0

For comparison, the calibration graph of phenobarbital obtained using the same conditions as described for the low-boiling barbiturates was calculated. The difference in the correlation coefficients and the reproducibility data are noticeable.

Determination of phenobarbital in plasma

A 1-ml volume of a spiked plasma sample containing 5 μg/ml of phenobarbital and hexobarbital was extracted according to a modification of an earlier published method[10]. The evaporated extracts were dissolved in DMF and injected into the gas chromatograph using the proposed conditions for phenobarbital. Fig. 7 shows a chromatogram obtained from a plasma extract after derivatization. No interfering peaks were observed in the plasma blank. It should be noted that the plasma sample contained phenobarbital at the low therapeutic concentration level. This means that improved specificity and sensitivity are obtained using the described derivatization technique combined with a glass capillary column.

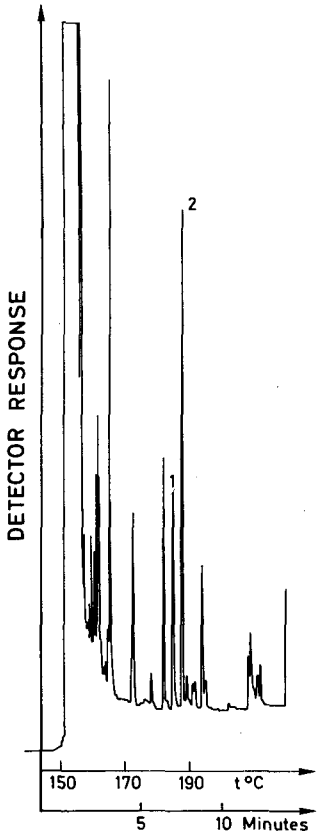


Fig. 7. Chromatogram obtained after flash-heater derivatization of a plasma extract containing hexobarbital (1) and phenobarbital (2) (5 μg/ml in plasma).

CONCLUSION

It is clear that DMF dimethylacetal is a valuable derivatization reagent for the determination of barbiturates. The advantages lie in its rapid reaction and relative mildness, producing only one product. The reagent is to be preferred to the strongly basic alkylating reagents as there is overwhelming evidence to indicate that the barbiturates undergo decomposition in the latter instance[7,8].

This investigation has shown that the flash-heater derivatization technique with DMF dimethylacetal as derivatization reagent can be used successfully for the determination of barbiturates. There is no risk of the presence of water, which must be excluded in all instances, in the reaction medium[11].

Derivatization carried out by simultaneous injection of the sample and the reagent has earlier been reported to be an unfavourable technique[12]. However, by exact control of parameters such as the injection technique, injection port temperature, initial column temperature and the solvent used to dissolve the substances, an accurate method with good sensitivity and reproducibility can be obtained. The specificity and the sensitivity are improved by the use of glass capillary columns of

high separating efficiency. This makes the method suitable for the identification of complex mixtures and also for quantitative analyses of low-level plasma concentrations. No change in column performance was observed during the study after injecting large amounts of derivatization reagent with DMF as solvent.

Various DMF dialkylacetals which may be applied for particular separations are commercially available. Further investigations will be carried out on the application of the flash-heater derivatization technique to other compounds of pharmaceutical interest using DMF dialkylacetals as derivatization reagents.

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