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GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHY OF CARBOXYLIC ACIDS AFTER FLASH-HEATER TRIMETHYLSILYLATION

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SUMMARY

Glass capillary column gas chromatography has been used for quantitative and qualitative analyses of carboxylic acids. The acids are first converted into non-polar derivatives by flash-heater derivatization, to make them more suitable for gas chromatography. Benzoic, nicotinic, salicylic, acetylsalicylic and *p*-aminobenzoic acids were used as model substances, and reagents suitable for forming trimethylsilyl derivatives were studied. The method was successful for benzoic, nicotinic and salicylic acid with N,O-bis(trimethylsilyl)trifluoroacetamide as derivatization reagent. With acetylsalicylic acid some decomposition to salicylic acid was observed and with *p*-aminobenzoic acid two derivatives were occasionally formed. Calibration graphs in the concentration range 5–50 μg/ml and the linearity up to 300 μg/ml were evaluated. The relative standard deviation for the quantitative analyses was calculated for the different acids.

INTRODUCTION

Gas chromatography (GC) with glass capillary columns is considered to be the most effective separation method in situations where high resolving power is necessary. However, only a few papers have been published on its application to drug analyses[1,2]. Analyses with glass capillary columns in the nanogram range pose certain problems. Thus, even good capillary columns often show undesirable adsorption characteristics, and strong tailing or even irreversible adsorption may take place with compounds containing functional polar groups. Glass capillary columns behave towards acidic or basic samples like a buffer of high capacity, and a considerable part of the sample disappears. Attempts to produce, on the glass, a layer of an inorganic material in order to avoid adsorption were promising[3]. However, at the moment it seems that a better solution to the problem is to produce non-polar derivatives of the acids.

The technique of on-column derivatization was introduced for identification of drugs[4]. We have described this technique for qualitative and quantitative drug analyses using flame ionization and electron capture detection[5,6]. Packed columns were used in these studies and the derivatives of the drugs were formed by simulta-

neous injection of the sample and the reagent into the column. Recently, the technique has been successfully applied to glass capillary column GC. Narcotic drugs used as model substances were derivatized in the heated capillary injector (flash-heater derivatization), and the method was used for resolving complex drug mixtures and for quantitative analyses in the nanogram range[7].

The purpose of the present investigation was to determine whether carboxylic acids could be derivatized by the same technique. Aromatic carboxylic acids of pharmaceutical interest, which also contained other functional groups, were used as model substances, and reagents capable of forming trimethylsilyl derivatives were investigated. The effects of various sampling techniques were studied, calibration graphs constructed and the reproducibility of the quantitative analyses was evaluated for the different acids.

MATERIALS AND METHODS

Reagents

Benzoic acid (BA), nicotinic acid (NA), salicylic acid (SA), acetylsalicylic acid (ASA) and *p*-aminobenzoic acid (PABA) were of pharmacopoeial grade and supplied by Norsk Medisinaldepot (Oslo, Norway). Hexadecane used as internal standard was from Koch-Light (Colnbrook, Great Britain). Ampoules (1 ml) of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N,O-bis(trimethylsilyl)acetamide (BSA), N-trimethylsilylimidazole (TMSIM) and Sylon BTZ (mixed BSA, TMSIM and trimethylchlorosilane) used as derivatization reagents were purchased from Supelco (Bellefonte, Pa., U.S.A.). After opening, the ampoules were handled as described previously[6].

Analytical reagent grade ethyl acetate and pyridine were obtained from E. Merck (Darmsradt, G.F.R.). Stock standard solutions of the acids (1 mg/ml) were prepared in ethyl acetate. Nicotinic acid was dissolved in pyridine and diluted with ethyl acetate to the desired concentration. These solutions were diluted to give concentrations from 5 μg/ml to 300 μg/ml ethyl acetate for gas chromatography.

Gas chromatography

A Fractovap 2300 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector (FID) and a capillary column splitless injector was used. The glass capillary column (30 m $\times$ 0.35 mm I.D.) (H. and J. Jaeggi, Trogen, Switzerland) was wall-coated with OV-17. The injection port temperature was 200° and the samples were injected at an oven temperature of 70°. The temperature was programmed at 5°/min up to 220°. Nitrogen was used as the carrier gas at an inlet pressure of 0.6 kp/cm^2, which gave a flow-rate of 1.4 ml/min through the column. The splitting ratio of the injector was 1:40 for the identification tests. Samples for the calibration graphs and the reproducibility tests were injected without splitter. The splitter was closed before the injection and re-opened 30 sec after the injection to give a splitting ratio of 1:40. The sensitivity setting was 10 $\times$ 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 Varian Model 112 mass spectrometer (Varian-

MAT, Bremen, G.F.R.) combined with a Varian Model 1400 gas chromatograph (Varian, Walnut Creek, Calif., U.S.A.). The glass capillary column (12 m × 0.28 mm I.D.) (LKB, Stockholm, Sweden) was wall-coated with SE-30.

Influence of the amount of derivatization reagent

A 1-μl volume of the test solution containing 50 μg/ml of BA, NA and SA and 20 μg/ml of the internal standard was injected without splitting into the gas chromatograph. The test solution was studied by injecting 1,2, 3 and 4 μl of BSTFA. The peak area ratios (acid derivative to internal standard) were calculated.

Calibration graphs

Calibration graphs for the concentration range 5–50 μg/ml were constructed for BA, NA and SA using hexadecane as internal standard. The concentration of the internal standard was 20 μg/ml. A 2-μl volume of BSTFA was injected into the gas chromatograph together with 1 μl of the test solution. The peak area ratios (acid derivative to internal standard) were plotted against the acid concentration. Five assays of each solution were carried out and the regression lines and the correlation coefficients were calculated. The linearity up to 300 μg/ml was also investigated.

Calibration graphs for ASA and PABA in the same concentration range were also constructed using hexadecane as internal standard.

Reproducibility of quantitative analyses after derivatization

Test solutions containing 5 μg/ml or 50 μg/ml of BA, NA and SA and 20 μg/ml of the internal standard were analysed as described and the peak area ratios were calculated. Solutions containing 50 μg/ml of ASA and PABA and 20 μg/ml internal standard were also analysed. The mean and the relative standard deviations (RSD) of ten analyses were calculated.

RESULTS AND DISCUSSION

Silylation studies

The separation of the trimethylsilyl derivatives of a mixture of the acids is shown in Fig. 1. A column coated with OV-17 had to be used for separating the SA and ASA derivatives. They were eluted as a single peak when using a 20 m SE-30 column.

BSA, TMSIM and Sylon 8 were also investigated to determine the most useful derivatization reagent. No derivative formation was observed with TMSIM and Sylon 8. Only with BSA did the reaction seem to be satisfactory. Table I shows the mean values of the peak area ratios (acid derivative to internal standard) and the RSD for BA, NA and SA after five injections of a solution of 100 μg/ml of each after derivatization with BSTFA and BSA. From these results BSTFA seemed to react more quantitatively than BSA, and also with a noticeably lower RSD. Consequently, BSTFA was selected as the silylating reagent in the quantitative analyses. BSTFA and its by-products are also more volatile than BSA and do not interfere with the early eluting compounds. This reagent was reported to be superior to BSA when low-molecular-weight acids are derivatized off-column[8].

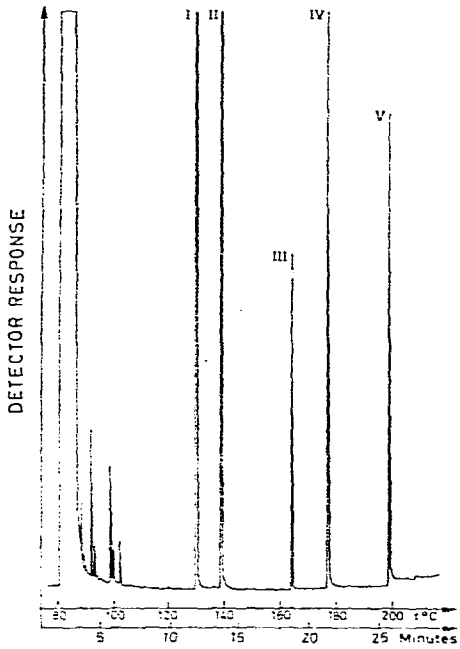

Fig. 1. Chromatogram obtained after flash-heater silylation by injecting 2 μl BSTFA together with 1 μl of an ethyl acetate solution containing the following components: I = benzoic acid; II = nicotinic acid; III = salicylic acid; IV = acetylsalicylic acid; V = *p*-aminobenzoic acid. For chromatographic conditions, see text.

TABLE I

BSTFA COMPARED TO BSA FOR TRIMETHYLSILYLATION

Acid	*Peak area ratio acid derivative (100 μg/ml) to internal standard*		*RSD (%)*	
	BSTFA	*BSA*	*BSTFA*	*BSA*
Benzoic acid	6.7	6.3	3.3	7.3
Nicotinic acid	4.5	4.2	3.9	9.3
Salicylic acid	6.5	4.1	3.1	20.3

Influence of BSTFA volume

The influence of the amount of BSTFA is shown in Fig. 2. It is apparent that at least 2 μl of reagent must be injected to obtain complete reaction. From the vertical error bars for each point on the curves, it would seem that 4 μl reagent should give the best precision. However, with this increased volume the probability of interfering peaks will also increase. Consequently, 2 μl of derivatization reagent were used in the quantitative analyses and a blank run showed no interfering peaks.

Sampling technique

It has been reported that quantitative results may be dependent on the sampling technique[9]. Thus, the rate of sample injection or delivery to the injector is important

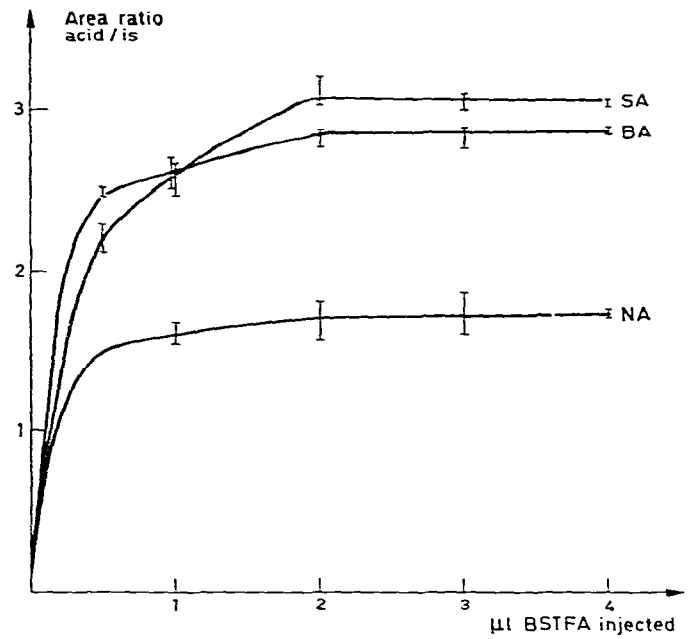

Fig. 2. Area ratio acid-TMS derivative/IS plotted against volume of BSTFA injected for a 1-µl solution containing 50 ng of benzoic acid (BA), nicotinic acid (NA) and salicylic acid (SA). For chromatographic conditions, see text.

in order to insure a uniform rate of sample vaporization and to avoid sample back-flash out of the injector insert. In the present work an injection rate of 5 sec was found to give the best chromatographic results when using the splitless mode. This was especially important for ASA and PABA. The relative peak areas of these derivatives were doubled when the injection rate was changed from 2 sec to 5 sec.

Yang *et al.*[9] have also investigated the optimal sampling time to insure quantitative sample recovery. The sampling time is defined as the period in which the syringe needle is resident in the heated region of the splitless injector. In our experiments a sampling time of 15 sec was found to give the best results. The results were independent of the time when the splitter was closed from 20 sec to 50 sec. However, when the splitter is re-opened too soon, a large fraction of the sample is vented out of the injector.

GC–MS identification of derivatives

The identity of the acid derivatives was checked by GC–MS. The mass spectra obtained showed that O-trimethylsilyl benzoate ($M^+ = 194$) and O-trimethylsilyl nicotinate ($M^+ = 195$) were formed. The mass spectrum of the SA derivative showed characteristic ions at *m/e* 267 ($M - 15^+$), 209 ($M - Si(CH_3)_3^+$), 193 ($M - OSi(CH_3)_3^+$) and 73 ($(CH_3)_3Si^+$). These data support the formation of O,O-bis(trimethylsilyl) 2-oxybenzoate and the ions are consistent with the previously published spectrum[10]. No mono derivative of SA could be detected, and it was concluded that the reaction was complete. The structure of SA suggests that it should be possible to obtain three individual trimethylsilylated derivatives[10]. Multiple gas–liquid chromato-

graphy (GLC) peaks have been obtained in attempts to silylate SA off-column[11]. In contrast, ASA can only form one silylated derivative, O-trimethylsilyl 2-acetoxybenzoate. The mass spectrum obtained showed characteristic ions at *m/e* 210 ($M - CH_2{=}C{=}O^+$), 195 ($M-42-15^+$) and 135 ($M-COOSi(CH_3)_3^+$) which support the derivative formation[10]. However, some ASA will also decompose to SA during injection. Therefore ions characteristic of disilylated SA were also detected. The ASA and SA derivatives were not separated on the SE-30 column used for GC–MS. A small peak with the same t_R as the SA derivative was detected in addition to the main peak when using the OV-17 column.

Silylation of PABA occasionally also gave rise to two peaks (Fig. 3). The GC–MS investigation showed that only the carboxylic group was silylated in the main product ($M^+ = 209$) giving O-trimethylsilyl 4-aminobenzoate. The small peak eluted at higher t_R was shown to be N,O-bis(trimethylsilyl) 4-aminobenzoate ($M^+ = 281$), where one proton in the amino group also had been substituted. Increasing the BSTFA volume had no influence on the derivative formation. The formation of the mono-TMS derivative of PABA confirms the low reactivity of aromatic amines having a deactivating carboxylic group attached to the ring, compared to aliphatic amines. Earlier findings showed that both protons were substituted after flash-heater silylation of aliphatic amines[7].

The decomposition of ASA

The decomposition of ASA was not influenced by variation of the injection

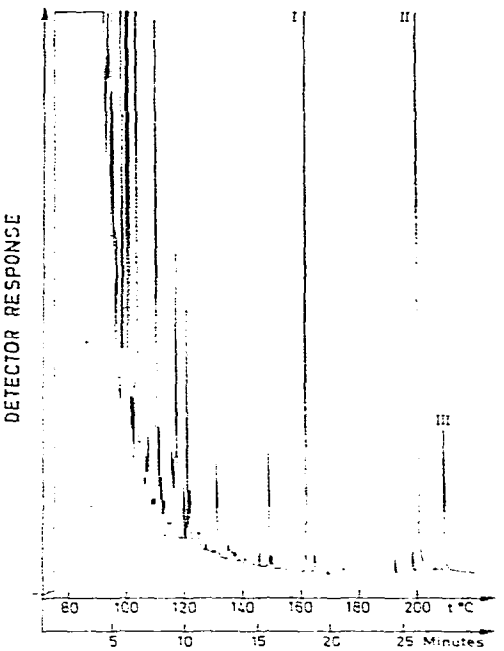

Fig. 3. Chromatogram obtained after flash-heater derivatization by injecting 2 μl BSTFA together with 1 μl of a solution containing 50 ng *p*-aminobenzoic acid. Peaks: I = hexadecane internal standard; II = mono-TMS-derivative; III = bis-TMS-derivative. For chromatographic conditions, see text.

port temperature from 150° to 250°. The amount of the decomposition of freshly prepared solutions varied from 3% to *ca.* 11% and was independent of the concentration. Solutions from different ASA batches showed the same values. We conclude that the flash-heater derivatization technique cannot be used for quantitative analyses of ASA from pharmaceutical preparations, because of the possibility of decomposition.

Calibration graphs

In order to check the linearity of the derivatization method in the temperature-programmed mode, calibration graphs were constructed for BA, NA and SA. The calibration graphs were calculated according to the method of least squares, relating y (the peak area ratio of the acid derivative to the internal standard) to x (the concentration of the acid solution in μg/ml). Table II gives the data for the different calibration graphs in the concentration range 5–50 μg/ml. The linearity was checked up to 300 μg/ml for the three acids and no change was observed (Fig. 4). The minimal detectable amount was *ca.* 500 pg for BA and SA, and *ca.* 1 ng for NA. Fig. 5 shows a chromatogram after flash-heater silylation of 10 ng NA, BA and SA where 20 ng hexadecane was used as internal standard.

TABLE II

DATA FROM THE CALIBRATION GRAPHS AND REPRODUCIBILITY TESTS AFTER DERIVATIZATION

Acid	*Calibration graph equation over range 5–50 μg/ml*	*Correlation coefficient*	*RSD (%)*	
			50 μg/ml	*5 μg/ml*
Benzoic acid	$y = 0.056x + 0.038$	0.9994	2.7	5.9
Nicotinic acid	$y = 0.036x - 0.086$	0.9934	3.1	9.9
Salicylic acid	$y = 0.060x + 0.111$	0.9986	3.2	7.3
Acetylsalicylic acid			5.1	
p-Aminobenzoic acid			8.9	

As a comparison, calibration graphs for ASA and PABA were also constructed in the same concentration range (Fig. 4). The response of these acids was markedly reduced compared to BA, NA and SA, and the minimal detectable amount was *ca.* 1 ng for both of them. The calibration graphs for ASA and PABA were not linear in the investigated concentration range with reduced response below 100 μg/ml. Decomposition to SA, adsorption to the column or loss during the decomposition cause the reduced response.

With PABA some adsorption of the underivatized amine group may occur. Some formation of the disilylated derivative may also cause reduced sensitivity. The amount of the disilylated derivative varied from 5% to 15% of the main derivative in repetitive injections of the same solution.

Reproducibility tests

The data obtained from the reproducibility test are also given in Table II. With the 50 μg/ml solution, the RSD for BA, NA and SA showed satisfactory low values. With the 5 μg/ml solution the RSD values were increased, but the results are considered satisfactory for the quantitation in this concentration range.

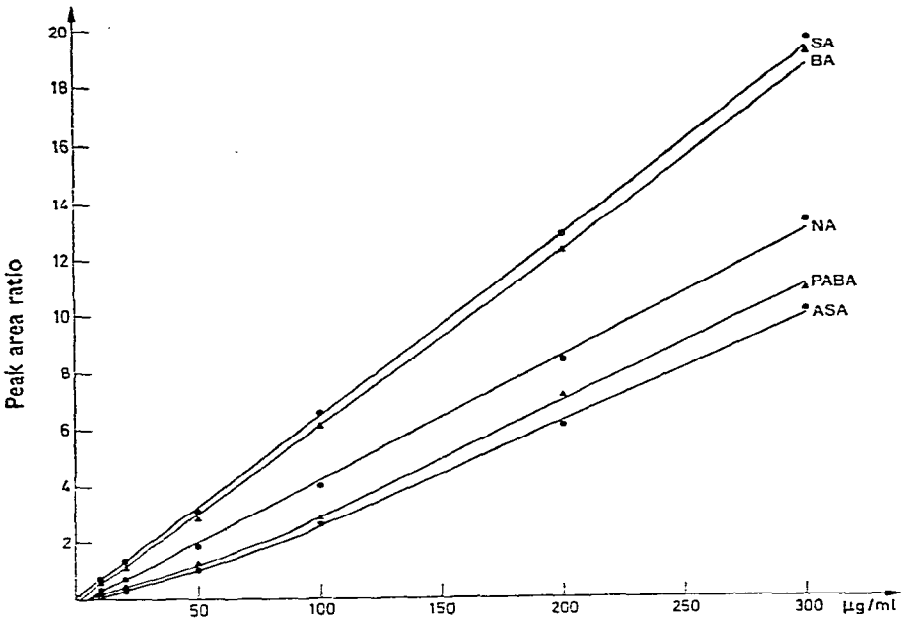

Fig. 4. Calibration graphs obtained after flash-heater silylation of benzoic acid (BA), nicotinic acid (NA), salicylic acid (SA), acetylsalicylic acid (ASA) and *p*-aminobenzoic acid (PABA) in the concentration range 5–300 µg/ml.

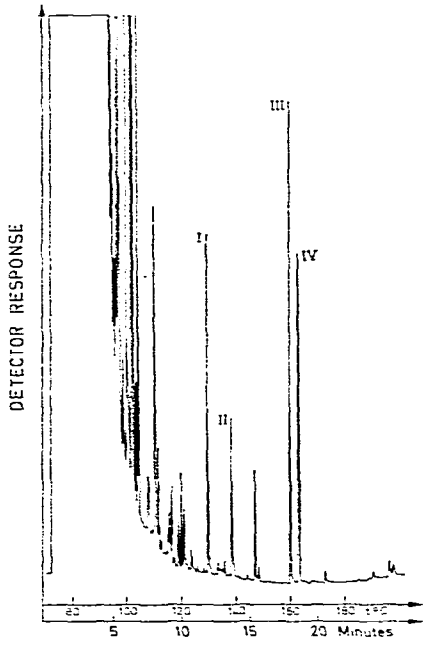

Fig. 5. Chromatogram of a sample containing 10 ng of benzoic acid (I), nicotinic acid (II) and salicylic acid (IV) after flash-heater silylation. Peak III is the internal standard hexadecane. For chromatographic conditions, see text.

As expected, the RSD values for ASA and PABA were higher compared to the other acids of the same concentration.

CONCLUSIONS

The flash-heater derivatization technique can be successfully used for carboxylic acids which do not decompose during the injection and which react rapidly with the derivatization reagent to form a single derivative. Less time is wasted in forming derivatives when the sample and the derivatization reagent are injected directly into the gas chromatograph. The specificity and the sensitivity are improved by use of glass capillary columns of high separating efficiency. This makes the method suitable for identification of complex mixtures and for quantitative analyses in the nanogram range.

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