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GAS CHROMATOGRAPHIC STUDIES OF DERIVATIVES OF CELLULINE-LIKE SUBSTANCES

EVA TOMORI
Research Institute for Pharmaceutical Chemistry, Budapest (Hungary)
and
H. KALÁSZ, J. NAGY and J. KNOLL
Semmelweis Medical University Department of Pharmacology, Budapest (Hungary)

SUMMARY

Different aspects of the gas chromatographic determination of the celluline-like substances exhibiting specific cardiotonic activity are discussed. As this substance is isolated from frog skin and bladder, many impurities have to be considered. Determination of these samples, having a broad range of boiling points, was performed by means of programmed-temperature gas chromatography. Identification of unknown peaks was executed with the help of pure standard substances and retention indices.

INTRODUCTION

Celluline, detected in 1952 by Knoll *et al.*[1], is an endogenous substance exerting a specific mode of action and plays a particular role in myocardial cell membrane transformations from the systolic to the diastolic phase. In the course of several years of research a number of celluline-like components were detected and isolated from different tissue perfusates (frog skin, frog bladder, frog liver, etc.). Biological activity can be demonstrated on isolated frog heart brought diastolically to hypodynamia by means of celluline-specific Ringer solution. This hypodynamia could not be reversed by known cardiotonic agents. Celluline, however, spontaneously caused immediate contractions in the isolated frog heart even in celluline-specific Ringer solution containing mainly potassium ions.

Raw materials isolated from frog skin and bladder as well as freeze-dried raw powders were purified and fractionated by means of gel chromatography, thin-layer chromatography, ion-exchange chromatography, and electrophoresis. The individual fractions were pooled according to their UV absorption spectra and their biological activity. The UV and IR spectra, as well as the gas chromatographic and mass spectrometric characteristics, of the purified products were studied. According to direct and indirect proofs[2,3] the principle responsible for biological activity is a calcium complex containing a variety of organic ligands. Structure determination studies

revealed that the substance may consist of the following components: (a) aromatic alkylamines, (b) carboxylic acids, and (c) other components.

Our primary aim was the qualitative and, if possible, quantitative determination of the main organic components of this agent.

THEORETICAL

Considering the broad retention range of the components to be investigated, the most up-to-date method was applied: programmed-temperature gas chromatography (PTGC), described in detail by Harris and Habgood[4]. PTGC reduced analysis time and consequently enabled the investigation of a relatively larger number of samples. Beyond this it was our task to separate the individual components, already assayed, by means of preparative gas chromatography for further structure elucidation purposes. In view of the negligible material requirement of the gas chromatographic method its utilization is highly advantageous, as the purification of celluline fractions leads to large losses of material. The substances were determined as derivatives. The components containing a carboxyl group were esterified in the presence of mineral acids, and the free amino groups were trifluoroacetylated. As ligands bound by calcium are liberated during reaction, this appears to be a highly satisfactory method. PTGC enables the utilization of the retention indices of McReynolds' ASTM system for the execution of measurements. Van den Dool and Kratz[5] defined the programmed retention index by the following equation:

$$I_{PTGC} = 100\left[\frac{T_{rx} - T_{rz}}{T_{rz+1} - T_{rz}} + z\right] \tag{1}$$

where

I_{PTGC} = PTGC retention index
T_r = retention temperature, °K
X = unknown material
z and $z + 1$ = normal hydrocarbons of carbon numbers z and $z + 1$, respectively
z = carbon number

By applying this equation, the index of the unknown component may also be calculated and identified by means of isothermic literature data. As only a very limited number of PTGC data are at our disposal, this means a significant progress, as there exist a variety of factors affecting measurements within an individual apparatus (flow-rate of carrier gas, inlet temperature, retention temperature, etc.).

It is well known, however, that the retention index–column temperature relationship, for a given stationary phase, may generally be determined by means of an Antoine-type[6] equation.

$$I^{\text{stationary phase}}_{\text{material}} = A + \frac{B}{T + C} \tag{2}$$

where I = isothermic retention index (index unit), T = column temperature in °K, and A, B and C are constants valid at constant temperatures and unchanged GC conditions and depending on the characteristics of the substance and the stationary phase, respectively, at the temperature range given.

As the column temperature is increased linearly and the retention index varies solely according to the Antoine-type equation, and assuming that the assay of the substance is started at T_0 and is finished at T_r, the following integral may be applied:

$$I_{\text{PTGC}}^{\text{stationary phase material}} = \frac{\int_{T_0}^{T_r} \left(A + \frac{B}{T + C}\right) dT}{T_r - T_0} \tag{3}$$

The integral may be resolved[7].

$$I_{\text{PTGC}}^{\text{stationary phase material}} = A + \frac{2.3 \cdot B \cdot \log \frac{T_r + C}{T_0 + C}}{T_r - T_0} \tag{4}$$

Utilizing this equation, as well as that of Van den Dool and Kratz, the index of the unknown peak may be determined and calculated. No retention index data of the assumed components obtained by previous measurements on 3% OV-1 stationary phase were at our disposal in the required temperature range. Therefore, the indices were determined at three different temperatures and subsequently constants A, B and C were calculated.

EXPERIMENTAL

Qualitative assays were performed on the stationary phases 10% neopentyl glycol succinate (NPGS) and 3% OV-1. Experimental details are summarized in Table I.

TABLE I

EXPERIMENTAL CONDITIONS

Gas chromatograph:	Model F-21 (Perkin-Elmer, Norwalk, Conn., U.S.A.)
Detector:	flame ionization
Injected sample:	1 μl
Carrier gas:	nitrogen
Flow-rate:	45 ml/min at 23.2 °C and at a pressure of 759.0 Torr
Injection temperature:	250 °C
Column temperatures:	140, 150, 160, 190 °C
Stationary phases:	3% OV-1 on Gas-Chrom Q, 80–100 mesh 10% NPGS on Gas-Chrom Q, 80–100 mesh

A chromatogram made at 150 °C on 3% OV-1 stationary phase under the conditions given in Table I is shown in Fig. 1.

Previous studies permitted us to assume that the calcium complex responsible for biological activity may contain substances having a phenylethyl skeleton, as for instance phenylethylamine, aromatic alkylcarbonic acids, etc. From these unknown components four were characterized by the above method. The retention data of these are listed in Table II.

Table III summarizes values of the constants required for the solution of the integral in the case of known retention indices.

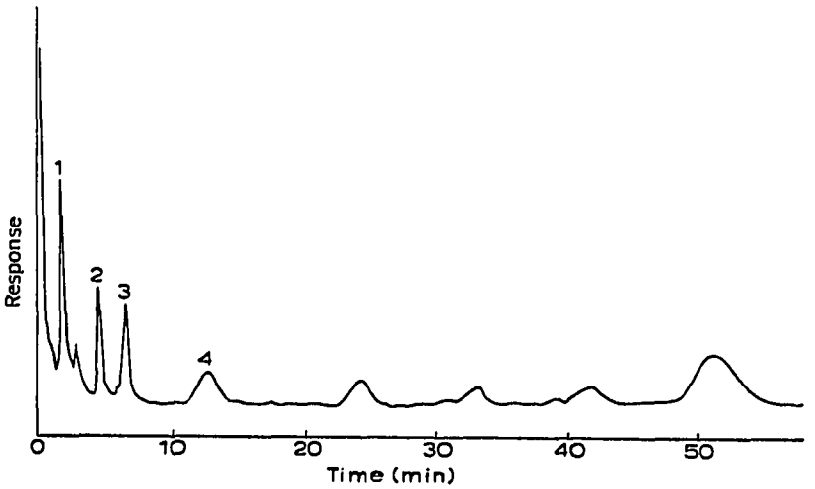

Fig. 1. Chromatogram of components isolated from frog skin and bladder and transformed into suitable derivatives, at 150 °C on 3% OV-1 stationary phase.

TABLE II

RETENTION INDEX DATA OF STANDARD MATERIALS USED FOR IDENTIFICATION

No.	*Material*	*Retention index data*		
		140 °C	*150 °C*	*160 °C*
1	Methylphenyl acetate	1163	1168	1177
2	Methylphenyl propionate	1259	1272	1284
3	Methyl N-trifluoroacetylphenylalanine	1446	1455	1462
4	N-Trifluoroacetyltyramine	1597	1601	1603

Calculation of the constants was performed according to the following equations:

$$C = \frac{(T_2 - T_1)(I_3T_3 - I_1T_1) + (T_3 - T_1)(I_1T_1 - I_2T_2)}{(T_3 - T_1)(I_2 - I_1) - (T_2 - T_1)(I_3 - I_1)} \quad (5)$$

$$A = \frac{(I_3T_3 - I_1T_1) + C(I_3 - I_1)}{T_3 - T_1} \quad (6)$$

$$B = (T_3 + C)(I_3 - A) \quad (7)$$

TABLE III

CONSTANTS CALCULATED FROM STANDARD MATERIALS AND USED FOR IDENTIFICATION

Components	*Constant*		
	A	*B*	*C*
Methylphenyl acetate	1145	−800	−458
Methylphenyl propionate	2016	−443,592	173
Methyl N-trifluoroacetylphenylalanine	1486	−1,200	−383
N-Trifluoroacetyltyramine	1609.3	−258.3	−392

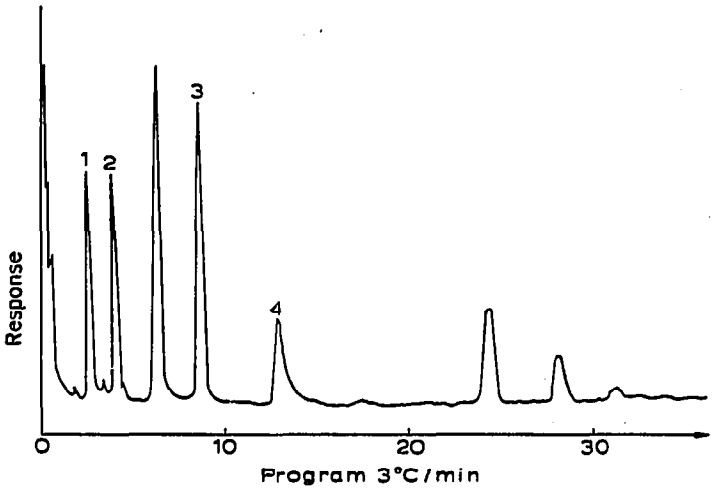

Fig. 2. Programmed-temperature gas chromatogram of components isolated from frog skin and bladder and transformed into suitable derivatives. Temperatures: (1) 127.8 °C, (2) 132 °C, (3) 146.5 °C, and (4) 160.5 °C. Column: 2 m × 4 mm with 3% OV-1 coated on 100–120 mesh Gas-Chrom Q.

Fig. 2 shows a chromatogram made by PTGC.

From the retention temperature data obtained by chromatography the I_{PTGC} values of some components were calculated by means of the method of Van den Dool and Kratz. Calculation of the retention index of one of the unknown components on 3% OV-1 according to eqn. 1 is demonstrated in the following example:

$$I_{PTGC} = 100\left(\frac{127.8 - 120}{129 - 126} + 11\right) = 1160 \text{ index units} \quad (8)$$

Table IV summarizes the retention temperature and I_{PTGC} data of a number of unknown components.

The retention index figures of some standard materials were calculated by means of eqn. 4 using the known values of constants.

As an example, the calculation procedure in the case of methylphenyl acetate on 3% OV-1 is given in eqn. 9.

$$I_{PTGC} = 1145 + \frac{2.3\,(-800)\log\frac{400.8 - 458}{393 - 458}}{400.8 - 393} = 1158 \text{ index units} \quad (9)$$

TABLE IV

RETENTION TEMPERATURE AND RETENTION INDEX DATA OF COMPONENTS ISOLATED FROM FROG SKIN AND BLADDER

Component	*Retention temperature (°C)*	I_{PTGC}
1	127.8	1160
2	132.0	1250
3	146.5	1442
4	160.5	1585

The good agreement of the figures proves the reliability of both methods of calculation.

Table V demonstrates the identification of four components according to the methods described above.

TABLE V

CALCULATED AND MEASURED I_{PTGC} DATA OF COMPONENTS ISOLATED FROM FROG SKIN AND BLADDER

No.	*Component*	I_{PTGC}		
		Calculated	*Measured*	*Deviation*
1	Methylphenyl acetate	1158	1160	−2.0
2	Methylphenyl propionate	1246	1250	−4.0
3	Methyl N-trifluoroacetylphenylalanine	1444	1442	+2.0
4	N-Trifluoroacetyltyramine	1585	1585	0.0

As certain carboxylic acids (phenylacetic and phenylpropionic acids) play an important role in biological processes, and as both components are important principles of the calcium complex responsible for biological activity, their levels were determined with the help of standards. The figures required for the calculation are summarized in Table VI. Methyl cinnamate was used as internal standard.

TABLE VI

PROPORTION OF PHENYLACETIC ACID AND PHENYLPROPIONIC ACID IN THE CALCIUM COMPLEX RESPONSIBLE FOR BIOLOGICAL ACTIVITY

Abbreviations: Aaa = counts of components on experimental chromatogram (cm² or counts); Saa = slope factor for component; Ais = counts of internal standard peak on experimental chromatogram (cm² or counts); Maa = molecular weight of amino acid × 10^{-3}; Sis = slope factor for internal standard; μmoles-is = μmoles of internal standard added to sample.

Component	*Sample weight (mg)*	*Aaa*	*Saa (counts/ μmole)*	*Ais*	*Sis (counts/ μmole)*	*Maa (mg)*	*μMoles-is*	*Found (%)*
Phenylacetic acid	25	43.5	9.6	48	12.3	0.136	6.7	19.2
Phenylpropionic acid	25	97.5	11.5	48	12.3	0.145	6.7	19.32

From the above data it can be concluded that the presence of the two broad bands in organic ligands may be considered important from the point of view of biological processes.

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