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Short Communication

Gas chromatographic screening for neostigmine and physostigmine using temperature-programmed retention indices

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ABSTRACT

Temperature-programmed gas chromatographic retention indices, relative to *n*-alkane (C standard) and *n*-alkylbis(trifluoromethyl)phosphine sulphide (M standard) homologous series, were determined for two low-volatile, highly toxic carbamates, neostigmine and physostigmine on SE-54 fused-silica capillary columns. The influence of temperature programme and sample concentration on the absolute values of the indices were evaluated. Reproducibility of the indices was good in both linear and multistep temperature programmes, but the reproducibility and peak shapes were better in the multistep temperature programme. When the compounds were present in high concentration, C standards and a flame ionization detector were used successfully, but multidetector M standards, which also can be seen with the selective alkali thermoionization detector, were superior when concentrations were low. Similarly M standards which can be seen also with other selective detectors (electron-capture, flame photometric and photoionization) could be a suitable index standard series for screening of different types of compounds containing heteroatoms.

INTRODUCTION

A retention index system based on homologous *n*-alkanes has been widely adopted in analytical toxicology, where the rapid identification of unknown substances is of key importance. When a great many samples must be analysed, retention index monitoring in GC analysis improves efficiency by reducing the number of samples needing to be reanalysed by another independent technique (MS or Fourier transform IR). Retention index monitoring also allows use of the retention index data determined in another laboratory.

The first compilations of the GC retention indices of toxicologically relevant compounds were based on measurements done by the packed columns with dimethylpolysiloxane stationary phases as SE-30 or OV-1 and *n*-alkanes as index standards [1,2]. The programmed and isothermal indices of hundreds of drugs have been used in combination with the differences between the retention indices measured on two different columns, OV-1 and OV-17 and found to allow reliable identifications [3,4]. Different column types like SE-30 equivalent narrow-bore thin-film capillary columns, wide-bore thick-film capillary columns and packed columns have been used and high correlation between the isothermal retention indices of physostigmine and of other compounds of toxicological interest have been found [5]. A major disadvantage of *n*-

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alkanes in screening for drugs is that they cannot be seen by selective detectors [6]. 1-Nitroalkanes have been proposed as alternative retention index standards for drug analysis in isothermal runs since these compounds can be detected with electron-capture (ECD), alkali thermoionization (ATD) and flame ionization (FID) detection in GC and they can also be used in HPLC [7]. Structurally, however, they are very different from most drugs. Selected mixtures of drugs are to be used as reference standards for optimum results. Their retention indices based on *n*-alkanes as reference substances have to be determined beforehand. This procedure is regarded as a standard practice in systematic toxicological analysis. Extensive evaluations in different laboratories have shown that for packed and capillary columns dimethylpolysiloxane is the preferred phase for screening in analytical toxicology. Only for special purposes, *e.g.* the differentiation of substances within a certain group, can other phases be useful, such as OV-17 or OV-225 [2,8].

In this work the usefulness of *n*-alkylbis(trifluoromethyl)phosphine sulphides (multidetector M standards), and for comparison *n*-alkanes (C standards), was investigated for the GC retention index monitoring of neostigmine and physostigmine. These low-volatile highly toxic carbamates were chosen as test compounds because they are cholinesterase inhibitors and potential chemical warfare agents. Except at extreme exposure and concentration the symptoms of carbamate poisoning are not as severe as those associated with organophosphorus compounds and so it was possible to investigate safely the effect of concentration of the test compounds on the retention indices.

The usefulness of multidetector M series as index standards was investigated when the concentration of neostigmine and physostigmine was changed from 5 ng/μl to 2 μg/μl and also when multistep temperature programmes were used. The retention indices were determined by two-channel GC with two similar columns connected to the same injector and to two similar or two different detectors.

Most drugs have heteroatoms which can be detected with the same selective detectors like M standards. A particular advantage of the M standards is that they can be used at trace level: they are detectable at levels of 0.4–0.6 pmol with FID, 2–7 fmol with ATD and 3–5 fmol with ECD [9].

EXPERIMENTAL

Instrumentation and chromatographic conditions

The gas chromatograph was a Micromat HRGC 412 microcomputer-controlled instrument (HNU-Nordion) with two-channel integration and printing software. Two fused-silica capillary columns 15 m × 0.32 mm, 0.25 μm cross-linked SE-54 film (dimethylpolysiloxane, 5% phenyl with 1% vinyl groups), were used, with FID and ATD. Standard chromatographic conditions were as follows: injector and detector temperature (FID or ATD) 250°C, carrier gas (He) flow-rate 2 ml/min, splitting ratio 1:10, septum purge 10 ml/min, starting point of the temperature programme 40°C (or 100°C), temperature-programming rate 10°C/min (or multistep), and end temperature of the programme 300°C.

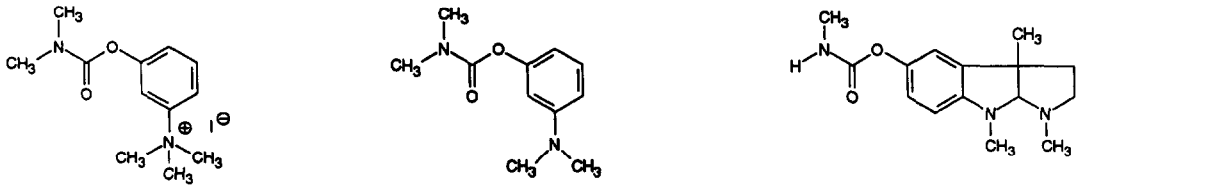
Chemicals

Alkylbis(trifluoromethyl)phosphine sulphides, $(CF_3)_2P(S)(CH_2)_nCH_3$, $n = 5 \ldots 19$ (M standards), were diluted in ethyl acetate, *n*-alkanes (C standards) in hexane. The M series is commercially available from HNU-Nordion. Neostigmine iodide (**Ia**) and physostigmine (**II**) (Fig. 1) were diluted in acetone.

Calculations and computer programmes

Retention indices (I) were calculated according to Van den Dool and Kratz [10] with the formula, in both linear and multistep temperature programming, $I_{C\ (or\ M)} = 100C_n + 100(C_{n+i} - C_n)(t_{R(x)} - t_{R(n)}) \ / \ (t_{R(n+i)} - t_{R(n)})$, where C_n and C_{n+i} are carbon numbers of the C standards (or carbon numbers of the alkyl chain of M standards) eluted on either side of the unknown compound, $t_{R(x)}$ is the retention time of the unknown and $t_{R(n)}$ and $t_{R(n+i)}$ are the retention times of C_n (or M_n) and C_{n+i} alkanes (or M_{n+i}), respectively.

SC-Workstation software (Sunicom, Helsinki,



Ia Ib II

Fig. 1. Structural formulas of neostigmine iodide (**Ia**), 3-(N,N-dimethylaminophenyl) dimethylcarbamate (**Ib**) and physostigmine (**II**).

Finland) was used for creating the method, generating the compound library, data acquisition and integration, identification and quantification of compounds. SC-software is based upon MS-Windows (Microsoft) and it can be used also with systems which have their own data acquisition and basic chromatogram manipulation software.

To set up a retention index-based identification method you need to introduce standard compounds to every sample. Index standards may also be normal sample components, provided that these components can be found in all samples. Normally a small amount of index standard mixture is added to the sample, preferably just before injection. Many of the autosamplers can do this.

The first and most critical step of the identification process is finding the retention index standard peaks among all other peaks. This is accomplished automatically by the programme through sophisticated pattern recognition algorithm. After that index values are calculated for each peak. During the final step the programme compares the calculated retention index values with the compound-specific retention index values stored in the library and if the calculated value is within the selected identification limit, the compound is tentatively identified.

Normally the internal standard method was used for quantification. It requires quantitative calibration which means calculation of response factors. Each compound quantified must have a reference compound (internal standard). Both the standards and the compounds have been calibrated, so that they have response factors. Mean or linear response factors can be calculated for tens of data files.

It is possible to produce quality control reports directly from the chromatographic data both in textual and in graphic format. Also statistical reports on quantification parameters can be obtained easily.

RESULTS AND DISCUSSION

Physostigmine is not thermally stable and it slowly decomposes on standing in air, particularly at pH < 5 [11]. Neostigmine iodide loses methyliodide on heating and elutes as 3-(N,N-dimethylaminophenyl)dimethylcarbamate (**Ib**) in GC, as shown by an analysis of the mass spectra. Fig. 2 shows a typical gas chromatogram for a sample containing neostigmine and physostigmine together with the M standards.

Study was made of the influence of the starting point (40 and 100°C) of the linear temperature programme on the retention indices of neostigmine and physostigmine. The results were compared with indices obtained in multistep temperature programmes (Table I). The differences in retention indices when the initial temperature was 40 and 100°C were less than 1.0 index unit (i.u.), but the peak shape of physostigmine was better when the temperature programme was started at 100°C. The absolute values of the indices were higher when the multistep programmes were used; the reproducibility was as good as with the linear temperature programme; peak shapes were improved for more accurate integration, and the time required for analysis was shorter.

The influence of the concentration of the carbamates on the retention indices has been investigated. Studies by Rijks [12] on hydrocarbons have shown that when the peak is

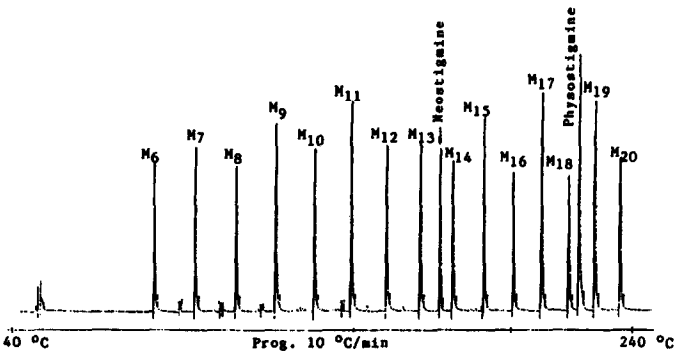


Fig. 2. Chromatogram of neostigmine and physostigmine together with M standards. Column, fused silica, cross-linked SE-54, 15 m × 0.32 mm, film thickness 0.25 μm; detection, ATD.

symmetrical, even a ten-fold increase in the sample concentration has no significant effect on the isothermal retention indices. The quantity of the different compounds in his samples was between 10 ng and 100 pg. Yin *et al.* [13] found that, when the quantity of a compound was large, there was a large effect on nearby peaks and a slight variation in the isothermal retention indices. However, the thicker the film the smaller the differences in the retention indices.

For a number of drugs a strong dependence of retention indices on concentration has been found. For example, the retention indices differed by 5 to 130 i.u. when the amount was increased from 1 ng to 4 μg [14]. The final amount was probably too large, causing overloading of columns. The film thickness, the retention time, and the similarity in polarity between the solute and the stationary phase have been shown to determine the maximum amount of compound that can be present in the sample. In a temperature-programmed run, the amount is lower for compounds with large retention indices than for compounds with small retention indices. Wang and Sun [15] recommend a sample size of 10 ng per compound for measurement of retention index values to an accuracy of ± 1 i.u. on common capillary columns (film thickness, 0.1–0.5 μm, I.D. 0.20–0.32 mm), although in some cases the amount of compound in a sample for retention index measurements can be as much as 100 ng.

Usually sample preparation sets limits on the concentration levels achievable. The practical limits of concentration should be known, therefore, before retention index monitoring is ap-

TABLE I

EFFECT OF THE TEMPERATURE PROGRAMME ON THE RETENTION INDICES OF NEOSTIGMINE AND PHYSOSTIGMINE

Temperature programmes: (I) from 40 to 300°C at 10°C/min; (II) from 100 to 300°C at 10°C/min; (III) from 40 to 200°C at 20°C/min, then from 200 to 300°C at 10°C/min; (IV) from 40 to 200°C at 20°C/min, then from 200 to 300°C at 5°C/min.

		I		II		III		IV	
		I, 5 runs	S.D.	*I*, 5 runs	S.D.	*I*, 5 runs	S.D.	*I*, 5 runs	S.D.
Neostigmine	I_C	1805.9	0.4	1806.3	0.4	1812.7	0.2	1812.2	0.2
	I_M	1360.9	0.1	1361.4	0.2	1368.8	0.2	1367.5	0.2
Physostigmine	I_C	2279.2	1.1	2278.3	0.8	2286.1	0.4	2278.7	0.6
	I_M	1839.9	0.4	1838.9	0.5	1849.5	1.4	1838.5	0.6

TABLE II

EFFECT OF THE CONCEENTRATION OF THE LOW-VOLATILE CARBAMATES ON THE RETENTION INDICES

Standard chromatographic conditions (see Experimental section).

Concentration	Neostigmine		Physostigmine	
	I_M	I_C	I_M	I_C
5 ng/μl	1360.7		1853.3	
25 ng/μl	1360.5		1845.0	
50 ng/μl	1360.6, 1360.0	1803.9	1840.1, 1840.4	2275.3
250 ng/μl		1804.1		2276.9
500 ng/μl		1805.0		2281.5
1 μg/μl		1806.8		2281.6
2 μg/μl	1367.9		1847.4	

plied. In this investigation the concentration of neostigmine and physostigmine was varied from 5 ng/μl to 2 μg/μl and the injection volume was 1 μl. ATD was used at lower concentrations of 5 ng/μl–50 ng/μl and FID at concentrations 50 ng/μl–2 μg/μl. Table II shows the results.

At concentrations of neostigmine between 5 and 250 ng/μl, the difference in the retention indices was less than 0.8 i.u. At higher concentrations the value began to increase: at 1 μg/μl the retention index was about 3 i.u. higher than at 5 ng/μl, and at 2 μg/μl about 7 i.u. higher indicating overloading of the column.

For physostigmine the situation was more complex. At concentration levels 50–250 ng/μl the retention indices were within 1.6 i.u. of each other. But when the concentration was lower or higher the retention indices increased. The structure of the compound and the nature of the stationary phase evidently were the dominant factors in determining this behaviour. Both at low and at high concentration physostigmine shows a slightly tailing peak on SE-54.

CONCLUSIONS

The results indicate the usefulness of the retention index method in GC screening for neostigmine and physostigmine. The reproducibility of the indices was good in each of the temperature programmes (0.2–1.4 i.u.), though the absolute values differed between programmes. Fast and reliable identifications are thus possible when the temperature programme used in the analytical run is the same as that used to record the library data. At concentration level 5–500 ng/μl the retention index window ± 2 i.u. is suitable for neostigmine but for physostigmine a larger window ± 6 i.u. may be necessary. With a different temperature programme, a larger index window has to be used also for neostigmine. Mixtures of selected drugs as retention index standards surely resemble more closely drugs to be analysed and this procedure is regarded as a standard practice in systematic toxicological analysis. But selected drugs cannot be added into the mixture in cases when some of them is among compounds to be analyzed quantitatively. M standards may then be one possibility to resolve the problem. Relative to the C standards, M standards allowed retention indices to be determined for much lower concentrations of neostigmine and physostigmine. Since many drugs possess heteroatoms, the M standards, used with selective detectors, may be useful for toxicological analysis, where concentration levels often are very low. Positive identifications by GC retention index monitoring must, of course, always be confirmed by another independent technique such as MS or Fourier transform IR.

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