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JOURNAL OF CHROMATOGRAPHY

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EXPERT SYSTEM FOR PHARMACEUTICAL ANALYSIS

II. RELATIVE CONTRIBUTION OF AND RULE VALIDATION FOR AMPEROMETRIC DETECTION (OXIDATION MODE)

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(First received May 13th, 1986; revised manuscript received July 21st, 1986)

SUMMARY

The expert system predicted correctly whether a drug could be detected with an amperometric detector (oxidation mode) in 93% of the compounds investigated. The validation was performed for drugs possessing a low molar extinction coefficient and for pharmaceuticals containing only small amounts of drugs. For 50% of this group of drugs, the amperometric detector could be used and the sensitivity was significantly increased in comparison with UV detection. The remaining groups of drugs, *i.e.*, for those for which neither UV detection nor the amperometric detector offered a solution, are also reported, together with some UV-inactive drugs that could be determined only by amperometric detection.

INTRODUCTION

Expert systems as a part of artificial intelligence will become a powerful tool in analytical chemistry[1,2] and also in column liquid chromatography[3,4]. They are software products that offer intelligent advice for problems requiring some expertise. Since the development of high-performance liquid chromatographic (HPLC) methods for pharmaceutical and biomedical analysis is often rather complex, it is our intention to build an expert system that takes intelligent decisions about the selection of parameters for chromatographic analysis of drugs.

The design of this expert system consists of different decision nodes that are structured in the form of a decision tree (Fig. 1). The expert system tries to find an initial selection of parameters in the space of possibilities by reasoning from one node to another. It first selects the appropriate detection system. This decision depends on the required sensitivity and selectivity, and on the characteristics of the substances to be quantified. Since an universal detector with sufficient sensitivity is not available in liquid chromatography, three native detection systems are incorporated into the expert system: UV, amperometric and fluorescence detection. This series of detectors is chosen because their properties complement each other: the range of application

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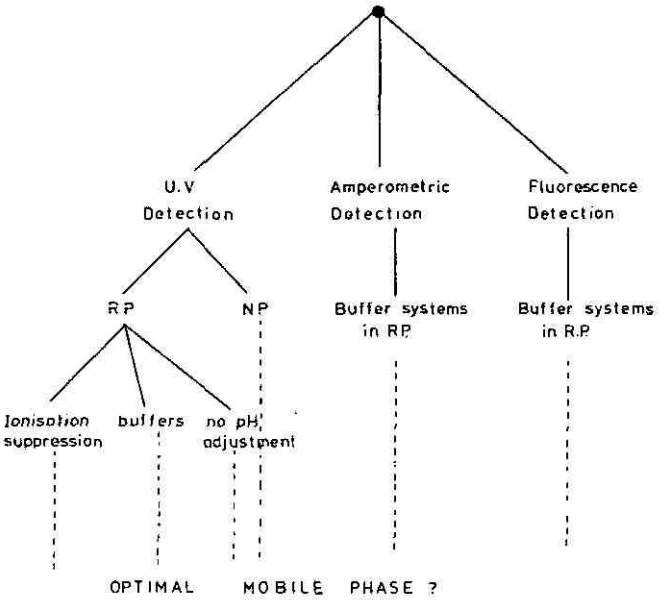


Fig. 1. An example of a decision tree in HPLC. R.P. = Reversed phase; N.P. = normal phase.

is the largest for UV, the selectivity for fluorescence detection and the sensitivity for amperometric detection[5–10].

The selection of a suitable detector has implications for the composition of the chromatographic system: amperometric and fluorescence detectors are preferably used with buffer systems, while this is rarely necessary with UV detection. In our strategy the stationary phase is always a cyanopropyl bonded phase[11] so that the expert system only decides in the second node whether a reversed phase, normal phase or buffers have to be used. Afterwards one can optimize the mobile phase composition. The scope of this article is restricted to the first node: UV *versus* amperometric detection.

The construction of an expert system involves three important stages. First the knowledge base used by the "expert" must be set up. This consists of facts (data) and rules. The second step is the validation of these rules and the last one consists in the implementation of the validated rules. The aim of this work is to develop a list of electro-oxidizable functions so that the system is able to decide whether a compound can be oxidized and detected with an amperometric detector by looking at the presence of certain functional groups in the molecule. This is only necessary if UV detection does not yield acceptable results[12].

In the expert system, UV detection is preferred and amperometric detection is used only when UV detection is not sufficient. In order to make an assessment of the contribution of amperometric detection to the detection of drugs with a low UV response, the validation is performed here on drugs possessing a low molar extinction coefficient and on pharmaceuticals containing small amounts of drugs. This permits the determination of the relative contribution of amperometric detection compared to UV detection: in which cases does amperometric detection offer a solution when

UV detection does not? For all redox active compounds of the test set selected, a voltammogram was recorded and the gain in sensitivity using an amperometric *versus* a UV detector was determined.

EXPERIMENTAL

Instrumentation

The HPLC apparatus included a Varian 8500 liquid chromatograph equipped with a Valco injector (50 μl). Two detection systems were coupled in series: a Varian UV detector with fixed wavelength 254 nm (optical pathlength 1 cm, cell volume 8 μl) and an LKB 2143 amperometric detector (glass–carbon electrode, cell volume 5.5 μl). A two-pen Kipp and Zonen BD9 recorder was used.

An HPLC system consisting of a Varian 5000 liquid chromatograph equipped with a Rheodyne injector (100 μl) and a Hewlett-Packard 1040 A diode-array detector (optical pathlength 0.6 cm, cell volume 4.5 μl) was used as a variable wavelength detector and also to monitor the spectra of all the compounds investigated. The absorptions were calculated with an HP 85 B and the chromatograms were recorded with a Varian Vista CDS 401 instrument.

Chromatographic conditions

A (250 mm × 4 mm I.D., particle size 5 μm) LiChrosorb CN column was used with a mobile phase of acetonitrile–phosphate buffer (pH 3, ionic strength = 0.05) (40:60) containing 0.001 *M* sodium chloride. The buffer solution was filtered through a 0.2-μm membrane filter and the mobile phase was thoroughly degassed before use. The flow-rate was 1 ml/min and all experiments were performed at room temperature.

Chemicals and reagents

The stock solutions were prepared in the mobile phase and stored at 4°C. Standard solutions were diluted in the mobile phase and prepared fresh daily. All drugs were of pharmacopoeial purity. Acetonitrile (Merck, Darmstadt, F.R.G.) was of liquid chromatographic grade. Phosphoric acid, sodium dihydrogenphosphate and sodium chloride were also obtained from Merck.

Software

The software for the expert system is being developed with an expert system tool kit called KES on an Apollo workstation. This will be described elsewhere[13].

RESULTS AND DISCUSSION

There is a great body of empirical information concerning amperometric detection in HPLC. In general, one can state that the electrochemical behaviour of a compound depends primarily on the molecular structure, but that it can be influenced by other parameters such as the mobile phase composition (pH, organic modifier, etc.) and the amperometric cell used (design, material from which the working electrode is made, etc.).

All the experiments were performed using the same eluent, for reasons described in Part I of this series[12]. An amperometric detector equipped with a thin-

layer cell and a glass–carbon electrode as working electrode is used, since its characteristics as a detection for the oxidation of organic compounds have been described in detail[10,14]. The eluent was selected first because it is compatible with all detection systems of the expert system, and secondly since its use with a cyanopropyl bonded phase can be considered as a general chromatographic system in drug analysis[12].

In earlier work[12] we reported that compounds containing a phenol, a primary or secondary aromatic amine, an aromatic methoxy or ethoxy or a thiol function are detectable by amperometric detection. Based on our experiments in the search for electroactive drugs, this list of electro-oxidizable functions can be expanded by adding the following functions: a phenothiazine sulphur, a secondary or tertiary aliphatic amine, piperazine and dioxazine. Exceptions are tertiary alicyclic amines (such as dipipanol, diphenylpyraline and procyclidine) and secondary aliphatic amines, bonded to two atoms that each form a double bound with another atom (as in diazepam, amobarbital, cloxacillin and dicloxacillin) (Table I).

The molecular structure of the drug, for which the expert system is asked to select a suitable detection system, is scanned for the presence of at least one oxidizable

TABLE I

LIST OF OXIDIZABLE FUNCTIONS

R is radical. X, Y, V and W are variables.

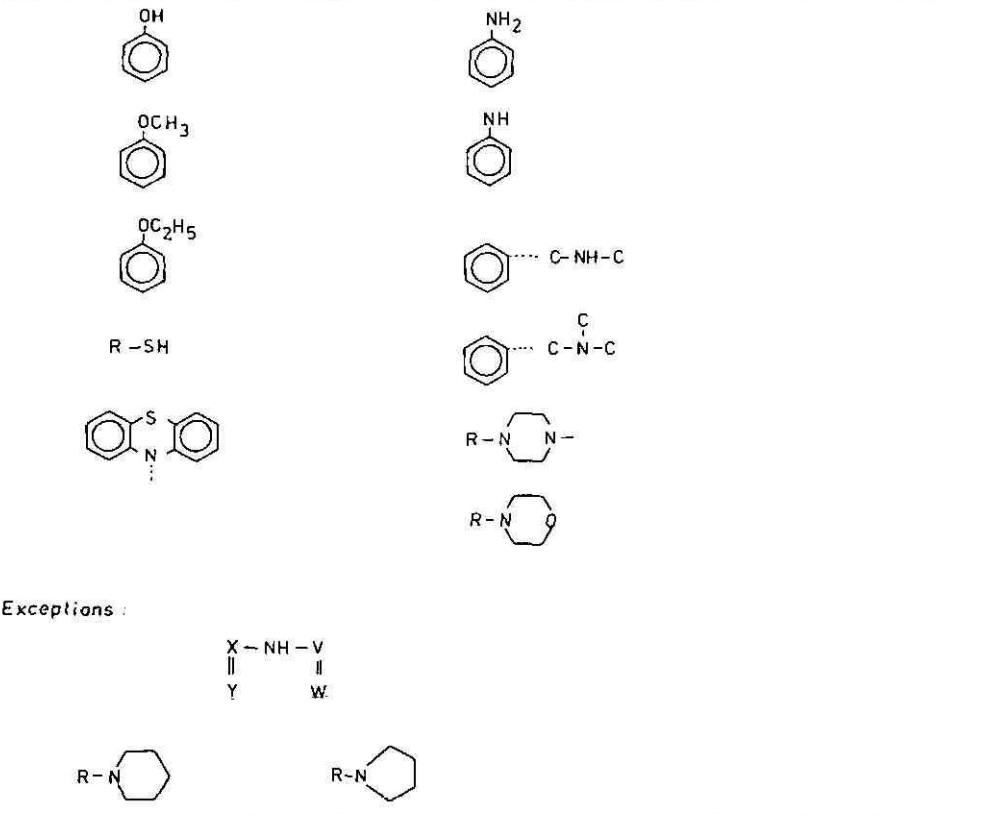
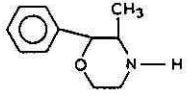
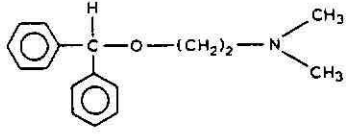
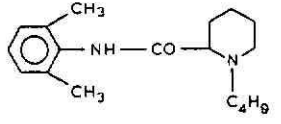
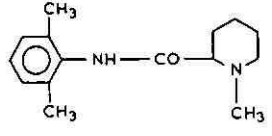
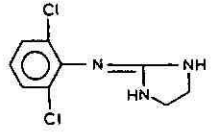


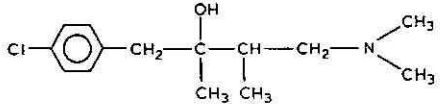
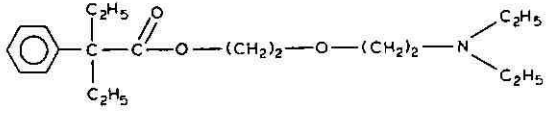
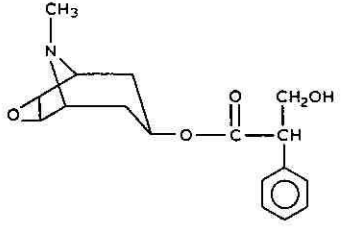
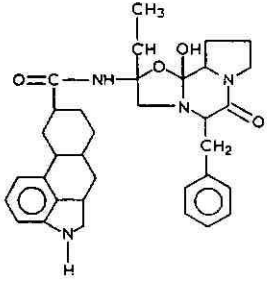
TABLE II

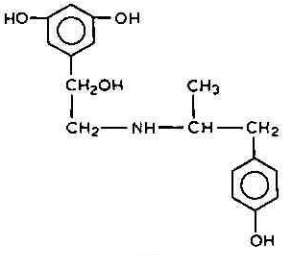
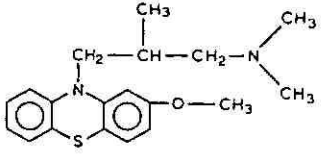
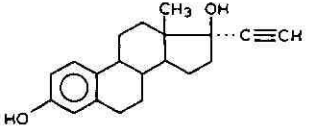
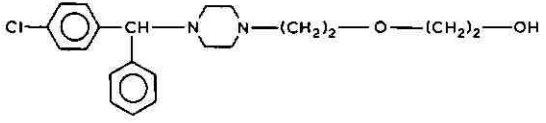
COMPARISON BETWEEN MINIMUM DETECTABLE QUANTITIES WITH UV DETECTION AND ELECTROCHEMICAL DETECTION (ED) FOR DRUGS POSSESSING LOW MOLAR EXTINCTION COEFFICIENTS

Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
(a) Theoretical prediction of a positive oxidative response is confirmed by the experiments							
Phenmetrazine		1.5	769	1155	26.5	1.2	29
Diphenhydramine		2	599	847	128.2	1.1	4.7
Bupivacaïne		1.8	643	1156	24.6	1.2	26
Mepivacaïne		1.7	455	790	17.7	1.2	25.7
Clonidine		1.8	910	733	8.6	1.2	85.2

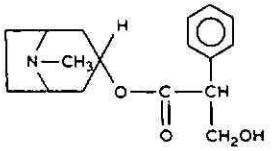
(Continued on p. 6)

TABLE II (*continued*)

Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
Clobutinol		1.8	1818	1653	69.4	1.1	23.8
Oxeladine		0.6	204	75.5	6.3	1.2	12
Scopolamine		1.1	1282	1960	50	1.1	25.6
Dihydroergotamine		1.5	253.2	173.8	2.7	1.1	64.4

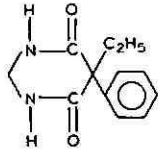
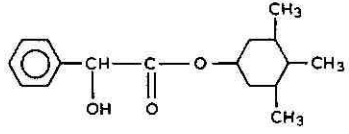
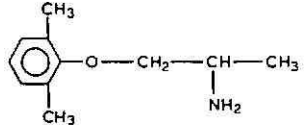
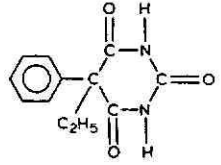
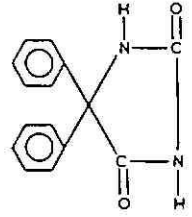
Fenoterol		1	376.8	118.5	1.1	1.1	107.7
Levomepromazine		2.4	7.8	14.5	0.4	1.2	19.5
Ethinylestradiol		0.7	468	113	1.1	1.1	102.7
Hydroxyzine		2	360	45.2	7.4	1.2	6

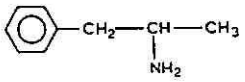
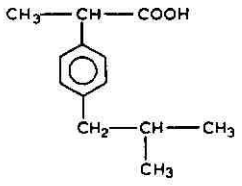
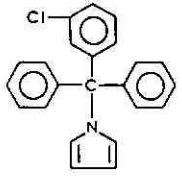
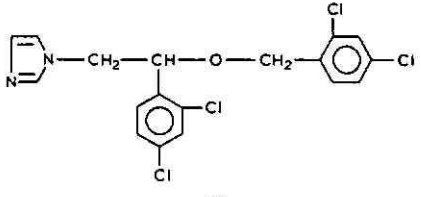
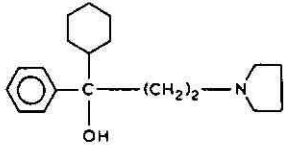
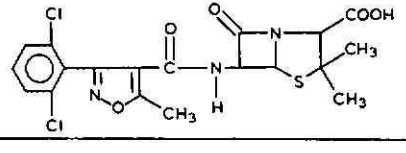
(b) Theoretical prediction of a negative oxidative response is confirmed by the experiments

Atropine sulphate		1	1124	–	–	–	–

(*Continued on p. 8*)

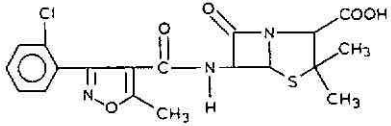
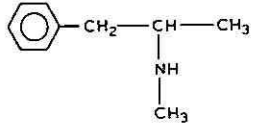
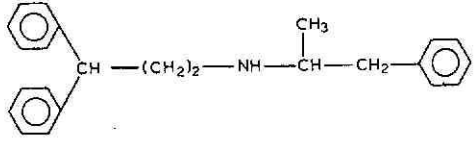
TABLE II (*continued*)

Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
Primidone		0.4	487	–	–	–	–
Cyclandelate		0.7	709	–	–	–	–
Mexiletine		0.9	636	824	–	–	–
Phenobarbital		0.4	510	–	–	–	–
Phenytoïne		0.75	142	–	–	–	–

Amphetamine		0.8	622	–	–	–	–
Ibuprofen		0.6	420	580	–	–	–
Clotrimazol		1.6	352	503	–	–	–
Miconazol		1.75	1341	1143	–	–	–
Procyclidine		1.5	1667	–	–	–	–
Dicloxacillin		0.5	231	–	–	–	–

(*Continued on p. 10*)

TABLE II (*continued*)

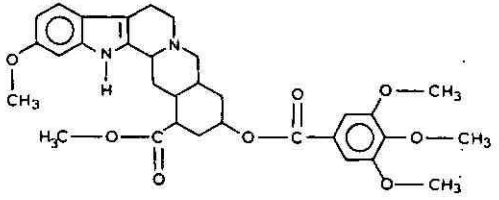
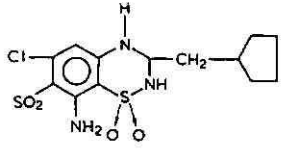
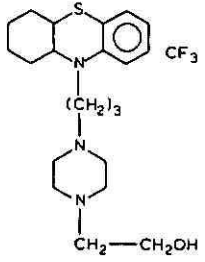
Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
Cloxacillin		0.45	155	–	–	–	–
(c) Theoretical prediction of a positive oxidative response is in contradiction with the experiments							
Metamphetamine		0.8	656	–	–	–	–
Prenylamine		1.9	595	–	–	–	–

* Minimum detectable concentration (on column).

** The factor gain in sensitivity with an amperometric detector.

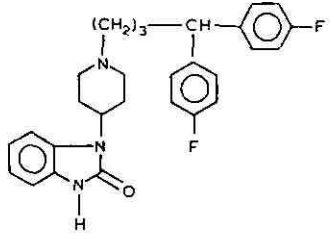
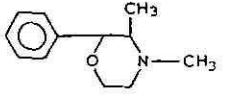
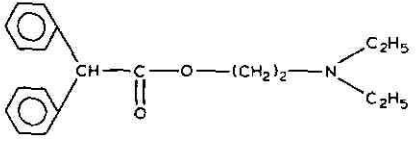
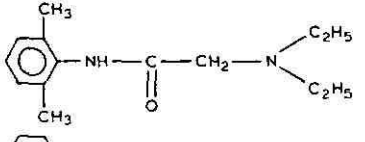
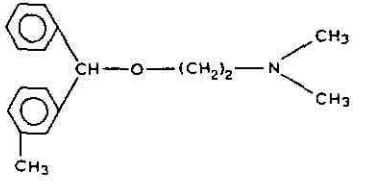
TABLE III

COMPARISON BETWEEN MINIMUM DETECTABLE QUANTITIES OBTAINED WITH UV DETECTION AND ELECTROCHEMICAL DETECTION FOR PHARMACEUTICALS CONTAINING SMALL AMOUNTS OF DRUGS

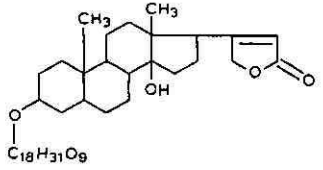
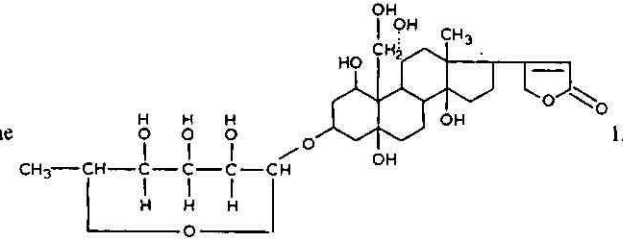
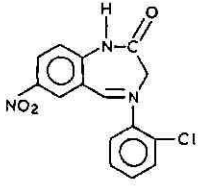
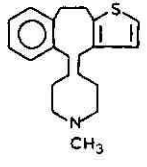
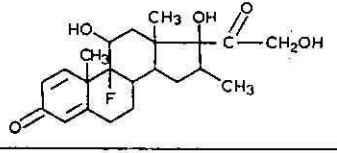
Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
(a) Theoretical prediction of a positive oxidative response is confirmed by the experiments							
Reserpine		2.5	51.2	60.1	4.2	0.9	14.3
Cyclopenthiazide		1	41.5	19.4	19.6	1.2	1
Fluphenazine		2.6	22.2	34.3	2.7	1.1	8.2

(*Continued on p. 12*)

TABLE III (*continued*)

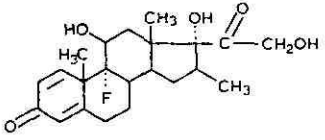
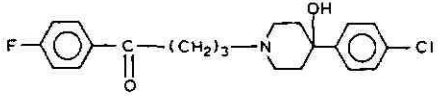
Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
Pimozide		2.1	321.1	119.1	10.1	1.2	11.8
Phendimetrazine		1.4	750	1587	16.7	1.2	45
Adiphenine		1.8	909	1587	36	1.1	25.3
Lidocaïne		1.7	403	–	1.9	1.2	212
Orphenadrine		2	563	847	75.2	1.1	7.5

(b) Theoretical prediction of a negative oxidative response is confirmed by the experiments

Digitaline		0.5	741.3	–	–	–	–
Ouabaïne		1.75	972.4	–	–	–	+
Clonazepam		0.6	17.8	16.8	–	–	–
Pizotifen		1.6	28.6	28.4	–	–	–
Betamethazon		0.5	16.4	19.8	–	–	–

(Continued on p. 14)

TABLE III (*continued*)

Compound	*Molecular structure*	*k'*	*MDC* (ng o/c)*			*Applied potential (V)*	*Gain** ED*
			UV 254 nm, l = 1 cm	*λ_{max}, l = 0.6 cm*	*ED*		
Dexamethazon		0.5	15	18.3	–	–	–
(c) Theoretical prediction of a negative response is in contradiction with the experiments							
Haloperidol		1.35	36.6	46.4	3.5	1.0	10.4

* Minimum detectable concentration (on column).

** The factor gain in sensitivity with an amperometric detector.

function, in order to predict whether it can be detected by amperometric oxidation or not. These decisions were compared with experimental results for 43 drugs. Since at the same time we wanted to assess the relative contribution of amperometric detection, *i.e.*, to find in which cases it solves detection problems that cannot be solved by UV detection, the drugs selected all possessed a low UV response.

The test set comprised two different groups. The first one was obtained from an atlas of UV spectra of drugs[15]. From all compounds possessing a molar extinction coefficient lower than 1000 (measured in methanol at the absorption maximum) one

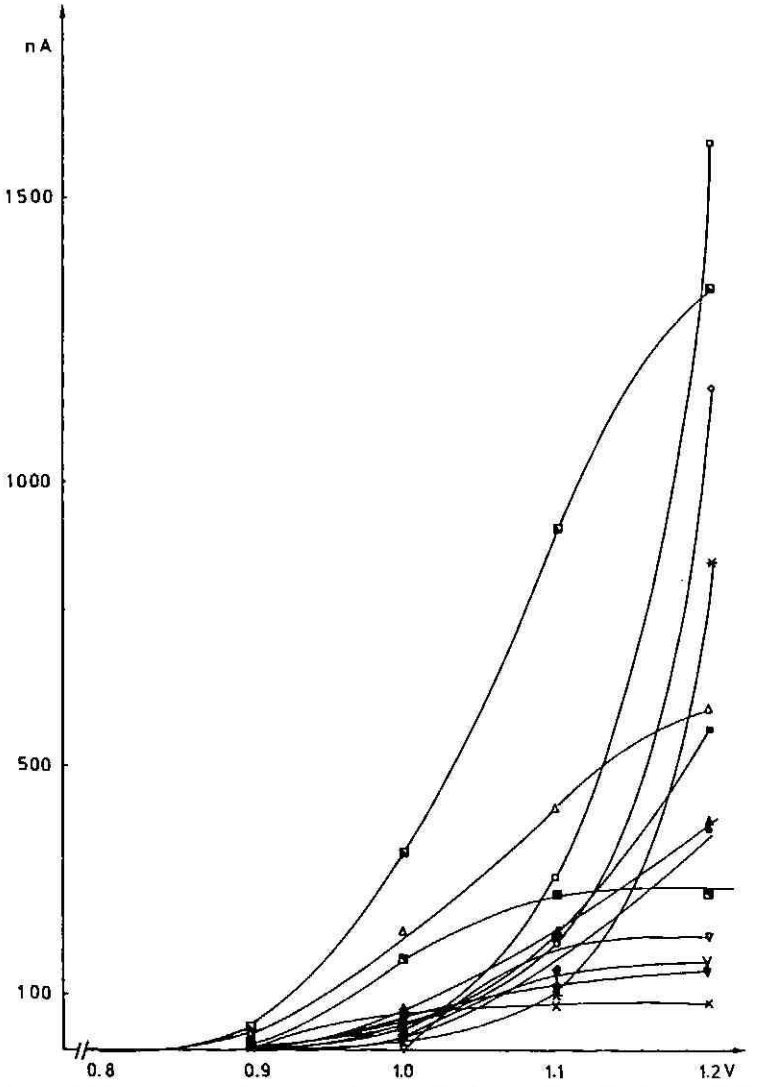


Fig. 2. Voltammogram of compounds possessing low molar extinction coefficients: ●, phenmetrazine; ×, diphenhydramine; ▲, bupivaceine; ■, mepivacaine; ○, clonidine; ▼, clobutinol; □, oxeladine; ▽, scopolamine; △, phendimetrazine; ◪, hydroxyzine; ✳, lidocaine; ◹, radiphenine; Y, orphenadrine. The concentration of all the solutes was 10 ppm, except for lidocaine, 1 ppm.

third was selected at random. These 28 drugs, listed in Table II, were used to verify the decisions made by the expert system. The second group comprised drugs that are usually present in low concentrations in pharmaceuticals, *i.e.*, less than 1 mg per formulation unit. These drugs were found in the Belgian drug compendium[16]. Half were selected for study (Table III). The amperometric detector is set at 1.2 V by the operator at a range of 200 nA full scale. When an electrochemical signal was obtained under these conditions the substance was considered to be electroactive. For each such compound a voltammogram was recorded. The potential just before the start

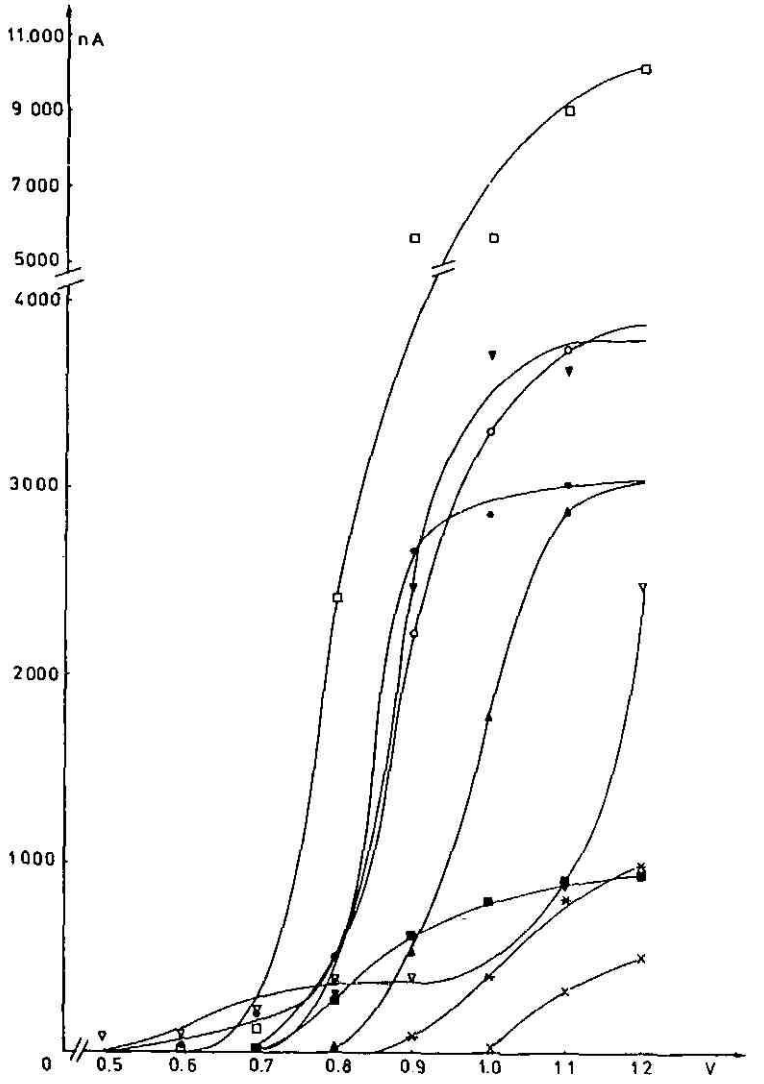


Fig. 3. Voltammogram of compounds present in low concentrations in pharmaceuticals: ●, reserpine; ×, cyclopenthiazide; ▲, haloperidol; ✳, pimozide; ○, dihydroergotamine; □, fenoterol; ▼, fluphenazine; ▽, levomepromazine; ■, ethinylestradiol. The concentration of all the solutes was 10 ppm, except for ethinylestradiol, 1 ppm.

of the limiting-current plateau was applied since at this point the signal-to-noise ratio is the most advantageous. At this potential the amount of drug providing a signal equivalent to 20 nA is considered to be the minimum detectable concentration (MDC-ED) and is compared with the amount of drug giving a signal equivalent to 0.002 absorption units (MDC-UV) (Tables II and III). The lowest MDC-UV of the two UV detection systems mentioned is compared with the MDC-ED value to calculate the gain in sensitivity when an amperometric detector is applied (Tables II and III).

For 15 of the 28 compounds with low molar extinction coefficients (Table II) the expert system predicted a positive electrochemical response. The experiments showed that for 13 drugs the decision was correct (Fig. 2); for the other 2 compounds however no electrochemical signal was obtained. These exceptions are secondary and tertiary amines, namely prenylamine and methamphetamine. Analogous structures such as pro- and nortriptyline, propranolol and pindolol are electroactive[12].

For the 13 drugs listed in Table II, no response was expected and indeed not obtained. Barbiturates and primary aliphatic amines represent the most important classes of drugs in this group. A second possible reason for an expected low UV

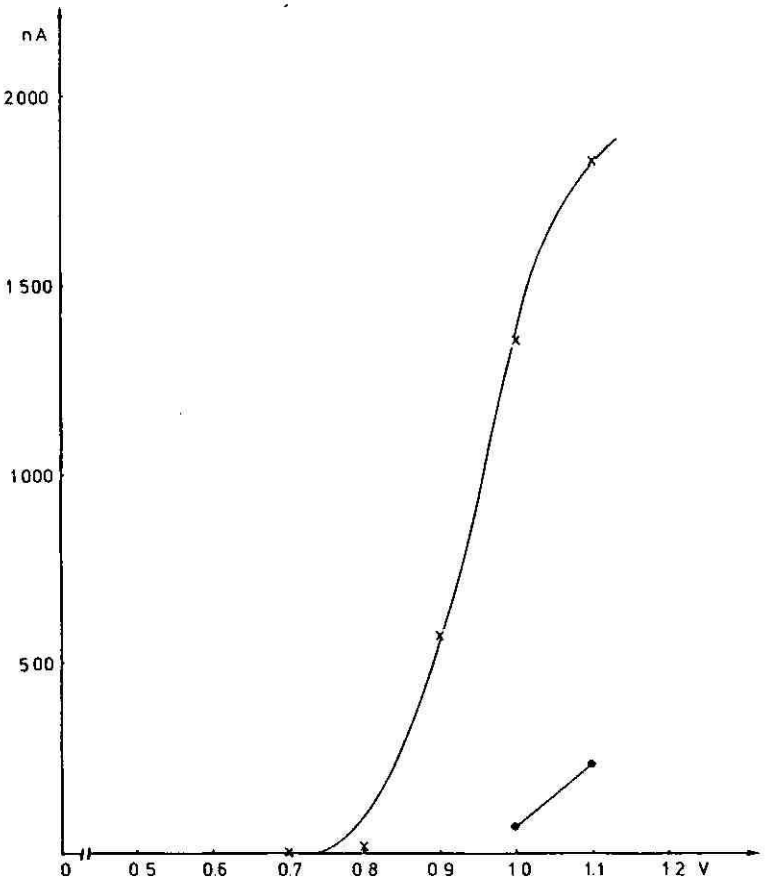


Fig. 4. Voltammogram of UV-inactive compounds: ●, piperazine (5 ppm); ×, penicillamine (0.5 ppm).

response is a low concentration. The utility of amperometric detection was also investigated for this group. For 9 compounds out of the 15 a voltammogram was recorded (Fig. 3). In only one case, namely haloperidol, an unexpected positive electrochemical signal was obtained. This is probably due to the position of the alcohol function near the aromatic ring (pseudo phenol function). Although the molar extinction coefficients are not low, the gain in sensitivity was significant for the amperometric detector, especially for phenolic structures such as fenoterol and ethinylestradiol (Table IIIa and c). For cyclopenthiazide however no improvement was obtained with an amperometric detector. Relatively low MDC-ED values were obtained for reserpine, fluphenazine, dihydroergotamine, haloperidol and levomepromazine. For the drugs listed in Table IIIb, no electroactive response was obtained.

CONCLUSIONS

Looking at the total test set, it is seen that the expert system predicted correctly whether a compound could be detected with an amperometric detector with the general chromatographic conditions used in 40 of the 43 cases investigated (93%). The percentage of correct decisions is acceptable for use in expert systems in the sense that an human expert would certainly not score better.

Amperometric detection offers a possible solution for 50% of this group of drugs with a low UV response since the sensitivity is nearly always enhanced. For some compounds that are not UV active at all, such as piperazine and penicillamine (Fig. 4), amperometric detection also offers a solution.

Phenols, primary aromatic amines, phenothiazines and indoles are most suitable for amperometric detection in the oxidation mode. The drugs with detection problems, *i.e.*, insufficient UV and amperometric response, are mainly barbiturates, corticosteroids, male hormones, amphetamines and analogous compounds and some antibiotics such as cloxacillin.

As a general conclusion one can state that amperometric detection is useful for pharmaceutical and biomedical analysis, but that for some compounds other detection systems must be investigated if the sensitivity and/or the selectivity is to be enhanced.

In Part I of this series in which we developed the rules[1,2], 72 electroactive substances were investigated. Applying the rules as described here to these substances and the others used in this study we find that of the 94 cases where the drug is electroactive this is recognized by the rules, except in the cases of haloperidol, prenylamine and metamphetamine.

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GENERAL CHROMATOGRAPHIC PURIFICATION PROCEDURE BASED ON THE USE OF HETEROBIFUNCTIONAL AFFINITY LIGANDS

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SUMMARY

A new method for affinity purification is described. A heterobifunctional ligand in a soluble form is added to the sample containing the substance to be purified. After binding of the substance with one of the functional groups of the ligand, the complex formed is isolated by passage through a sorption column having affinity for the second part of the heterobifunctional ligand. In a stepwise elution process, first the substance of interest is isolated, followed by the ligand which can then be reused. Model studies on the purification of lactate dehydrogenase using a heterobifunctional ligand containing Cibacron Blue and soy bean trypsin inhibitor are described. The affinity matrix used was trypsin immobilized on Sepharose.

INTRODUCTION

Affinity chromatography has been a very successful technique on the laboratory scale[1,2], but so far very few large scale applications have been described[3]. The procedure used today is, in essence, the same as that described 15 years ago. Few attempts to develop modified affinity purification procedures have been published. The affinity purification of protein by partitioning in aqueous two-phase systems has been studied with great interest and has high potential[4,5]. Affinity precipitation has been attempted and seems to be an attractive alternative when specific interactions are used and the concentration of the compound to be purified is known[6]. Affinity interactions in conjunction with membrane technology have also been studied[7].

However, if affinity interactions are to be useful on a large scale at a competitive cost, then systems for sanitation of the affinity system and, when applicable, for recycling of the solid phase must be developed. Sanitation often involves exposure to extreme conditions, *e.g.*, temperature or chemicals. Autoclaving is normally out of the question in these systems. Chemical treatment has to be harsh in order to be effective. A recommended treatment is the use of 0.5 *M* sodium hydroxide for periods of 5 h[8]. Today there are gels that will withstand this treatment, but the ligands used in affinity chromatography are often much too sensitive.

A step towards achieving a system with higher stability and potential to withstand the sanitation conditions was attempted by us by use of heterobifunctional

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affinity ligands which could first bind a substance in solution and then interact with a solid support. After elution of the substance to be purified, the heterobifunctional ligand was eluted from the column. By this approach it is possible to keep the sensitive ligand in solution and remove it before sanitation.

MATERIALS AND METHODS

Sepharose CL-4B, Sephadex LH-20, Dextran T40 and T500 were obtained from Pharmacia (Uppsala, Sweden), Reactive Blue 2 (Cibacron Blue F3G-A), soy bean trypsin inhibitor (Type II), trypsin (Type IX) (E.C. 3.4.21.4) and $NaBH_3CN$ from Sigma (St. Louis, MO, U.S.A.). Purified bovine heart lactate dehydrogenase (E.C. 1.1.1.27) was a generous gift from P. Wikström, Lund. All other chemicals used were of reagent grade.

Preparation of trypsin-Sepharose

Sepharose CL-4B was activated with cyanogen bromide according to the general procedure[9]. The immobilization reaction was performed for 1 ml of matrix with 8 mg trypsin in 1 ml of 0.1 *M* sodium bicarbonate, pH 8.3 and 0.5 *M* sodium chloride. The suspension was gently mixed for 4 h at 20°C after which the remaining active groups were blocked with ethanolamine (1 *M*, pH 8.0). The adsorbed trypsin was removed by washing in 0.1 *M* acetate buffer, pH 4.0, 0.5 *M* sodium chloride and coupling buffer, respectively.

Synthesis of the heterobifunctional affinity ligand Cibacron Blue–dextran–soy bean trypsin inhibitor

Cibacron Blue F3G-A was purified by chromatography on Sephadex LH-20 as described[10]. The substitution of dextrans with the dye was carried out as described by Ashton and Polya[11]. The amount of dye bound to the dextran was determined spectrophotometrically, assuming no difference in the extinction coefficients of free and covalently bound dye. The dye-substituted dextrans (100 mg dextran in 3 ml of water) were activated with sodium periodate. Solid sodium periodate was added to the dextran solution (ratio of sodium periodate to glucose residues was 0.08 when Dextran T40 was used, 0.03 when Dextran T500 was used) and the solution was stirred for 2 h at room temperature. Soy bean trypsin inhibitor (STI) and $NaBH_3CN$ were dissolved in 1 ml of 10 m*M* phosphate buffer pH 6.0 containing 0.15 *M* sodium chloride, and the solution was added to the activated dextran (STI/Dextran T40 = 1.05; STI/Dextran T500 = 5; $NaBH_3CN$/STI = 30).

After incubation for 14 h at room temperature, 27 μl ethanolamine and 2.7 mg $NaBH_3CN$ in 1 ml phosphate buffer pH 6.0 were added and allowed to react for 3 h. The ligand solution was dialyzed several times against 50 m*M* Tris–HCl, pH 8.0. Dye–dextran without bound STI was removed by passing the solution over the trypsin-Sepharose column. Free STI was not separated from the STI–dye–dextran complex.

Preparation of bovine heart extract

Bovine heart (100 g) was homogenized in a Waring blender with 200 ml phosphate buffer pH 7.0 containing 1 m*M* β-mercaptoethanol and 50 g ice; the homogenate was stirred for 10 min and then centrifuged at 20000 *g* for 15 min. The super-

natant was filtered through glass wool and brought to 30% saturation with ammonium sulphate. After stirring for 15 min, the mixture was centrifuged at 20000 *g* for 15 min. The supernatant was then brought to 65% saturation with ammonium sulphate, stirred for 30 min and centrifuged again for 15 min at the same speed. The pellet was dissolved in 50 ml water and dialyzed against 2 × 2 l of 50 m*M* Tris–HCl, pH 8.0.

Chromatographic procedure

All chromatographic steps were carried out at room temperature. The enzyme solution or homogenate was mixed with the heterobifunctional affinity ligand (STI–dye–dextran) in 50 m*M* Tris–HCl, pH 8.0 containing 2 m*M* calcium chloride, 5 m*M* magnesium chloride, 0.4 m*M* EDTA and 2 μM β-mercaptoethanol and the solution was stirred for 0.5 h. The mixture was applied on the trypsin-Sepharose column (5.6 cm × 1.5 cm) which had been equilibrated with the same buffer at a flow-rate of 1 ml/min. After washing the column with coupling buffer, the bound enzyme was eluted with either (0.5 m*M* NADH, or 0.5 *M* sodium chloride in 0.1 *M* ethylenediamine hydrochloride buffer pH 6.0 containing 2 m*M* calcium chloride. Thereafter, the column was washed to remove sodium chloride. The STI–dye–dextran was then eluted with a pulse of 10 m*M* hydrochloric acid. Fractions were assayed for enzyme activity[12].

Assay of enzyme activity

Lactate dehydrogenase (LDH) was assayed by following the oxidation of NADH at 340 nm in the presence of pyruvate. The assay mixture, in a total volume of 3 ml, contained 0.2 *M* Tris–HCl, pH 7.3, 1.0 m*M* sodium pyruvate and 0.22 m*M* NADH.

Gel electrophoresis

Electrophoretic analyses of the enzyme preparations were carried out on 12.5% polyacrylamide gel in the presence of sodium dodecyl sulphate according to the method of Laemmli[13]. Proteins were stained by the basic silver stain procedure[14,15].

RESULTS AND DISCUSSION

The traditional affinity chromatographic procedure for the preparation of LDH involves the use of an affinity matrix with the ligands bound to the polymeric backbone, or spacer arms attached to the polymer. Upon passage of the homogenate through the column, binding takes place. In a sequential process, washing and elution as well as reconditioning take place in the column. The procedure is thus very much based on interactions between macromolecules in free solution and the immobilized ligand. This traditional process is usually carried out with an excess of immobilized ligands. Because of steric hindrance, only a fraction of these ligands can be utilized. Furthermore, the ligands are attached to the sorbent, which means that when the sorbent has to be treated for sanitation then also the ligands are so treated. When the ligands lose their activity a new sorbent has to be produced. Exceptions are when reversible immobilization is used[16].

The present paper deals with an alternative procedure in the sense that the technology described makes it possible to operate with the subtle affinity interactions

in free solution. Furthermore, the ligands may be removed from the solid phase when harsh treatment is used. The principles of the heterobifunctional affinity chromatography are demonstrated. In a subsequent paper, some theoretical background and experimental evaluations will be presented.

The synthesis of the heterobifunctional affinity ligand was carried out in two stages. First, Cibacron Blue was attached to the dextran molecule. After separation of the complex from unreacted dye, the dextran molecule was activated using periodate and STI was added. After coupling, the complex containing STI was separated from unreacted dye–dextran by means of affinity chromatography on a column of trypsin-Sepharose. A typical elution pattern is shown in Fig. 1. To purify further the complex from unreacted STI, it was possible to pass the complex over a column of concanavalin A-Sepharose. However, no harm due to the presence of traces of free STI was foreseen, hence this latter purification step was not included in the process. The degree of derivatization was measured for the final heterobifunctional complex. The amount of dye bound was measured spectrophotometrically, assuming that the specific absorptivity was not changed when the dye molecules were immobilized. STI was quantified using the absorbance at 280 nm.

The heterobifunctional affinity ligand was then tested in model experiments. Addition of the complex to a solution of purified LDH from beef heart with a subsequent pass over a trypsin column revealed that the heterobifunctional ligand functioned as predicted. Fig. 2 shows a typical elution pattern. The first peak is a result of a slight overloading of the column. When no more protein was present in the effluent, the elution conditions were changed. The addition of 0.50 *M* sodium chloride has been reported to be an efficient way to dissociate the Cibacron Blue–lactate dehydrogenase complex[17]. This was shown to be the case in our experiment as well. It should be stressed that this treatment is specific in the sense that the heterobifunctional ligand is still bound to the column. To rinse the column, a shift in pH was used. In blank experiments when the heterobifunctional ligand added to LDH was passed over an unmodified Sepharose column, the elution pattern was as shown in Fig. 3b. Addition of Cibacron Blue–dextran to LDH and passage over a trypsin column was also tested (Fig. 3). Fig. 2 clearly demonstrates the feasibility of using an heterobifunctional affinity ligand together with an affinity column to isolate spe-

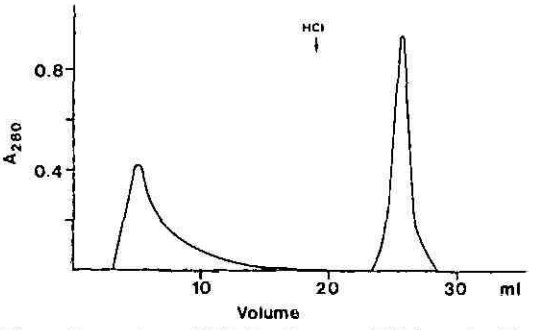

Fig. 1. Separation of STI–dye–Dextran T40 from dye–Dextran T40 on trypsin-Sepharose. Sample applied: 31.5 mg dextran. Flow-rate: 2.0 ml/min. Washing buffer: 50 m*M* Tris–HCl, pH 8.0 containing 2 m*M* calcium chloride, 5 m*M* magnesium chloride, 0.4 m*M* EDTA and 2 μM β-mercaptoethanol. Elution buffer: 10 m*M* hydrochloric acid.

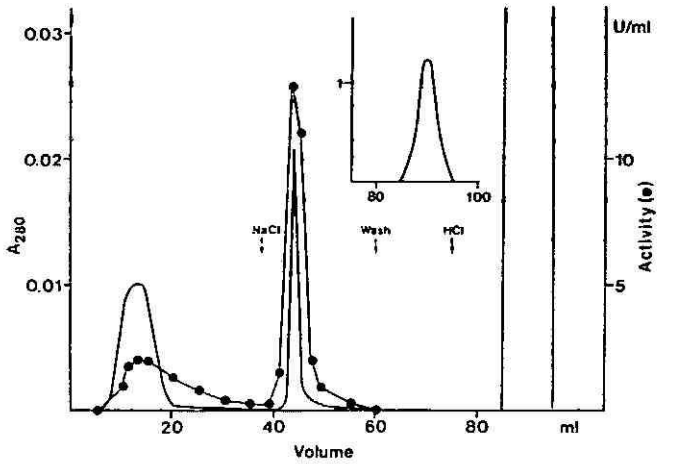

Fig. 2. Chromatography of a pre-equilibrated mixture of LDH and STI–dye–Dextran T40 on trypsin-Sepharose. Sample applied: LDH (88 units) and STI–dye–Dextran T40 (32.5 μM dye). Flow-rate 1.0 ml/min. LDH was eluted with a pulse of 0.5 M ethylenediamine hydrochloride buffer pH 6.0 containing 2 mM calcium chloride. The ligand was eluted by a pulse of 10 mM hydrochloric acid.

cifically a certain molecule.

A crucial point in such a procedure will, of course, be the yield of the substance to be isolated as well as the recovery of the heterobifunctional ligand. The recovery of the ligands when using Dextran T40 as the core onto which the two different affinity ligands were attached is shown in Table I. It is seen that the percentage recovery increased on repeated use. This was interpreted in terms of selection of the most suitable heterobifunctional complexes, the less suitable ones being lost in the initial cycles. It is, of course, important to use appropriate densities of the ligands on the polymer core, both in relation to each other and in absolute terms.

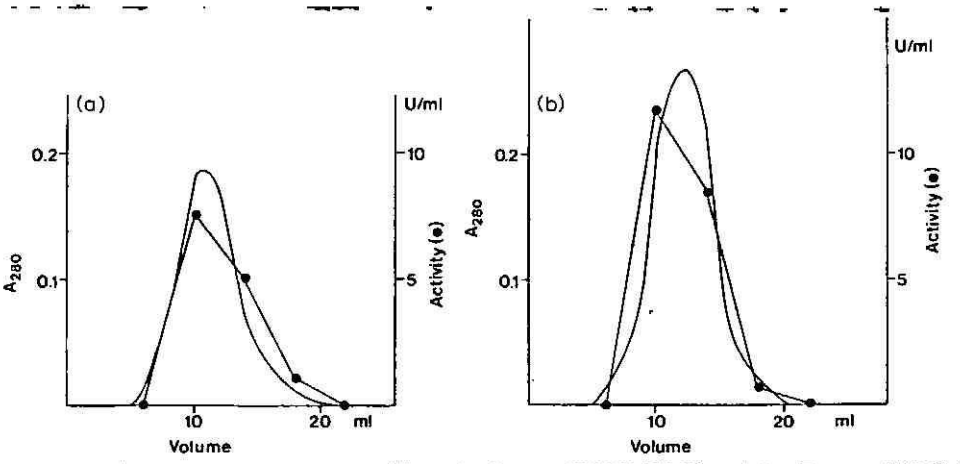

Fig. 3. (a) Chromatography of a pre-equilibrated mixture of LDH (50 U) and dye–Dextran T40 (0.11 μmol dye) on Sepharose-immobilized trypsin. Flow-rate 2.0 ml/min. (b) Chromatography of a pre-equilibrated mixture of LDH (75 U) and STI–dye–Dextran T40 (0.17 μmol dye) on Sepharose CL-4B. Flow-rate 2.0 ml/min.

TABLE I

RECOVERY OF STI-DYE-DEXTRAN AND YIELD OF LACTATE DEHYDROGENASE

Dye concentration (μM)	*LDH (units)*	*Recovery of ligand (%)*	*Yield of enzyme (%)*
Dextran T40			
35.3	65.0	81	72
18.9	43.3	81	59
15.9	32.5	81	52
19.3	21.7	78	44
16.0	21.7	89	37
Dextran T500			
22.3	103	76	63.0
16.8	75	68	92.5
13.2	100	67	58.7
11.2	50	70	52.0
12.1	25	88	44.0

It could be argued that Dextran T40 with a molecular weight of about 40 000 is too small and that a larger dextran would have superior ability to bind several of the different ligands, to bind protein in solution and still to be able to expose one or more STI molecules for interaction in the column chromatographic step. In Table I are given data for recovery of the ligand as well as of the enzyme when LDH was isolated from a buffer solution with Dextran T40 and T500.

The yield of the enzyme was also measured. In Fig. 4 are plotted measured values of the yields as well as calculated values. The latter were obtained by calculating the yields in the binding reactions. The dissociation constant between LDH and Cibacron–dextran was set at $6.7 \cdot 10^{-6}$ M[11].

The same preparation of ligands was used to purify LDH from a crude homogenate. As expected, the yield of enzyme was lower (23% as compared to the calculated value of 73%). Since the dye is not very specific for LDH but merely a general ligand for many dehydrogenases and kinases and other proteins as well, one has to take this into consideration and not expect too high a purity in one single early step[18], Fig. 5. In a series of experiments, Dextran T500–Cibacron Blue–STI was used to isolate LDH from a crude homogenate. In Fig. 6 it is seen that the observed yield

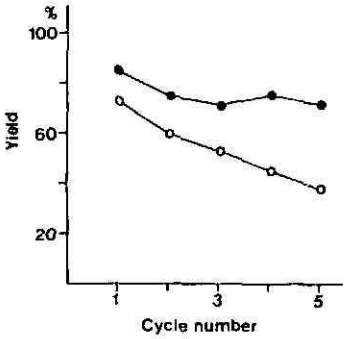

Fig. 4. A plot of measured (O) and calculated (●) yields of LDH when using STI–dye–Dextran T40. Data in Table I were used.

is closer to the theoretical values than when Dextran T40 was used.

A prerequisite for a method like the one described here to be successful is that it should be simple, cost effective and general. Simplicity and generality lie in the fact that, provided the appropriate affinity pairs are used, one and the same column can be used for almost any affinity chromatographic process. Selectivity in the elution process is an advantage, but not a necessity. Hence, by reducing the consumption of gel material in the chromatographic procedure there is a substantial reduction in cost. Furthermore, the ligand used in the primary interaction is much more efficiently used in free solution as compared to that immobilized on the solid chromatographic material. The binding strength and yield will be discussed in a subsequent paper[19].

When applying affinity chromatography under normal conditions, a separate column is needed for each substance to be purified. This may turn out to be a very expensive investment for purification on a large scale. One way to avoid this is the use of general ligands, *e.g.*, immobilized NAD^+ or derivatives thereof in the purification of dehydrogenases[20].

Furthermore, on a large scale, sterility is of crucial importance, and this means that column sanitation is a requirement. This, in turn, leads to the use of stable affinity ligands. During recent years there has been a marked increase in the use of semiaffinity ligands. Substances like hydrophobic groups and dyes have been shown to interact, more or less, specifically with certain proteins and have thus been utilized as affinity ligands. Some of these are stable and can thus withstand the sanitation treatment. Another extreme would be to use ion exchange resins —a quite realistic possibility when working with heterobifunctional ligands.

Fig. 5. Gel electrophoresis of crude homogenate. LDH reference and LDH-rich fraction purified with STI–dye–Dextran T500.

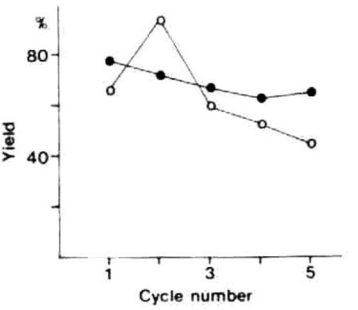

Fig. 6. A plot of observed (○) and calculated (●) yields of LDH when using STI–dye–Dextran T40. Data in Table I were used.

A different line of development in downstream processing has been taken by the genetic technologists. To facilitate the isolation of a target protein, a certain sequence of amino acids has been fused to the protein of interest. The added sequence having extreme properties makes the fused product markedly different from the other protein molecules in the sample. This makes it easy to isolate the fused product from all the other cell components. In a subsequent step the added sequence is split off and the pure protein isolated. The addition of a sequence of arginine residues made it possible to use ion-exchange chromatography[21] and the use of cysteinyl residues enabled the exploitation of covalent chromatography with a subsequent selective elution step[22]. Fusion with protein A made it possible to use an immunoglobulin column as a general affinity column[23]. There are certainly several more posibilities. The technique seems very useful, the only limitation being that the protein has to be a genetically modified one.

When isolating a substance from a complex biological homogenate, affinity purification possesses the highest resolving power. However, it is still characterized by some minor drawbacks in the laboratory scale. On a larger scale, these limitations are even more severe. It would be natural to use affinity interactions as early as possible in a purification process. This is of course governed by a desire to reduce volumes and remove unwanted proteins. Modern affinity chromatographic techniques were mainly developed to serve as a final purification step towards the end of a separation sequence. If, however, more robust systems can be developed, then they will be a natural choic early in the purification process.

ACKNOWLEDGEMENT

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THEORETICAL AND EXPERIMENTAL EVALUATION OF THE USE OF HETEROBIFUNCTIONAL AFFINITY LIGANDS IN GENERAL CHROMATOGRAPHIC PURIFICATION SYSTEMS

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SUMMARY

A new general procedure for purification of proteins is evaluated. The procedure is based upon the interaction in free solution between the protein to be purified and a heterobifunctional ligand having an affinity for the molecule to be purified and also for the solid used as an affinity support. The binding constants needed in the different steps in order to obtain an efficient purification are evaluated on a theoretical basis, and some experimental data are provided to support the conclusions drawn. This new technique is efficient, simple and cost effective.

INTRODUCTION

In a previous paper[1] we demonstrated the feasibility of using a heterobifunctional ligand when purifying an enzyme from a cell homogenate. The system is based on the use of an heterobifunctional ligand that is added to the homogenate. Affinity binding takes place on one of the moieties of the heterobifunctional compound. The mixture is then passed over an adsorption column with affinity for the other moiety of the complex, the idea being that by this latter interaction the protein bound to the first affinity moiety will be trapped on the column. After washing to remove all impurities, a specific elution is carried out and the protein isolated. In a subsequent step the heterobifunctional ligand is dissociated from the column and eluted in a concentrated peak.

This procedure seems to carry many useful properties in that it is easy to operate and is general as the same column may well be used to purify a broad spectrum of substances. One drawback might be its complexity, since two different affinity interactions are involved. The present paper deals with a theoretical treatment of this system and also presents some experimental data to confirm what is shown theoretically.

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THEORETICAL

The primary affinity binding reaction may be represented

$$AB + C \rightleftharpoons ABC \tag{1}$$

where AB is the heterobifunctional affinity ligand and C the protein to be purified. The corresponding equilibrium constant is

$$K_a = \frac{x}{(p - x)(L - x)} \tag{2}$$

where x is the concentration of complex (ABC) formed and p and L denote the initial concentrations of the protein (C) and the ligand (AB), respectively.

The second affinity interaction takes place when the complex formed in free solution is trapped on an affinity sorbent

$$(ABC + AB) + D \rightleftharpoons ABD + ABCD \tag{3}$$

where D is the affinity ligand on the solid support to which the soluble is to be bound. The corresponding equilibrium constant is

$$K_b = \frac{y}{(L - y)(d - y)} \tag{4}$$

where y is the concentration of the complex (ABD) + (ABCD), L is the initial concentration of the ligand (AB) and d is the initial concentration of the ligand D.

A crucial parameter of an affinity purification is the yield. To evaluate the conditions for achieving high yields, some values were chosen in order to investigate whether the system would be able to achieve these values. The yield in the first step was set to 90% and in the last step to 99.9%, *i.e.*, very high values in relation to what is normally achieved in most affinity purification processes. With a 90% yield in the first step, eqn. 2 can be rearranged to

$$K_a = \frac{9}{L - 0.9\,p} \tag{5}$$

and eqn. 4 to

$$K_b = \frac{999}{d - 0.999\,L} \tag{6}$$

$$L = \frac{K_b d - 999}{0.999\,K_b} \tag{7}$$

Multipoint binding of heterobifunctional compound

To increase the amount of bifunctional ligand that is bound to the matrix, it is possible to use more than one ligand on the core. Consider an heterobifunctional

compound A, which contains n sites for the ligand D. Each site has the same microscopic ligand dissociation constant, k, and the sites are assumed to be independent. The equilibria may be written as

$$A_0 + D \rightleftharpoons A_1$$

$$A_{n-1} + D \rightleftharpoons A_n \tag{8}$$

and the macroscopic dissociation constants as:

$$K_1 = \frac{[A_0]\,[D]}{[A_1]}$$

$$K_n = \frac{[A_{n-1}][D]}{[A_n]} \tag{9}$$

Eqn. 9 gives:

$$[A_i] = \frac{[A_0][D]^i}{\prod_{j=1}^{i} K_j} \tag{10}$$

There are $\Omega_{n,i}$ ways to put i ligands on n sites

$$\Omega_{n,i} = \frac{n!}{(n-i)!i!} \tag{11}$$

and the relationship between K_i and k is:

$$K_i = \frac{\Omega_{n,i-1}}{\Omega_{n,i}} \cdot k \tag{12}$$

Rewriting eqn. 10 by use of eqn. 12 gives:

$$[A_i] = [A_0]\left[\prod_{j=1}^{i} (n-j+1)/j\right] \cdot \left(\frac{[D]}{k}\right)^i \tag{13}$$

The unbound fraction of the bifunctional ligand is given by:

$$P_f = \frac{[A_0]}{[A_{tot}]} = \frac{[A_0]}{[A_0] + \sum_{i=1}^{n} [A_i]} \tag{14}$$

Substitution of eqn. 13 into eqn. 14 gives:

$$P_f = \frac{1}{1 + \sum_{i=1}^{n} \left[\prod_{j=1}^{n} (n-j+1)/j \right] \cdot \left(\frac{[D]}{k} \right)^i} \quad (15)$$

The product term in eqn. 15 is identical to $\Omega_{n,i}$, thus eqn. 15 can be rewritten as:

$$P_f = \frac{1}{1 + \sum_{i=1}^{n} \frac{n!}{(n-i)!\, i!} \cdot \left(\frac{[D]}{k} \right)^i} \quad (16)$$

The denominator of eqn. 16 is the binomial expansion of $\left(1 + \frac{[D]}{k}\right)^n$, thus we finally obtain:

$$P_f = \frac{1}{\left(1 + \frac{[D]}{k}\right)^n} \quad (17)$$

MATERIALS AND METHODS

Sepharose 4B, Sephadex G-75, Dextran T10 and T40 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Jack beans, $NaBH_3CN$, trypsin (Type IX from hog pancreas), soy bean trypsin inhibitor (STI) (Type II) and α-methyl-D-glucopyranoside were obtained from Sigma (St. Louis, MO, U.S.A.), sodium periodate and sodium borohydride from Merck (Darmstadt, F.R.G.).

Isolation of concanavalin A (Con A)

Jack beans (500 g) were swollen in 1.25 l of 0.9% sodium chloride solution overnight. The beans were then homogenized in a Turrax mixer in 1.5 l of Tris–HCl buffer (10 m*M*, pH 7.0 also containing 0.9% sodium chloride, 1 m*M* magnesium chloride 1 m*M* manganese chloride and 1 m*M* calcium chloride). The homogenate was stirred for 5 h at 4°C, and was then filtered through cheese cloth. The solution was centrifuged for 15 min at 24 500 *g*. The supernatant was collected and the fat was removed by passage through a layer of glass wool. The extract was then mixed with 400 ml of pre-swollen Sephadex G-75 and gently stirred overnight at 4°C. The gel material was then washed with 5 l of the Tris buffer described above before transferring it to a glass filter where it was washed with the same buffer until A_{280} of the eluate was *ca.* 0.05. The Con A bound to the gel was eluted by a pulse of 1 *M* glucose dissolved in 10 m*M* Tris-HCl, pH 7.5 also containing 0.9% sodium chloride. All fractions with $A_{280} > 0.1$ were pooled and dialyzed against the above mentioned buffer.

Preparation of Con A-Sepharose 4B and trypsin-Sepharose 4B

Sepharose 4B was activated using cyanogen bromide as described[2]. After

thorough washing with 0.1 *M* sodium bicarbonate, pH 8.3, containing 0.5 *M* sodium chloride and 0.1 *M* acetate buffer, pH 4.0, containing 0.5 *M* sodium chloride, coupling was performed by adding 5 mg Con A per ml gel or 8 mg trypsin per ml gel. Coupling proceeded at 20°C for 4 h.

Preparation of STI–Dextran T40

Dextran T40 (100 mg) was dissolved in 2.5 ml of 10 m*M* phosphate buffer pH 6.0 containing 0.15 *M* sodium chloride. Sodium periodate (10.7 mg per 0.5 ml buffer) was added and the reaction allowed to proceed for 2 h at room temperature with gentle mixing. Then, 60 mg STI and 6 mg $NaBH_3CN$ in 1 ml of buffer were added. After reaction at room temperature for 14 h, the pH was adjusted to 9–9.5 with 0.5 *M* sodium carbonate. Sodium borohydride (7.5 mg) was added and the reaction continued overnight at 4°C. The reaction mixture was then dialyzed against 0.05 *M* Tris–HCl, pH 8.0.

Purification of STI–Dextran T40 from the reaction mixture

Underivatized dextran was removed by passing the sample over a column of trypsin-Sepharose (10 ml, diamter 1.0 cm) pre-equilibrated with 0.05 *M* Tris–HCl, pH 8.0. After washing the column with buffer, the STI–dextran was eluted with a pulse of 10 m*M* hydrochloric acid. Unreacted STI was removed by passing the sample over a Con A-Sepharose column (10 ml Con A-Sepharose equilibrated with 50 m*M* Tris–HCl pH 7.0 containing 20 m*M* calcium chloride). The STI–dextran was applied on the column at a flow-rate of 0.25 ml/min. After washing with buffer, the STI–dextran was eluted using 0.1 *M* α-methyl-D-glucopyranoside. The pure ligand was then removed by a second passage over the trypsin column.

Chromatographic procedure

All chromatographic steps were carried out at room temperature. The trypsin-Sepharose was equilibrated with 50 m*M* Tris–HCl, pH 8.0 containing 20 m*M* calcium chloride. Con A and STI–dextran were mixed in the same buffer as above and the mixture was stirred for 45 min before being passed over the trypsin gel at a flow-rate of 0.4 ml/min. After washing with coupling buffer off non-specifically bound protein, the Con A was eluted by a pulse of 0.1 *M* α-methyl-D-glucopyranoside. After complete elution the column was washed with coupling buffer to remove the glucopyranoside. The STI–dextran was eluted by a pulse of 10 m*M* hydrochloric acid.

Determination of K_a for Con A and dextran by zonal affinity chromatography

A column containing 10 ml of Sephadex G-75 was equilibrated with 50 m*M* Tris–HCl pH 8.0 and 20 m*M* calcium chloride containing various concentrations (10–2.5 m*M*) of Dextran T10. A 5-mg amount of Con A in 1 ml of each of the above dextran solutions was applied on the column at a flow-rate of 0.22 ml/min and was eluted with the equilibration buffer.

RESULTS AND DISCUSSION

The heterobifunctional ligand consists of two parts, the STI with affinity for trypsin-Sepharose and the dextran which acts both as a matrix and a ligand with

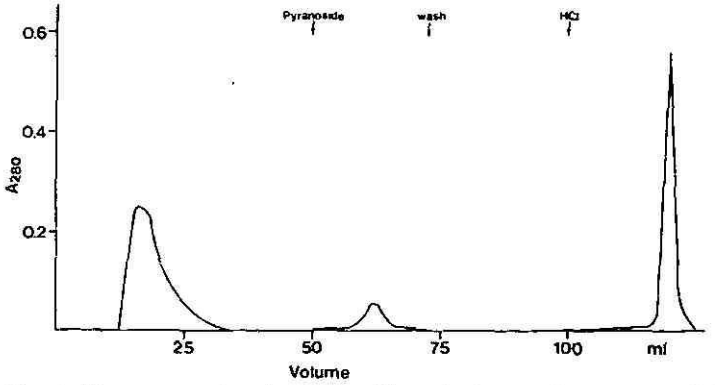


Fig. 1. Chromatography of a pre-equilibrated mixture of Con A and STI–dextran on Sepharose-immobilized trypsin. Sample applied: STI–dextran (25 μM) and Con A (2.7 μM). Flow-rate: 0.4 ml/min. Elution of Con A was performed with 0.1 M α-methyl-D-glucopyranoside and that of STI–dextran with 10 mM hydrochloric acid.

affinity for concanavalin A. When a pre-equilibrated mixture of con A and STI–dextran is passed over the trypsin-Sepharose column a chromatogram like that shown in Fig. 1 is obtained. The first peak is the result of overloading of the column. The affinity bound Con A was eluted with α-methyl-D-glucopyranoside subsequent to the washing of any non-specifically bound protein from the column. The sugar was then removed before the heterobifunctional ligand was eluted from the column. This system is not ideal, but offers certain advantages in this study.

As can be seen in Table 1, the major part of the Con A present is not bound by the STI–dextran. Assuming that the affinity binding takes place only during the incubation with STI–dextran and not during the passage through the column, then a value of K_a for the interaction between Con A and STI–dextran can be determined as $6 \cdot 10^3\ M^{-1}$. A value of the equilibrium constant for the interaction between Con A and dextran was estimated using zonal affinity chromatography. In Fig. 2 is shown an elution profile for con A from a column containing Sephadex G-75. The column was equilibrated with buffer containing various concentrations of Dextran T10. Every time the column was loaded with the same amount of Con A, which was then eluted using the equilibration buffer. Increasing concentrations of dextran resulted in reduced elution volumes down to a value V_0, after which there was no further reduction in elution volume with increase in dextran concentration. The elution volume, V_i, at the absorption maximum A_{280}, was measured for the different dextran concentra-

TABLE 1

YIELD OF CON A FROM A COLUMN OF TRYPSIN-SEPHAROSE AFTER EXPOSURE OF THE CRUDE HOMOGENATE TO STI-DEXTRAN T40

STI–Dextran T40 (μM)	*Con A (μM)*	*Yield of Con A (%)*
25.0	2.7	13
18.6	0.48	10

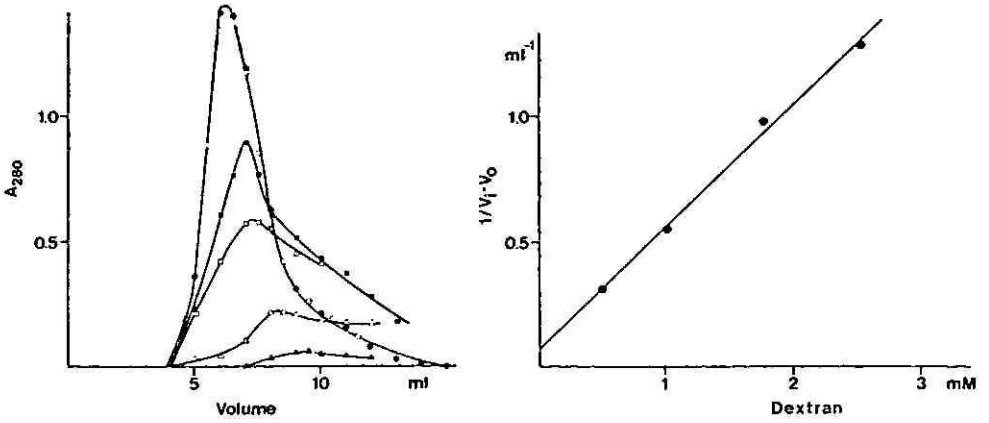


Fig. 2. Chromatography of Con A on Sepharose G-75. Different Dextran T10 concentrations in the eluting buffer were used. The same quantity of Con A (5 mg) was applied to the column, equilibrated with 50 m*M* Tris–HCl buffer containing 0.02 *M* calcium chloride, pH 8.0, and Dextran T10 in the concentrations 10 (●), 7.5 (○), 2.5 (■), 1.75 (□), 1.0 (△) and 0.5 m*M* (▲). Fractions of 0.5 ml were collected while eluting with the corresponding equilibrating buffer.

Fig. 3. Plot of $1/(V_i - V_0)$ (ml^{-1}) *versus* the concentration of Dextran T10 in the eluent. The data in Fig. 2 were used.

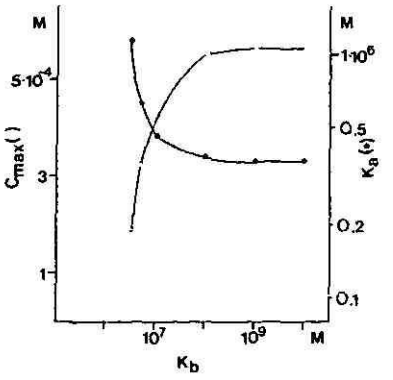
tions. In Fig. 3 is plotted $1/(V_i - V_0)$ *vs.* the dextran concentration. The slope of the line is $1/K_i(V_0 - V_m)(L/K_L)$ and the intercept on the $1/(V_i - V_0)$ axis is $1/(V_0 - V_m)(L/K_L)$, Where V_m is the void volume and K_L is the dissociation constant of the complex of Con A and Sephadex G75. The value of K_a (*i.e.*, $1/K_i$) was derived from the quotient slope/intercept, *i.e.*, $6 \cdot 10^3\ M^{-1}$. This value was obtained by using zonal affinity chromatography, with the assumption that each dextran molecule can bind only one Con A, and is in good agreement with the one calculated earlier (see Table II).

The yield of C, the protein to be purified, depends on several factors: [C], [AB], [D], K_a and K_b. The choice of D (ligand bound to the solid support) and B (the compound with affinity for D) is of great importance, since the concentration of D normally varies between 1 and 10 mg/ml, which in turn leads to the fact that K_b must be very high. This can be seen from eqn. 7 which is derived from eqn. 6, where $K_b[D] > 999$ gives a yield of 99.9%. The calculations are based on interactions in free solution in a batch experiment. A smaller value of K_b is needed when the reaction

TABLE II

CALCULATED YIELDS OF CON A IN THE CHROMATOGRAPHIC PURIFICATION PROCEDURE USING STI-DEXTRAN AND A TRYPSIN-SEPHAROSE AFFINITY COLUMN AT THREE DIFFERENT ASSUMED K_a VALUES

STI-Dextran T40 *(μM)*	*Con A* *(μM)*	K_a (M^{-1})		
		$6 \cdot 10^3$	$12 \cdot 10^3$	$18 \cdot 10^3$
25.0	2.7	13	23	30
18.6	0.48	10	18	25



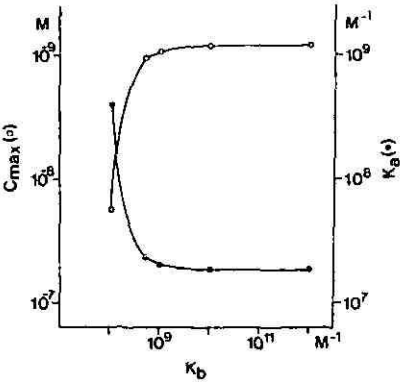


Fig. 4. Plot of $[C]_{max}$ and K_a *versus* K_b for [D] = 0.5 mM. K_a is calculated for [C] = 0.95$[C]_{max}$.

Fig. 5. Plot of $[C]_{max}$ and K_a *versus* K_b for [D] = 10 μM. K_a is calulated for [C] = 0.95$[C]_{max}$.

takes place in a column, but then restrictions due to solid phase–liquid interactions have to be taken into consideration. Normally, K_a is reported only for reactions in free solutions, and this makes such calculations even more approximate, since K_a may decrease for either one or both of the reactants upon immobilization.

Figs. 4 and 5 show K_a and $[C]_{max}$ as functions of [D] and K_b. From Fig. 4 it is seen that K_b cannot be less than $3 \cdot 10^6$ M^{-1} if a 99.9% yield is to be achieved. At high [D], very high concentrations of C can be used with K_a values in the region $2 \cdot 10^5$–10^6 M^{-1}. At lower [D], *e.g.*, when using antibodies, the requirements for a higher K_b ($> 10^8$) are increased. $[C]_{max}$ and K_a decreases and increases, respectively, when decreasing [D]. One way to lower the demands of either a high K_b or a high [D] is to use more than one ligand on the core that could interact with the solid phase, Fig. 6 (it is assumed that [D] $\gg$ [A]).

It is reasonable to desire high recoveries of the heterobifunctional ligand in order to reduce the costs of the process. The use of a heterobifunctional ligand ought to be less expensive than traditional technology, based on several reasons. The need for gel is drastically reduced, since the new method allows the same gel preparation to be used for several different separations. Consequently, the number of columns required is also drastically reduced. Another reason is that the need for ligand is

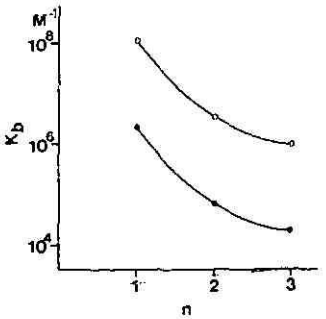


Fig. 6. Plot of the values of K_b needed to achieve a 99.9% yield of bifunctional ligand *versus* the number of ligands interacting with the solid phase. [D] = 0.5 mM (●); 10 μM (○).

reduced, especially when dealing with low-molecular-weight ligands. When using a ligand like 2-iminobiotin in the immobilized state, an excess has to be used in order to obtain a high concentration of ligand under the operating conditions. If, however, the ligand is immobilized on a soluble polymer, then there is no steric hindrance to the interaction with avidin, as observed in the immobilized state[3].

It is difficult to compare the costs of the different methods, due to a lack of relevant information in the literature. A consideration of recycling must deal not only with the ligand as such but also with sanitation of the column. In many affinity chromatographic processes there is a need for good control of the aseptic conditions in the column. It would be possible in many cases to wash the column with, *e.g.*, sodium hydroxide, but such a treatment is often detrimental to the affinity column. The use of heterobifunctional ligands opens up the possibility of using very stable compounds for the affinity interaction on the column, and thus to create systems that can withstand column sanitation.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS FOR THE ANALYSIS OF ANALGESIC AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN FORENSIC TOXICOLOGY

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SUMMARY

High-performance liquid chromatography retention data are presented for over 40 analgesic drugs on an ODS-silica packing material to assist in the identification of these compounds. Three isocratic eluents prepared from isopropanol, formic acid and an aqueous phosphate buffer have been used. One eluent has been used for the analysis of paracetamol in whole blood.

INTRODUCTION

Within the last decade the number of analgesic and non-steroidal anti-inflammatory drugs available to the public has increased markedly. This has enhanced the possibility of such drugs occurring in toxicological cases. Many of these compounds are polar, making high-performance liquid chromatography (HPLC) a particularly suitable technique for the purposes of identification and/or quantification.

The scientific literature contains many specific HPLC methods for the analysis of analgesic drugs in various matrices including pharmaceuticals and body fluids. A review of this general area has been published[1] while some detailed surveys for particular drugs have also appeared (*e.g.* salicylates[2], paracetamol[3], phenacetin[3]). Most papers present retention data for only a small number of analgesic drugs, and the available information covers a wide range of different packing materials and eluents. A few papers have presented retention data for groups of ten or more compounds[4,5] often when considering interferences with specific assays, but no attempt has been made to measure the retention properties of a large group of these drugs on a single packing material.

Most workers have used reversed-phase HPLC systems for the analysis of analgesic drugs. The forensic science laboratories in the U.K. have recently standardized on a particular brand of ODS-silica (ODS-Hypersil), to facilitate the rapid transfer of HPLC methods between laboratories. Current work involves the provision of a large data base of retention properties on this material. To date data have been published for barbiturates[6,7], amphetamines[8], cocaine and related local anaesthet-

ics[9], LSD and other ergot alkaloids[10] and benzodiazepines[11]. It was the intention of the present work to record the retention properties of a large group of analgesic drugs on ODS-Hypersil using suitable eluents.

EXPERIMENTAL

Apparatus

HPLC was carried out using a Waters M6000 pump, a Rheodyne 7125 injection valve (fitted with a 10-μl loop) and a Pye-Unicam LC-UV variable-wavelength detector operated at 240 nm. The stainless-steel column (16 cm × 5 mm I.D.) was packed with 5 μm ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.) by a slurry procedure, dispersing the packing material in isopropanol with hexane as the pressurizing solvent.

Materials

Isopropanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Potassium dihydrogen orthophosphate (AnalaR), perchloric acid, 72% w/w (AristaR), and formic acid, 98–100% (AnalaR) were obtained from BDH (Poole, U.K.). All other chemicals used were of analytical grade.

Pure samples of paracetamol, its glucuronide, sulphate, cysteine, and mercapturic acid metabolites, and a pure sample of N-propionyl-*p*-aminophenol were obtained from the Sterling Winthrop Research Centre (Alnwick, U.K.). The remaining analgesic drugs were from the drug collection of the Central Research Establishment (Home Office Forensic Science Service, Aldermaston, U.K.).

Chromatography

Three eluents were required to elute the analgesic drugs. The eluents were prepared by mixing aqueous potassium dihydrogen phosphate (1000 ml, 0.1 *M*) with formic acid (1 ml) and isopropanol (x ml), where x = 17 ml, 176 ml and 540 ml for eluents 1, 2 and 3 respectively. The eluents had isopropanol contents of 1.7%, 15% and 35% respectively. All eluents were thoroughly degassed with helium before use. Flow-rates were 2 ml/min for eluent 1 and 1.5 ml/min for eluents 2 and 3. The column was allowed to equilibrate for one hour with each eluent before beginning retention measurements.

Drug samples were dissolved in a minimum volume of methanol or isopropanol and diluted with the appropriate eluent prior to injection on to the HPLC column. The paracetamol metabolites were injected in water.

Analysis of paracetamol in whole blood

The blood precipitant containing internal standard was prepared by diluting perchloric acid (72% w/w, 4 ml) with water (100 ml) and adding N-propionyl-*p*-aminophenol (5 mg).

Each blood sample was given gentle vortex mixing before analysis to ensure homogeneity and then 100 μl was transferred to a small glass test tube (50 × 6 mm). The blood precipitant containing internal standard was added and the liquid vortex mixed for 20–30 s. (The internal standard was added at a concentration equivalent to 100 μg/ml in the blood.) The mixture was centrifuged at 9000 *g* for approximately

45 s and the supernatant liquid decanted into a clean test tube where it was subjected to a further centrifugation (approximately 20 s). Aliquots (5 μl) of the clear supernatant were injected on to the HPLC system (eluent 1).

Quantification was performed using peak height ratio measurements (paracetamol–internal standard) with changes to the detector sensitivity where necessary. The calibration graph was prepared by conducting the assay with swine serum spiked with the drug.

RESULTS AND DISCUSSION

Initial experiments with various eluents on the ODS-Hypersil column revealed that the analgesic drugs show a very wide range of retention properties and that a series of isocratic eluents would be required. Paracetamol and its metabolites were found to be amongst the most polar compounds showing low retention on the column. As paracetamol is frequently encountered in forensic toxicology casework[12] it was considered desirable that one of the isocratic eluents selected should be appropriate for the analysis of this drug. An HPLC system published by Buchanan *et al.*[13] involving an eluent containing isopropanol–0.1 *M* potassium dihydrogen phosphate–formic acid (17:1000:1, v/v/v) was found to be particularly suitable and this was adopted as eluent 1. The experimental work confirmed that the parent drug was well resolved from its metabolites with good peak shapes (Fig. 1A). The capacity ratios of the compounds eluted on this system are given in Table I in order of increasing retention.

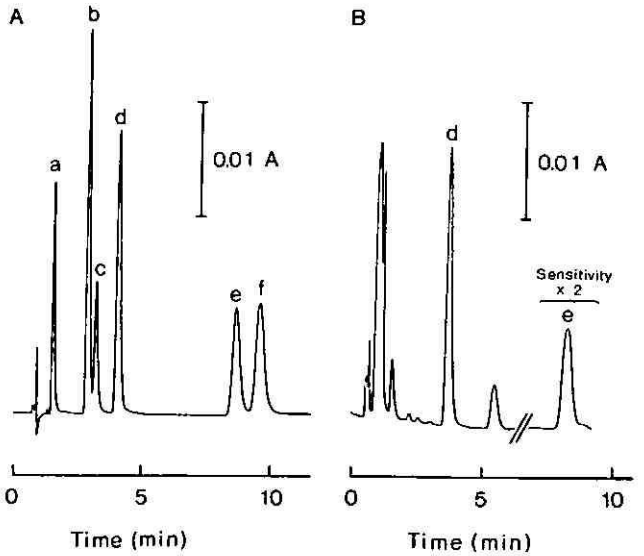

Fig. 1. Separation of paracetamol and its metabolites using eluent 1. (A) Standard mixture; (B) post mortem blood containing 300 μg/ml paracetamol. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 1.7% isopropanol containing a formic acid–phosphate buffer; flow-rate: 2 ml/min; detection: 240 nm. Peaks: a = paracetamol glucuronide; b = paracetamol sulphate; c = paracetamol cysteine; d = paracetamol; e = N-propionyl-*p*-aminophenol (internal standard for blood analysis); f = paracetamol mercapturide.

TABLE I

HPLC RETENTION DATA FOR PARACETAMOL AND ITS METABOLITES, IN ORDER OF INCREASING RETENTION, USING ELUENT 1

Eluent 1 = isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (17:1000:1, v/v/v).

Compound	*Capacity ratio (k')*
Paracetamol glucuronide	0.7
Paracetamol sulphate	2.1
Paracetamol cysteine	2.3
Paracetamol	3.2
N-propionyl-*p*-aminophenol*	7.9
Paracetamol mercapturide	8.6

* Internal standard used for the quantification of paracetamol in blood.

All the other analgesic drugs considered in the present study gave capacity ratios (k') greater tha 10 with eluent 1 except for amidopyrine which gave a broad tailing peak at the same retention time as paracetamol mercapturide. Two further isocratic eluents containing higher proportions of isopropanol were found to be necessary to elute the other compounds with satisfactory retention properties. These eluents were prepared from the same components as eluent 1 but with 15% and 35%

TABLE II

HPLC RETENTION DATA FOR ANALGESIC DRUGS, IN ORDER OF INCREASING RETENTION, USING ELUENT 2

Eluent 2 = isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (176:1000:1, v/v/v).

Compound	*Capacity ratio (k')*
Amidopyrine	0.32*
Paracetamol	0.32
Dipyrone	0.45*
Nifenazone	0.45*
Phenazone	0.95*
Morazone	2.05*
Acetanilide	2.3
Salicylamide	2.5
Acetylsalicylic acid	2.7
Phenacetin	4.4
Etenzamide	4.6
Salicylic acid	4.6
Choline salicylate	4.8
Glycol salicylate	7.3
Piroxicam	7.7
Propyphenazone	11.0
Benorylate	22.4

* Tailing peaks.

TABLE III

HPLC RETENTION DATA FOR ANALGESIC DRUGS, IN ORDER OF INCREASING RETENTION, USING ELUENT 3

Eluent 3 = isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (540:1000:1, v/v/v).

Compound	*Capacity ratio (k')*	*Compound*	*Capacity ratio (k')*
Dipyrone	0.1	Bufexamac	1.95
Nifenazone	0.1	Oxyphenbutazone	1.95
Paracetamol	0.1	Tolmetin	2.05
Phenazone	0.1	Ketoprofen	2.4
Amidopyrine	0.2	Famprofazone	2.5
Morazone	0.4	Alclofenac	2.6
Salicylamide	0.4	Naproxen	3.3
Acetanilide	0.5	Salsalate	3.6
Acetylsalicylic acid	0.5	Zomepirac	3.7
Etenzamide	0.55	Methyl salicylate	3.9
5-Hydroxyindoprofen	0.6	Fenbufen	4.0
Phenacetin	0.6	Diflunisal	4.1
Piroxicam	0.6	Phenylbutazone	6.5
Benorylate	0.7	Indomethacin	6.95
Choline salicylate	0.7	Fenoprofen	7.9
6-Hydroxyindoprofen	0.7	Benoxaprofen	11.3
Salicylic acid	0.7	Diclofenac	11.5
Glycol salicylate	1.0	Ibuprofen	15.1
Indoprofen	1.2	Phenyl salicylate	15.6
Propyphenazone	1.25	Flufenamic acid	19.7
Sulindac	1.25	Mefenamic acid	21.2

isopropanol for eluents 2 and 3 respectively. Capacity ratios for the drugs and metabolites with these two eluents are presented in Tables II and III in order of increasing retention. The complete collection of retention data is given in Table IV where the compounds are listed in alphabetical order allowing the rapid selection of the appropriate eluent for a given drug.

The selection of eluents 2 and 3 followed a series of experiments to examine the influence of isopropanol concentration on chromatographic separation. Fig. 2 shows the results for eight drugs selected to be representative of those having short to intermediate retention. Overall the effect is as expected, with k' values decreasing with increasing isopropanol concentration. Where data are available all compounds eluted in the same order at different isopropanol concentrations except for 5-hydroxyindoprofen, 6-hydroxyindoprofen and salicylic acid which show changes between 25% and 30% organic modifier and for sulindac and oxyphenbutazone in the same region.

Generally all the compounds examined using eluents 2 and 3 gave satisfactory peak shapes except for amidopyrine, dipyrone, morazone, nifenazone and phenazone which showed slight tailing. Fig. 3 shows the separation of a standard mixture of six compounds using eluent 2 while the separation of a standard mixture of twelve analgesics using eluent 3 is shown in Fig. 4.

TABLE IV

HPLC RETENTION DATA FOR ANALGESIC DRUGS, ARRANGED IN ALPHABETICAL ORDER

Eluents: isopropanol–potassium dihydrogen phosphate (0.1 *M*)–formic acid (x : 1000:1, v/v/v), x = 17, 176 and 540 ml for eluents 1, 2 and 3 respectively.

Compound	*Capacity ratio (k')*		
	Eluent 1	*Eluent 2*	*Eluent 3*
Acetanilide		2.3	0.5
Acetylsalicylic acid		2.7	0.5
Alclofenac			2.6
Amidopyrine	8.6	0.32	0.2
Benorylate		22.4	0.7
Benoxaprofen			11.3
Bufexamac			1.95
Choline salicylate		4.8	0.7
Diclofenac			11.5
Diflunisal			4.1
Dipyrone		0.45	0.1
Etenzamide		4.6	0.55
Famprofazone			2.5
Fenbufen			4.0
Fenoprofen			7.9
Flufenamic acid			19.7
Glycol salicylate		7.3	1.0
5-Hydroxyindoprofen			0.6
6-Hydroxyindoprofen			0.7
Ibuprofen			15.1
Indomethacin			6.95
Indoprofen			1.2
Ketoprofen			2.4
Mefenamic acid			21.2
Methyl salicylate			3.9
Morazone		2.05	0.4
Naproxen			3.3
Nifenazone		0.45	0.1
N-propionyl-*p*-aminophenol*	7.9		
Oxyphenbutazone			1.95
Paracetamol	3.2	0.32	0.1
Paracetamol cysteine	2.3		
Paracetamol glucuronide	0.7		
Paracetamol mercapturide	8.6		
Paracetamol sulphate	2.1		
Phenacetin		4.4	0.6
Phenazone		0.95	0.1
Phenylbutazone			6.5
Phenylsalicylate			15.6
Piroxicam		7.7	0.6
Propyphenazone		11.0	1.25
Salicylamide	14.0	2.5	0.4
Salicylic acid	21.6	4.6	0.7
Salsalate			3.6
Sulindac			1.25
Tolmetin			2.05
Zomepirac			3.7

* Internal standard used for the quantification of paracetamol in blood.

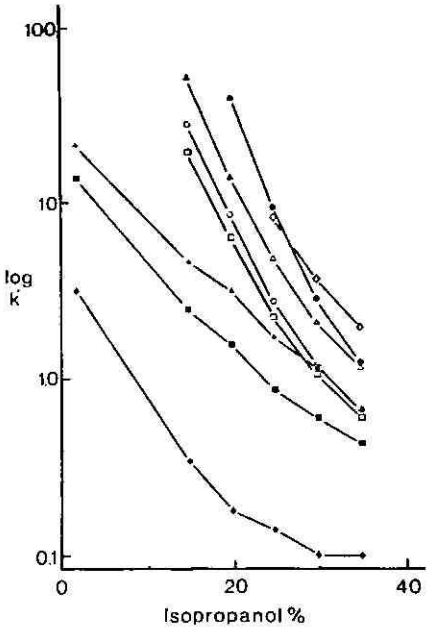

Fig. 2. The effect of isopropanol concentration on the retention of various analgesic drugs. Eluents: x% isopropanol containing a formic acid–phosphate buffer. Drugs: ◆ = paracetamol; ■ = salicylamide; ▲ = salicylic acid; ● = sulindac; ◇ = oxyphenbutazone; □ = 5-hydroxyindoprofen; ○ = 6-hydroxyindoprofen; △ = indoprofen.

Analysis of paracetamol in whole blood

The work of Buchanan *et al.*[13] describing one of the HPLC eluents adopted in the present study also describes a rapid sample preparation procedure for the quantification of paracetamol in human plasma. No information was presented in the original paper on the applicability of the method to the type of blood sample

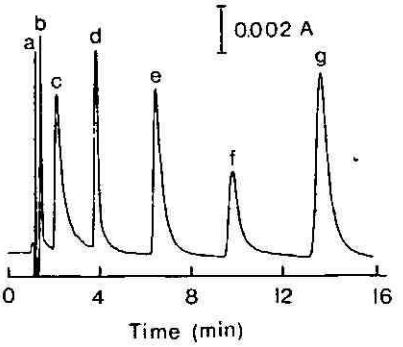

Fig. 3. Separation of analgesic drugs using eluent 2. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 15% isopropanol containing a formic acid–phosphate buffer; flow-rate: 1.5 ml/min; detection: 240 nm. Peaks: a = solvent disturbance; b = paracetamol; c = phenazone; d = salicylamide; e = salicylic acid; f = piroxicam; g = propyphenazone.

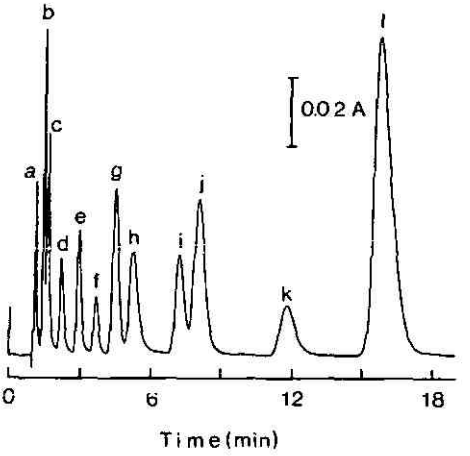

Fig. 4. Separation of analgesic drugs using eluent 3. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 35% isopropanol containing a formic acid–phosphate buffer; flow-rate: 1.5 ml/min; detection: 240 nm. Peaks: a = paracetamol; b = salicylamide; c = phenacetin; d = propyphenazone; e = oxyphenbutazone; f = alclofenac; g = salsalate; h = diflunisal; i = phenylbutazone; j = indomethacin; k = diclofenac; l = phenylsalicylate.

likely to be encountered in forensic toxicology where the isolation of plasma is often impossible because of haemolysis and/or putrefaction[14]. As an extension of the present study the applicability of eluent 1 for the analysis of paracetamol in whole blood using the approach of Buchanan *et al.*[13] has been examined.

The published procedure involved the addition of perchloric acid (0.33 *M*, 100 μl) to plasma (100 μl) causing the precipitation of protein which was removed by centrifugation at 9000 *g* before injection of the supernatant on to the HPLC column. Experiments showed that the replacement of plasma by whole blood gave incomplete precipitation and the method required minor modification involving an increase in the volume (200 μl) and concentration (0.49 *M*) of the perchloric acid. The modified method has been successfully tested on a wide range of different whole blood samples including specimens provided for the investigation of road traffic offences and also putrefying post mortem material. Fig. 1B shows a typical chromatogram arising from a fatal case involving paracetamol overdose. Interferences with the assay by endogenous compounds have not been encountered and the retention data presented in this paper for other analgesics indicate that no interferences occur from these drugs.

Quantification was performed by peak height ratio measurements of paracetamol relative to the internal standard (*N*-propionyl-*p*-aminophenol) which was added with the perchloric acid. Calibration graphs were constructed by conducting the assay with spiked swine serum samples. Using the assay procedure with external calibration, the recoveries of paracetamol added to water, swine serum and whole blood were measured as 97.2%, 102.7% and 91.7% respectively at 10 μg/ml and 102.5%, 105.1% and 88.0% respectively at 200 μg/ml. Similarly recoveries of the internal standard (100 μg/ml) were found to be 100.0%, 100.0% and 82.7% respectively. A linear relationship between the paracetamol–internal standard peak height ratio and the concentration of paracetamol in blood up to 250 μg/ml was demon-

strated for the method. In a collaborative study between 11 U.K. forensic science laboratories two samples of blood were spiked with paracetamol at 13.0 μg/ml and 235 μg/ml respectively. The results showed mean values of 13.3 μg/ml and 238.7 μg/ml with coefficients of variation of 16.8% and 5.61% respectively.

CONCLUSIONS

The present paper presents a series of three isocratic eluents on a single HPLC column (ODS-Hypersil) which are suitable for the analysis of a wide range of analgesic, anti-inflammatory drugs and should prove useful for the identification and quantification of these drugs in forensic science laboratories. A rapid sample preparation procedure for paracetamol analysis in whole blood has also been examined which can be used with one of the HPLC eluents. Similar protein precipitation procedures have been used for the analysis of other analgesic drugs in blood when concentrations are relatively high (*e.g.* aspirin and metabolites[15,16] and diflunisal[15]. However, experience has shown that some analgesic drugs give poor recoveries with such procedures and further work is required to explore alternative sample preparation procedures to facilitate the full exploitation of these HPLC systems for blood analysis.

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FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF SOME DICARBOXYLIC ACIDS PRESENT IN CONDENSATION-TYPE POLYMERS

II*. BIS(TRIMETHYLSILYL) ESTERS

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SUMMARY

Isothermal retention indices of some dicarboxylic acid bis(trimethylsilyl) esters at four temperatures of a non-polar capillary column with a cross-linked polymethylsiloxane stationary phase are presented. The separation of particular esters is discussed and the chromatographic behaviour of bis(trimethylsilyl) esters is compared with that of dimethyl esters. The characteristic features of electron impact and methane and isobutane chemical ionization mass spectra and their usefulness for the identification of the trimethylsilyl derivatives are discussed.

INTRODUCTION

In contrast with the use of methyl esters as convenient derivatives for the gas chromatography (GC) of carboxylic acids in condensation-type polymers after degradation of the polymer chain and derivatization of the liberated acids[1], trimethylsilyl (TMS) esters have been used in only a limited number of applications[2–5]. On the other hand, the silylation of aliphatic carboxylic acids is widely used in biochemical analyses[6], and TMS derivatives being chromatographed on non-polar capillary columns[7–9]. The isothermal[7] and methylene unit (MU)[8,9] retention indices for saturated and unsaturated aliphatic bis(TMS) esters have been published.

The reason for the wide use of TMS derivatives ensues not only from the versatility of the silylation reaction but also from the convenient character of the mass spectra of TMS esters. The presence of the ion $(M-15)^+$ in the electron impact

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(EI) mass spectrum of TMS derivatives enables the relative molecular mass of a compound to be determined without employing soft ionization methods[10–12].

This paper deals with the chromatographic behaviour of 23 dicarboxylic acid bis(TMS) esters in terms of retention indices with respect to the chemical structure of the particular compound. The dominant and characteristic ions in the EI and chemical ionization (CI) mass spectra are discussed with respect to their use for reliable identification.

EXPERIMENTAL

The TMS esters were prepared by silylation of individual acids with a 20-fold molar excess of BSTFA (Pierce, Rockford, IL, U.S.A.) in screw-capped vials with PTFE-lined silicone-rubber septa. The vials were heated in an aluminium block at 60°C for 30 min. The silylation of aromatic acids required a longer reaction time.

Gas chromatography

The GC experiments were performed on a Model 3700 gas chromatograph with a flame ionization detector (Varian Aerograph, Palo Alto, CA, U.S.A.) fitted with a 25 m × 0.20 mm I.D. Ultra 1 fused-silica capillary column (Hewlett-Packard, Palo Alto, CA, U.S.A.), with a film thickness of the polymethylsiloxane cross-linked stationary phase of 0.32 μm. The splitting ratio was 1:140 and the linear velocity of the carrier gas (nitrogen) was *ca.* 17 cm/s. To ensure that the column capacity was not exceeded, not more than 10 ng of each component were introduced into the column. The retention times were measured with an Autolab System IV (Spectra-Physics, Mountain View, CA, U.S.A.) with an accuracy of 0.1 s. The Kováts retention indices were calculated from the data obtained from the GC analyses where all the necessary components, *i.e.*, methane, *n*-alkanes and the studied TMS esters, were injected simultaneously. In this way a high reproducibility, even after operation of the capillary column for 1 year, was achieved. The standard deviations of the retention indices given in Table I are *ca.* 0.3 index unit.

Gas chromatography–mass spectrometry

Mass spectrometric data were obtained on a Varian-MAT 44S GC–MS system coupled to a Model 3700 gas chromatograph via an open split interface. The introduction of compounds into the mass spectrometer was performed through the same polarity Ultra 1 column (25 m × 0.32 mm I.D.), film thickness 0.52 μm, to verify the elution order of coeluting solutes. The quadrupole mass spectrometer was operated at an ion source pressure (helium carrier gas flow) of 10^{-3} Pa with an ionization energy of 70 eV (EI) and 210 eV (CI) and an ion source temperature of 160–170°C. The pressure of the CI reaction gas, methane or isobutane, in the CI ion box was 25 Pa. All EI and CI mass spectra were taken at the maximum of the overloaded chromatographic peak to ensure an approximately constant pressure of the measured compound vapour in the ion source during scanning.

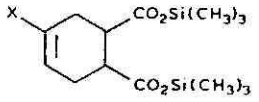
RESULTS AND DISCUSSION

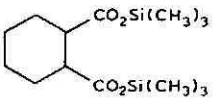
The studied TMS esters were divided into four classes according to the chem-

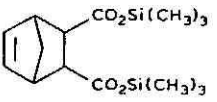
ical structure of the parent acid: aliphatic saturated (S), aliphatic unsaturated (U), aromatic (A) and alicyclic dicarboxylic acid bis(trimethylsilyl) esters:

(S2) $n = 0$
(S3) $n = 1$
(S4) $n = 2$
⋮ ⋮
(S12) $n = 10$

(U4Z) R = H (*Z*)
(U4E) R = H (*E*)
(U5Z) R = CH_3 (*Z*)
(U5E) R = CH_3 (*E*)
(U5I) isomer $-CH_2-C(=CH_2)-\ldots$







(THF) X = H
(MTHF) X = CH_3
(CTHF) X = Cl

(HHF)

(NAD)

Compounds A12, A13 and A14 are TMS phthalate, isophthalate and terephthalate, respectively.

Gas chromatography

The Kováts retention indices of the TMS esters, the capacity factors and the retention index dependence on temperature, dI/dT, are given in Table I.

As is apparent from the methylene retention index increments (Table II), the TMS esters of linear saturated alkanedioic acids do not form such an ideal homologous series as the *n*-alkanes and the values of methylene increments alternate strongly round the theoretical value 100 for the lowest homologues.

The homologous series of aliphatic saturated TMS esters can be efficiently separated by temperature-programmed analysis, 120°C for 2 min followed by an increase of 10°C/min up to 260°C, which ensures the separation of compounds with retention indices in the range 1000–2300 (Fig. 1).

The retention indices of aliphatic saturated TMS diesters are temperature dependent with negative values of dI/dT (*ca.* −0.22 for the studied compounds), which contrasts with the temperature-independent retention indices of long-chain aliphatic dimethylesters[13].

The introduction of a TMS group into the molecule of aliphatic unsaturated acids affected the chromatographic behaviour of these derivatives in comparison with the behaviour of dimethyl esters. TMS maleinate (U4Z) elutes before and TMS fumarate (U4E) elutes after the saturated analogue TMS succinate (S4). The values of ΔI for planar isomers, U4Z, U4E and U5Z, U5E, are *ca.* 50 index units (Table II)

TABLE I

KOVÁTS RETENTION INDICES, CAPACITY FACTORS AND dI/dT VALUES FOR DICARBOXYLIC ACID BIS(TRIMETHYLSILYL) ESTERS ON A NON-POLAR POLYMETHYLSILOXANE STATIONARY PHASE AT COLUMN TEMPERATURES OF 125, 150, 175 AND 200°C

TMS ester	*125°C*			*150°C*		
	I	*k'*	*dI/dT*	*I*	*k'*	*dI/dT*
S2	1105.0	1.64	−0.37	1095.4	0.73	−0.32
S3	1189.3	2.60	−0.24	1183.4	1.11	−0.22
S4	1302.1	4.78	−0.24	1296.3	1.89	−0.22
S5	1394.2	7.83	−0.26	1387.6	2.90	−0.24
S6	1498.6	13.59	−0.25	1492.2	4.70	−0.24
S7	1599.2	23.51	−0.27	1592.6	7.50	−0.24
S8	1695.1	38.56	−0.23	1689.5	11.71	−0.22
S9	–	–	–		–	–
S10	–	–	–	–	–	–
S11	–	–	–	–	–	–
U4Z	1288.2	4.38	−0.16	1284.6	1.80	−0.12
U4E	1341.8	5.86	−0.32	1333.4	2.26	−0.32
U5Z	1338.0	5.74	−0.15	1334.2	2.27	−0.13
U5E	1394.9	7.80	−0.33	1386.5	2.90	−0.33
U5I	1331.0	5.55	−0.19	1326.3	2.19	−0.19
THF	–	–	–	1594.7	7.63	+0.14
MTHF	–	–	–	1646.2	9.63	+0.07
CTHF	–	–	–	1746.5	15.38	+0.16
HHF	–	–	–	1602.9	7.89	+0.14
NAD	–	–	–	1655.4	10.09	+0.24
A12	–	–	–	1675.2	11.06	+0.10
A13	–	–	–	1744..8	15.30	–
A14	–	–	–	1784.0	18.19	+0.12

and the values ΔI for chain branching are similar for the *cis*-isomer pair U4Z, U5Z and the *trans*-isomer pair U4E, U5E.

Within the range 140–200°C the elution order of TMS fumarate (U4E), citraconate (U5Z) and itaconate (U5I) varies and for their complete separation a column with a more polar stationary phase should be used. The retention indices of *trans*-isomers show temperature dependence whereas with the *cis*-isomers this dependence is less apparent.

The elution order of aromatic TMS esters is TMS phthalate (A12), isophthalate (A13) and terephthalate (A14), with a high ΔI value between the particular isomers, which enables them to be separated on a relatively low-efficient packed column.

The temperature dependence of the retention indices of aromatic acid TMS diesters is relatively small in comparison with those of dimethyl esters, which have been shown to be strongly temperature dependent[13]. Owing to the different signs of dI/dT with aliphatic saturated (S) and aromatic (A) TMS esters, the coelution of TMS phthalate (A12) with TMS suberate (S8) at 193°C and TMS terephthalate with TMS azelate (S9) at 167°C occurs.The separation of alicyclic TMS esters on a non-

175°C			200°C		
I	*k'*	*dI/dT*	*I*	*k'*	*dI/dT*
1088.5	0.38	−0.30	1080.7	0.22	−0.26
1178.2	0.55	−0.22	1173.0	0.30	−0.20
1290.8	0.88	−0.22	1285.5	0.46	−0.22
1381.3	1.28	−0.23	1375.9	0.64	−0.22
1486.7	1.96	−0.22	1481.2	0.92	−0.21
1587.1	2.95	−0.22	1581.8	1.32	−0.20
1684.0	4.37	−0.20	1679.0	1.87	−0.20
1785.0	6.56	−0.20	1780.3	2.68	−0.20
1884.1	9.76	−0.21	1879.0	3.79	−0.20
1982.9	14.50	−0.20	1978.0	5.37	−0.19
1281.7	0.85	−0.10	1279.4	0.45	−0.09
1325.3	1.01	−0.32	1317.7	0.51	−0.32
1330.6	1.04	−0.12	1327.6	0.53	−0.11
1378.2	1.25	−0.33	1369.9	0.62	−0.33
1321.7	1.00	−0.19	1317.6	0.51	−0.19
1598.8	3.01	+0.14	1602.1	1.41	+0.14
1648.5	3.79	+0.10	1651.4	1.69	+0.12
1750.8	5.70	+0.22	1757.0	2.47	+0.26
1606.9	3.20	+0.17	1611.7	1.47	+0.21
1661.9	3.98	+0.26	1669.0	1.81	+0.28
1678.0	4.25	+0.12	1681.6	1.89	+0.15
1745.4	5.58	–	1745.1	2.37	–
1787.8	6.66	+0.13	1790.8	2.78	+0.13

polar capillary column is more favourable than the separation of the corresponding dimethyl esters because of the higher ΔI values for particular TMS esters. Moreover, the separation is performed with higher capacity factors and, therefore, at higher number of effective plates (Fig. 2).

As is obvious from the presented and published data[13], the chromatographic behaviour of the bis(TMS) esters and dimethylesters of dicarboxylic acids on a non-polar capillary column differs significantly. The introduction of the TMS group strongly affects the molecular structure of the compounds, which can be observed indirectly as a decrease in dI/dT with all the types of TMS esters studied relatively to the corresponding dimethyl esters. Further, the elution order of some solutes changes, *e.g.*, the elution of aromatic acid TMS esters on a non-polar capillary column follows the elution of higher *n*-alkyl esters[14]. The values of ΔI for the isomeric pairs fumarate–maleate and isophthalate–terephthalate increase and the separation of critical pair tetrahydrophthalate (THF)–hexahydrophthalate (HHF) is also possible (Fig. 2).

The ΔI values for two solutes for the bis(TMS) ester and dimethyl ester of the

TABLE II

RETENTION INDEX INCREMENTS FOR THE CH_2 GROUP IN THE HOMOLOGOUS SERIES OF ALIPHATIC SATURATED BIS(TMS) ESTERS, FOR CHAIN AND CYCLE BRANCHING, FOR PAIRS OF ISOMERS OF BIS(TMS) ESTERS OF DICARBOXYLIC ACIDS ON A NON-POLAR POLYMETHYLSILOXANE FUSED-SILICA CAPILLARY COLUMN AT 125, 150, 175 AND 200°C

TMS esters	*125°C*	*150°C*	*175°C*	*200°C*
S3–S2	84.3	88.0	89.7	92.3
S4–S3	112.8	112.9	112.6	112.5
S5–S4	92.1	91.3	90.5	90.4
S6–S5	104.4	104.6	105.4	105.3
S7–S6	100.6	100.4	100.4	100.6
S8–S7	95.9	96.9	96.9	97.4
S9–S8	–	–	101.0	101.1
S10–S9	–	–	99.1	98.7
S11–S10	–	–	98.8	99.0
U4E–U4Z	53.6	48.8	43.6	38.3
U5E–U5Z	56.9	52.3	47.6	42.3
U5Z–U4Z	49.8	49.6	48.9	48.2
U5E–U4E	53.1	53.1	52.9	52.2
THF–HHF	–	8.2	8.1	9.6
THF–MTHF	–	51.5	49.7	49.3
A13–A12	–	69.6	67.4	63.5
A14–A12	–	108.8	109.8	109.2
A14–A13	–	39.2	42.4	45.7

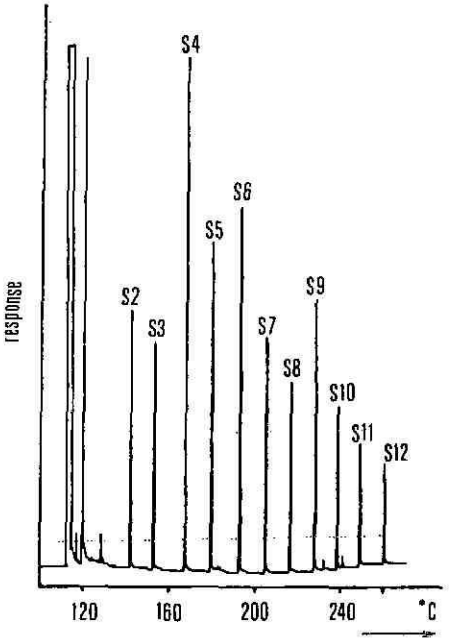


Fig. 1. Chromatogram of the separation of linear saturated dicarboxylic acid bis(trimethylsilyl) esters by temperature-programming (120°C for 2 min, increased at 10°C/min to 260°C) on an Ultra 1 non-polar fused-silica capillary column (25 m × 0.20 mm I.D.; 0.32 μm film thickness). S2 = bis(TMS) oxalate, S3 = bis(TMS) malonate, S4 = bis(TMS) succinate, ..., S12 = bis(TMS) dodecanedioate.

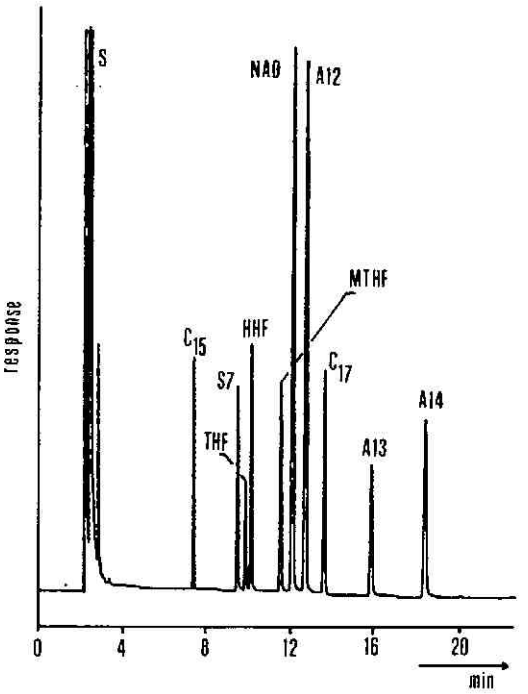


Fig. 2. Isothermal separation of bis(trimethylsilyl) esters of aromatic dicarboxylic acids (A12 = phthalate; A13 = isophthalate; A14 = terephthalate), aliphatic TMS ester (S7 = pimelate) and alicyclic bis(TMS) esters (THF = tetrahydrophthalate, HHF = hexahydrophthalate, MTHF = 4-methyltetrahydrophthalate, NAD = nadicate) at 183°C on an Ultra 1 non-polar fused-silica capillary column (25 m × 0.20 mm I.D.; 0.32 μm film thickness) in the region of C_{16}–C_{18} alkanes elution. S = BSTFA and its reaction products.

same acid on the same column at the same temperature (Table III) show that the increase in retention between these two derivatives, which ranges from 103.3 to 174.5 index units per ester group, is influenced by the type and the structure of the carboxylic acid. This retention index difference for two solutes can be used as a second identification parameter when two derivatization procedures, methylation and silylation, are used in the GC analysis of carboxylic acids in condensates.

Mass spectrometry

In contrast with dimethyl esters, the EI mass spectra of TMS esters show a very limited number of dominant ions. The main fragments at *m/z* 73, 75 and 147 indicate the presence of the TMS group in the molecule of the studied compound but do not state much about the character of the underivatized parent molecule. On the other hand, the EI mass spectra of TMS esters consist of a large number of low-intensity ions and it is possible to use them in the discussion of the identity of the derivatives.

In the upper of the part EI mass spectra of aliphatic saturated (S) TMS esters (Table IV), the characteristic fragment of TMS derivatives $(M-15)^+$ of medium intensity is present, enabling the relative molecular mass of the ester to be determined.

TABLE III

RETENTION INDEX INCREMENTS FOR TWO SOLUTES, DICARBOXYLIC ACID BIS(TRIMETHYLSILYL) ESTERS AND DIMETHYL ESTERS ON A NON-POLAR POLYMETHYLSILOXANE FUSED-SILICA CAPILLARY COLUMN AT 125, 150, 175 AND 200°C

Ester	*125°C*	*150°C*	*175°C*	*200°C*
S3	305.3	301.9	–	–
S4	306.9	302.6	–	–
S5	294.5	288.8	–	–
S6	293.2	288.4	284.9	–
S7	291.2	285.5	280.4	–
S8	284.8	279.9	274.7	269.7
S9	–	–	273.9	268.7
S10	–	–	271.2	265.5
S11	–	–	269.3	264.0
U4Z	308.9	306.3	–	–
U4E	349.0	340.5	–	–
U5Z	281.6	278.8	–	–
U5E	310.3	301.8	–	–
U5I	272.0	269.5	–	–
THF	233.0	229.5	226.1	221.1
MTHF	–	216.9	212.8	209.2
CTHF	–	216.4	211.2	206.6
HHF	–	236.6	232.3	227.7
NAD	236.8	232.7	228.1	224.1
A12	263.3	257.3	251.2	244.4
A13	–	264.5	256.9	246.3
A14	–	313.9	307.3	298.7

TABLE IV

DOMINANT AND CHARACTERISTIC IONS IN THE EI MASS SPECTRA OF DICARBOXYLIC ACID BIS(TRIMETHYLSILYL) ESTERS FOR m/z $\geqslant$ 50 WITH THEIR RELATIVE INTENSITIES AT AN ION SOURCE TEMPERATURE OF 170°C, MOLECULAR FORMULA AND RELATIVE MOLECULAR MASS OF THE ESTER

Ester	*Ions (m/z)*
S3, M = 248, $C_9H_{20}O_4Si_2$	59(6), 72(8), 73(84), 74(9), 75(27), 117(2), 147(100), 148(16), 149(8), 233(10)
S4, M = 262, $C_{10}H_{22}O_4Si_2$	55(27), 56(21), 57(5), 59(6), 61(6), 72(8), 73(100), 74(14), 75(46), 77(6), 116(3), 117(2), 129(5), 147(93), 148(14), 149(8), 172(3), 173(2), 247(4)
S5, M = 276, $C_{11}H_{24}O_4Si_2$	55(77), 59(5), 61(7), 72(12), 73(100), 74(12), 75(51), 76(4), 97(15), 116(11), 117(6), 129(11), 147(81), 148(14), 149(9), 158(18), 159(5), 204(6), 233(5), 261(23)
S6, M = 290, $C_{12}H_{26}O_4Si_2$	55(57), 56(7), 59(5), 61(8), 67(5), 69(6), 72(5), 73(100), 74(11), 75(82), 76(5), 77(8), 83(19), 111(54), 117(10), 129(7), 141(20), 147(24), 149(4), 159(8), 172(11), 217(3), 275(11)
S7, M = 304, $C_{13}H_{28}O_4Si_2$	55(52), 56(4), 59(5), 61(9), 69(35), 72(8), 73(100), 74(14), 75(89), 76(6), 77(5), 79(8), 81(10), 83(7), 97(13), 116(6), 117(11), 125(23), 129(9), 147(17), 149(5), 155(26), 173(12), 204(3), 217(4), 289(11)

TABLE IV (*continued*)

Ester	*Ions (m/z)*
S8, M = 318, $C_{14}H_{30}O_4Si_2$	55(62), 56(6), 59(5), 61(11), 67(8), 69(15), 72(7), 73(100), 74(14), 75(96), 76(7), 77(8), 79(4), 83(15), 93(9), 95(10), 97(9), 111(4), 117(13), 129(13), 138(7), 139(10), 147(10), 149(10), 169(13), 187(14), 204(5), 217(5), 303(16)
S9, M = 332, $C_{15}H_{32}O_4Si_2$	55(61), 56(5), 59(5), 61(9), 67(12), 69(8), 72(8), 73(100), 74(13), 75(91), 76(6), 77(5), 79(7), 81(8), 83(11), 93(5), 95(8), 97(8), 107(6), 111(6), 117(16), 129(15), 147(10), 149(13), 152(11), 201(15), 204(7), 217(5), 317(17)
S10, M = 346, $C_{16}H_{34}O_4Si_2$	55(49), 56(6), 57(6), 59(4), 61(8), 67(7), 69(19), 72(7), 73(100), 74(13), 75(91), 76(6), 77(5), 79(7), 81(9), 83(6), 93(7), 95(5), 97(10), 107(5), 116(5), 117(17), 125(5), 129(17), 138(4), 147(9), 149(11), 204(8), 215(14), 331(20)
S11, M = 360, $C_{17}H_{36}O_4Si_2$	55(44), 56(4), 57(8), 59(5), 61(7), 67(10), 69(24), 72(6), 73(100), 74(12), 75(83), 76(7), 77(7), 79(6), 81(8), 83(10), 93(7), 95(6), 97(8), 98(5), 107(3), 111(3), 116(5), 117(18), 129(15), 139(5), 147(8), 149(10), 152(4), 204(12), 217(11), 229(17), 345(20)
S12, M = 374, $C_{18}H_{38}O_4Si_2$	55(42), 56(5), 57(7), 59(5), 61(7), 67(11), 69(19), 72(7), 73(100), 74(11), 75(85), 76(6), 77(7), 81(11), 83(15), 93(8), 95(7), 97(8), 98(6), 111(4), 116(4), 117(22), 129(19), 147(8), 149(12), 204(19), 217(15), 243(15), 359(28)
U4Z, M = 260, $C_{10}H_{20}O_4Si_2$	54(4), 55(3), 58(4), 59(6), 61(2), 66(5), 72(3), 73(89), 74(8), 75(22), 83(4), 115(6), 126(2), 133(4), 143(3), 147(100), 148(17), 149(9), 170(3), 215(2), 245(21), 246(5)
U4E, M = 260, $C_{10}H_{20}O_4Si_2$	53(6), 54(3), 55(3), 58(5), 59(12), 61(5), 73(74), 74(7), 75(34), 83(6), 84(4), 115(8), 133(6), 143(15), 144(2), 147(31), 148(5), 149(5), 155(3), 157(2), 217(3), 245(100), 246(22), 247(10)
U5E, M = 274, $C_{11}H_{22}O_4Si_2$	59(4), 61(3), 67(11), 73(100), 74(8), 75(24), 83(2), 97(6), 112(2), 122(4), 133(3), 141(2), 147(38), 148(6), 149(5), 157(2), 169(2), 184(37), 185(6), 259(35), 260(9), 261(4)
U5I, M = 274, $C_{11}H_{22}O_4Si_2$	58(3), 59(6), 61(3), 67(6), 68(6), 72(5), 73(100), 74(8), 75(20), 83(3), 97(3), 98(2), 117(2), 122(2), 131(2), 133(5), 147(78), 148(14), 149(8), 215(6), 230(5), 259(12), 260(3)
THF, M = 314, $C_{14}H_{26}O_4Si_2$	59(4), 73(100), 74(9), 75(17), 77(5), 78(16), 79(8), 103(2), 107(2), 117(6), 118(2), 133(2), 147(28), 148(4), 149(3), 152(11), 153(2), 181(1), 196(7), 224(8), 225(2), 299(8), 300(2), parent 0.52
MTHF, M = 328, $C_{15}H_{28}O_4Si_2$	59(4), 61(2), 72(3), 73(100), 74(9), 75(19), 77(5), 91(5), 92(47), 93(19), 103(2), 117(6), 147(21), 148(3), 149(3), 166(8), 210(9), 211(2), 238(3), 313(5), parent 0.93
CTHF, M = 348, $C_{14}H_{25}ClO_4Si_2$	59(3), 61(2), 72(3), 73(100), 74(9), 75(19), 77(6), 78(36), 79(5), 93(2), 103(2), 112(2), 117(5), 118(2), 133(1), 147(26), 148(4), 149(2), 186(3), 188(1), 230(3), 232(1), 258(3), 260(1), 333(4), 335(1), parent 0.60
HHF, M = 316, $C_{14}H_{28}O_4Si_2$	53(4), 54(3), 55(8), 59(4), 61(3), 67(3), 72(4), 73(100), 74(9), 75(25), 79(5), 80(6), 81(31), 82(7), 108(2), 109(5), 110(4), 117(6), 133(3), 147(51), 148(9), 149(6), 154(4), 183(6), 198(9), 204(2), 217(2), 226(10), 227(3), 243(2), 257(2), 272(3), 301(12), 302(3), parent 1.58
NAD, M = 326, $C_{15}H_{26}O_4Si_2$	55(3), 59(3), 61(2), 65(6), 66(46), 67(3), 72(4), 73(100), 74(9), 75(21), 83(1), 91(8), 92(3), 99(2), 115(5), 118(4), 119(17), 120(3), 133(2), 147(24), 148(4), 149(3), 171(25), 172(3), 193(4), 208(4), 209(20), 210(4), 236(12), 237(2), 245(23), 246(5), 261(6), 311(1), parent 0.70
A12, M = 310, $C_{14}H_{22}O_4Si_2$	59(3), 61(1), 72(3), 73(80), 74(8), 75(9), 76(8), 77(4), 89(2), 91(3), 103(2), 104(2), 105(5), 119(3), 126(2), 131(2), 133(3), 135(2), 140(7), 147(100), 148(17), 149(9), 163(3), 193(2), 220(4), 221(7), 295(15), 296(4), parent 2.86
A13, M = 310, $C_{14}H_{22}O_4Si_2$	59(3), 61(2), 65(2), 72(3), 73(100), 74(10), 75(23), 76(29), 77(5), 89(5), 90(2), 91(4), 103(25), 104(17), 105(4), 118(3), 119(4), 121(2), 126(3), 133(4), 135(6), 140(17), 141(2), 147(4), 149(2), 177(6), 178(2), 193(3), 205(12), 206(2), 207(8), 221(13), 222(2), 251(3), 279(14), 280(2), 295(74), 296(17), 309(2), parent 7.51
A14, M = 310, $C_{14}H_{22}O_4Si_2$	59(3), 61(2), 72(2), 73(56), 74(6), 75(23), 76(31), 77(4), 89(7), 90(2), 91(3), 103(63), 104(30), 105(4), 118(4), 119(2), 126(3), 133(3), 134(3), 135(7), 140(19), 147(1), 149(2), 177(3), 178(6), 192(2), 193(5), 221(30), 222(5), 223(2), 251(17), 252(4), 294(2), 295(100), 296(25), 297(10), parent 7.74

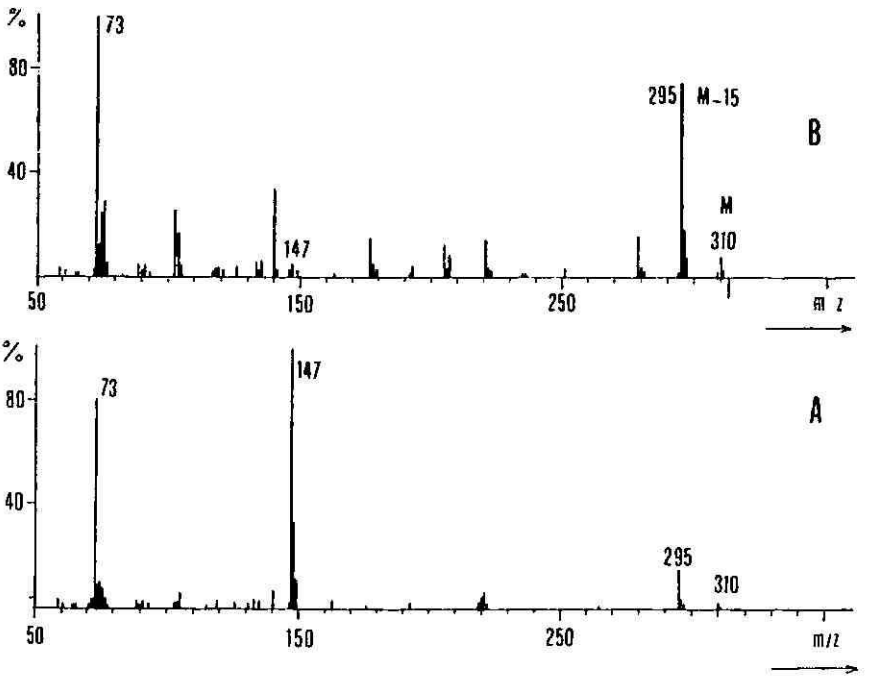


Fig. 3. Influence of the *ortho* effect on the molecular ion fragmentation on differences between the mass spectra of substituted isomers. The relative intensities of the ions at *m/z* 147 and 295 in the EI mass spectrum of bis(TMS) phthalate (A) are reversed in the mass spectrum of the *meta* isomer, bis(TMS) isophthalate (B).

The other ions present, $(M-131)^+$ and $(M-179)^+$ (S5–S8), resemble the ions $(M-73)^+$ and $(M-63)^+$ found in the EI mass spectra of aliphatic saturated dimethyl esters. In the lower part of the spectrum ions are apparent at *m/z* 55, 59, 61, 72, 74, 76, 77 and 117 and with the long-chain homologues ions are present at *m/z* 79, 81, 83, 95, 97, 129, 204 and 217.

The ions $(M-15)^+$ in the EI mass spectra of aliphatic unsaturated (U) TMS esters have medium intensity. Owing to a convenient planar *cis* configuration[11] of TMS maleate, the ion at *m/z* 147, originating from the ion $(M-15)^+$, thus constitutes the base peak of the spectrum.

The influence of the *ortho* position on the formation of the ion at *m/z* 147 from the ion $(M-15)^+$ is apparent in the EI mass spectra of isomeric TMS phthalate esters. The structure of the *ortho* isomer of TMS phthalate enables the facile elimination of the ion at *m/z* 147 that forms the base peak of the spectrum. With TMS isophthalate (A13) and terephthalate (A14) the ion at *m/z* 147 is of low intensity. This phenomenon supports the opinion[15] that the ion $(M-15)^+$ with *cis* and *ortho* isomers seems to have the form of a cyclic silyloxonium ion and the elimination of the ion at *m/z* 147 with two silicone atoms is possible. In TMS isophthalate and terephthalate, where the rigid molecular structure does not permit the formation of a cyclic ion, the ion at *m/z* 147 is of low intensity and the ion $(M-15)^+$ forms the dominant peak of the spectrum (Fig. 3).

In addition to the common ions at *m/z* 73, 75 and 147 and $(M-15)^+$ ions, the ions $(M-118)^{+\cdot}$ and $(M-90)^{+\cdot}$ are also present in the EI mass spectra of alicyclic 1,2-bis(TMS) esters. These ions may be considered as analogous to the ions $(M-60)^{+\cdot}$ and $(M-32)^{+\cdot}$ in the EI spectra of alicyclic dimethyl esters, assuming that the fragmentation routes of the TMS esters and dimethyl esters have some common features.

The influence of the *ortho* effect is obvious from the data in Table IV. The specific feature of the fragmentation of alicyclic 1,2-bis(TMS) esters is the origin of $(M-162)^{+\cdot}$ ions, corresponding in mass to positively charged anhydride ions, which have not been found in the mass spectra of alicyclic dimethyl esters[13].

Methane and isobutane mass spectra

The CI mass spectra of dicarboxylic acid TMS esters, monocarboxylic and tricarboxylic acid TMS esters and diol bis(TMS) ethers were measured in order to evaluate the ability of the CI method in both an individual and a group characterization of a compound. The dominant ions in the methane and isobutane CI mass

TABLE V

DOMINANT IONS IN THE METHANE CI MASS SPECTRA OF DICARBOXYLIC ACID BIS(TRIMETHYLSILYL) ESTERS AT AN ION SOURCE TEMPERATURE OF 160–170°C, A METHANE PRESSURE OF *ca.* 25 Pa AND AN ENERGY OF ELECTRONS OF 210 eV FOR $m/z \geq M-89$

Ester	*M−89*	*M−43*	*M−15*	*M−1*	*M*	*M+1*	*M+29*	*M+41*	*M+73*
S2	–	–	32	3	4	100	23	19	(34)
S3	8	–	31	–	3	100	18	14	(26)
S4	100	–	61	–	2	44	19	17	(43)
S5	100	–	80	–	3	42	35	27	(43)
S6	100	–	62	–	3	39	25	18	(30)
S7	100	–	61	3	3	43	23	15	(15)
S8	97	–	79	6	7	100	27	20	(8)
S9	80	–	65	7	8	100	24	15	(10)
S10	47	–	52	8	9	100	20	13	(6)
S11	44	1	53	11	13	100	20	14	(24)
S12	40	–	52	13	13	100	19	14	(20)
S14	42	4	47	18	15	100	17	13	(11)
U4Z	29	–	52	–	7	100	28	28	(24)
U4E	10	–	37	–	6	100	20	15	(61)
U5Z	83	–	100	–	7	100	36	37	(66)
U5E	25	–	40	–	11	100	19	14	(61)
U5I	84	–	55	–	4	79	24	16	(100)
THF	100	–	79	7	9	91	25	19	(60)
MTHF	100	–	49	3	9	67	10	4	(23)
CTHF	100	–	44	3	6	42	10	7	(7)
HHF	100	–	69	6	5	28	14	10	(7)
MHHF	100	–	46	3	6	39	11	8	(9)
NAD	100	–	88	7	21	93	29	35	(18)
A12	71	5	51	–	7	70	16	12	(100)
A13	15	7	52	–	9	100	19	14	(43)
A14	15	8	45	–	9	100	17	13	(32)

spectra are $(M-89)^+$, $(M-15)^+$ and $(M+1)^+$, the adduct ions resulting from the reaction between the alkane plasma and ester molecule, $(M+29)^+$, $(M+41)^+$ (methane) and $(M+41)^+$, $(M+43)^+$ (isobutane), and the ion $(M+73)^+$ (Table V).

The methane CI mass spectra show a greater fragmentation of the quasi-molecular ion $(M+H)^+$ compared with the lower energy isobutane CI mass spectra and with some esters (S4, S5, S6, S7, alicyclic esters) the quasi-molecular ion does not constitute the base peak of the spectrum. With increase in the number of CH_2 groups in the molecular of aliphatic saturated bis(TMS) esters, the intensity of the second quasi-molecular ion $(M-H)^+$ gradually increases. In the isobutane CI mass spectra the quasi-molecular ion $(M+1)^+$ forms the base peak of the spectrum.

The most striking feature of the CI mass spectra of TMS esters is the presence of the ion $(M+73)^+$, the intensity of which varies considerably with the vapour pressure of measured compound, the pressure of the chemical ionization reaction gas in the ionization box, the temperature of the ionization box and the structure of the studied ester. The relative mass of an $(M+73)^+$ ion corresponds to the relative molecular mass of a tris(TMS) derivative of a dicarboxylic acid and this ion is assumed to be the product of persilylation of one ester group via an intermolecular transfer of a TMS group. This phenomenon has also been observed in the mass spectra of monocarboxylic and tricarboxylic acid TMS esters (Fig. 4), but not with alkanediol bis(TMS) ethers.

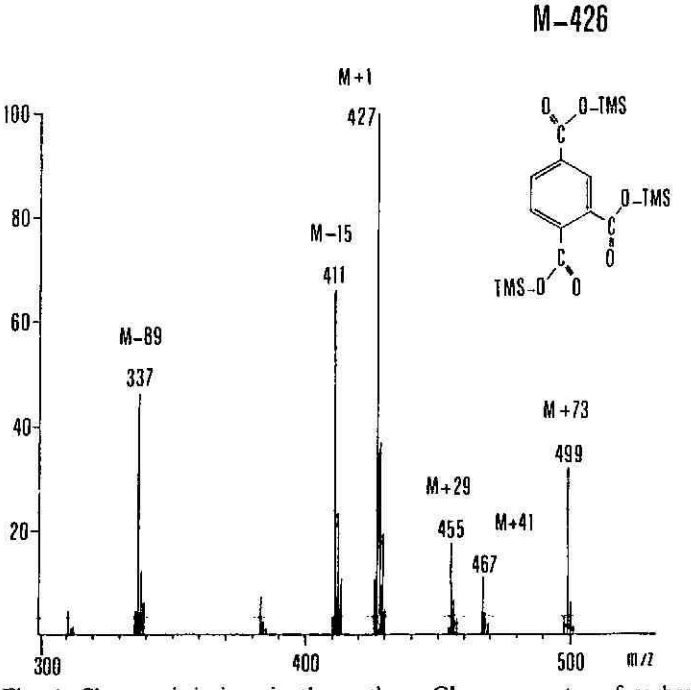


Fig. 4. Characteristic ions in the methane CI mass spectra of carboxylic acid trimethylsilyl esters, $(M-89)^+$, $(M-15)^+$, $(M+1)^+$, $(M+29)^+$, $(M+41)^+$ and $(M+73)^+$, in the methane CI mass spectrum of tris(TMS) trimellitate ($C_{18}H_{30}O_6Si_3$), relative molecular mass 426.

CONCLUSION

The TMS derivatives of dicarboxylic acids have been characterized with the aid of capillary GC and low-resolution MS. The data obtained together with the application of a GC–MS method permit the rigorous identification of polycarboxylic acids in condensation-type polymers and form a basis for developing methods of quantitative analysis.

From the point of view of the separation of isomers and critical pairs on a non-polar capillary column, TMS derivatives seem to be more convenient derivatives than methyl esters.

The EI mass spectra can be used for the reliable identification of the four types of bis(TMS) esters described here when a low-resolution mass spectrometer is connected to a gas chromatograph.

CI mass spectrometry has proved to be a versatile method not only for the identification of a particular derivative but also for a quick search of a chromatogram, which often consists of a large number of component peaks, resulting from the hydrolysis of polymers and derivatization of the liberated monomers, *e.g.*, solvents, blank components, derivatized and underivatized compounds. From the analytical point of view the presence of the $(M+73)^+$ ion indicates that the sample compound is a TMS ester and can serve as supporting evidence for the determination of the relative molecular mass of a compound.

The use of TMS derivatives in the analysis of carboxylic acids in condensation-type polymers is important in connection with the limitations of methods of acid- and base-catalysed condensate alcoholysis[16] and with the fact that effective methods for the quantitative hydrolysis of polymer and isolation of acids are continuously being developed[17].

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STEPWISE GRADIENT DEVELOPMENT IN THIN-LAYER CHROMATOGRAPHY

III*. A COMPUTER PROGRAM FOR THE SIMULATION OF STEPWISE GRADIENT ELUTION

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SUMMARY

A general equation for the final R_F value of a solute chromatographed under conditions of stepwise gradient elution with one void volume of mobile phase has been derived. The elution process and the distance travelled by the spot as a function of eluent volume are illustrated graphically for retention–eluent composition relationships typical of a displacement adsorption mechanism or for reversed-phase chromatography. A computer program (in BASIC) is given for the simulation of stepwise gradient thin-layer chromatography. The program can be used for the optimization of stepwise gradient programs by computer simulation of the elution process.

INTRODUCTION

In Parts I[1] and II[2] it was demonstrated that a sandwich chamber with a glass distributer[3,4] simplifies the use of continuous [5,6] and stepwise[2,6] gradient elution in thin-layer chromatography (TLC), as the eluent is delivered to the layer by a capillary siphon from a small container, or is introduced directly, in small portions, under the distributer[1,2,5,6]. Qualitative rules for the modification of the gradient profile were formulated in Part I[1]; however, it would be advantageous to have a mathematical model of the process involved.

Numerous mathematical considerations concerning gradient elution have been mostly restricted to continuous column chromatography[7–10]. Several workers[11–17] considered the movement of zones under conditions of stepwise elution and derived corresponding equations that took into account the delayed overtaking of the solute band by the consecutive zones of increased concentration of the modifier. In this paper analogous considerations are applied to the migration of consecutive zones of the mobile phase and the corresponding migration of the solute band in TLC where

* For Part II, see ref. 2.

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the elution stops when the front of the mobile phase has reached the far end of the plate. The mathematical relationship between the R_F values of solutes, the applied gradient program and the retention–eluent composition relationships involved are presented as a computer program that permits the study of the chromatographic process and the resulting separation of the components of the sample on the computer screen.

THEORETICAL

Gradient elution is used when the sample to be chromatographed contains components with a wide range of retention parameters. Consider a twenty-component mixture with capacity factors k' of the components forming a geometrical progression, the divisor being equal to 2 [$k'(j) = 0.5k'\ (j - 1)$] and exponentially dependent on the modifier concentration (molar or volume fraction c), in accordance with the Snyder–Soczewiński model of adsorption[16–22]:

$$a(j) = 25.6/2^j \text{ (solute No. } j = 1,2, \ldots, 20) \tag{1}$$

$$\log k'(j) = \log a(j) - m \log c;\ k'(j) = a(j)c^{-m};\ R_{F(j)} = 1/[1 + a(j)c^{-m}] \tag{2}$$

where $a(j) = k'(j)_{\text{mod}}$ for $c = 1.0$ (pure modifier).

The log k' *vs.* log c plots of the twenty solutes are given in Fig. 1, which has

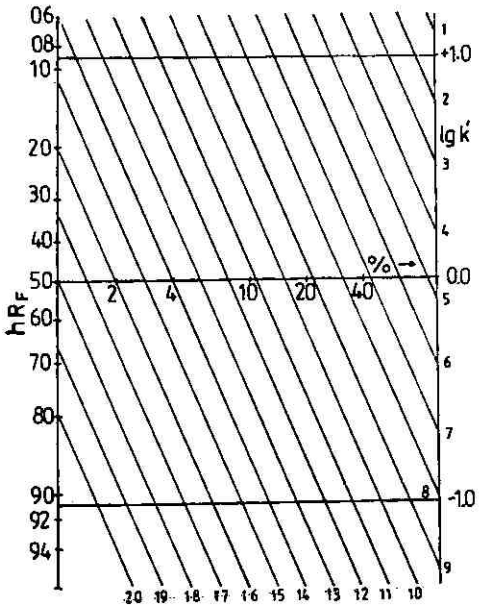


Fig. 1. Family of linear log k' *vs.* log c_{mod} plots for hypothetical solutes 1–20 with capacity factors forming a geometrical progression according to eqns. 1 and 2); slope = −2. For isocratic elution only ten solutes give R_F values in the range 0.04–0.96 (left-hand ordinate).

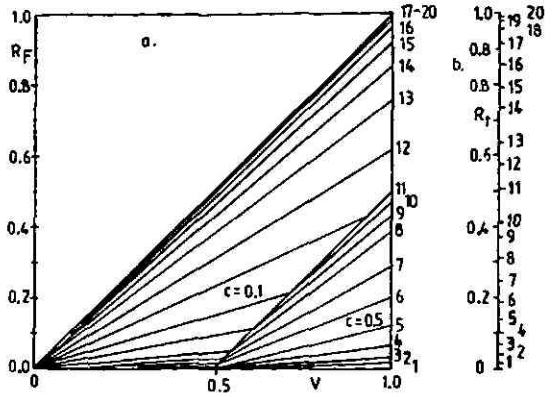


Fig. 2. (a) Two-step development of the hypothetical mixture (Fig. 1). Lines of unit slope: migration of the fronts of the first ($c = 0.1$) and second ($c = 0.5$) zones of the stepwise gradient; the remaining lines represent the migration of the individual compounds of the mixture, accelerated in the second zone. Solutes 9–11 are accumulated near the front of the more concentrated mobile phase. (b) R_F values of the hypothetical series of solutes after five-step development ($c = 0.05, 0.1, 0.2, 0.5, 1.0$).

a parallel R_F axis subordinated to the right-hand-side log k' axis; $m = 2$. It can be seen that no isocratic eluent can separate all the components: pure modifier ($c = 1.0$) separates well solutes 1–7 and the less polar solutes are accumulated near the solvent front; for $c = 0.1$ (10%), solutes 7–14 are well separated, the remaining ones being accumulated either near the start line or the front line; for $c = 0.02$ (2%), solutes 1–10 are accumulated on the start line. Thus, only half of the components can be satisfactorily separated by isocratic elution.

The separation can be greatly improved already by two-step gradient elution[23,24] (Fig. 2). However, the distribution of spots along the chromatogram is uneven, some spots being accumulated near the front of the more concentrated eluent

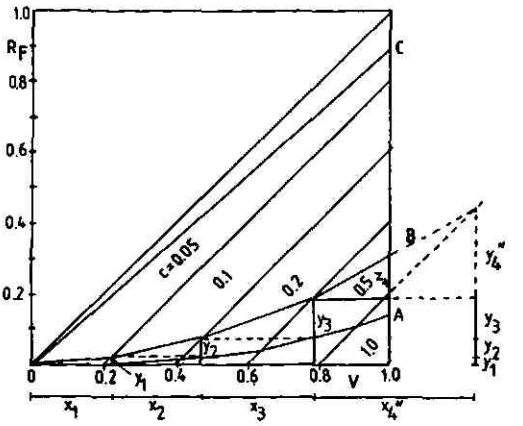


Fig. 3. Five-step development, equal volumes of the eluent fraction (0.2 of the void volume). Lines of unit slope: fronts of consecutive concentration zones. Exponential gradient program: 0.05, 0.1, 0.2, 0.5, 1.0; retention *vs.* modifier concentration relationships corresponding to eqn. 2 for $m = 1$.

owing to the steep gradient of elution strength. In the case illustrated in Fig. 2, the mixture (Fig. 1) was eluted to half the length of the plate with 10% modifier and the elution continued with 50% modifier ($c = 0.5$).

A still better distribution of spots can be obtained with multi-step gradients and especially with continuous gradient programs. In earlier papers[1,2,5,6], 8–10-step or continuous gradients were used; to find a compromise between the smoothness of the gradient profile and simplicity of the elution procedure, a five-step gradient seems to be the most convenient. As will be demonstrated below, such a profile does not cause accumulation of spots near the boundaries of the consecutive concentration zones. Such a process will be considered theoretically and then generalized.

Fig. 3 represents the migration of the fronts and zones of a mobile phase composed of five fractions of eluent of exponentially increasing concentration of the modifier (five-step gradient). Equal volumes ($v = 0.2$) of the five fractions are applied so that the consecutive fronts of the concentration zones are lower by 0.2 unit relative to the preceding ones. The ordinate axis represents the migration along the TLC plate and the abscissa the volume of eluent absorbed by the layer; the void volume of the layer is assumed to be equal to 1.

Consider the migration of the spot of solute B from the start line. The spot migrates in the first zone of eluent longer than would be expected from $v(1) = 0.2$, as the front of the next concentration zone must first overtake it[12,14,15]; the spot migrates a distances $y(1)$ in the first zone and the corresponding volume of mobile phase, $x(1)$, is equal to $0.2 + y(1)$. The coordinates of the first intersection point $[x(1), y(1)]$ can be calculated by analytical geometry from the equation of the migration of the spot [$y = R_{F(1)} \cdot x(1)$, where $R_{F(1)}$ is the R_F value of solute in the first concentration zone] and that of the front of the second concentration zone [$y(1) = x(1) - 0.2$]. The solution gives

$$x(1) = \frac{0.2}{1 - R_{F(1)}} \text{ and } y(1) = \frac{0.2\ R_{F(1)}}{1 - R_{F(1)}} = \frac{0.2}{k'(1)} \tag{3}$$

where $y(1)$ is the fractional ΔR_F value of the spot travelled in the first concentration zone of the mobile phase. It is evident from Fig. 3 that

$$x(1) = 0.2 + y(1) \tag{3a}$$

The discussion is repeated for the second concentration zone, in which the R_F value of the solute is $R_{F(2)}$. Transferring the origin of coordinates to the former intersection point $[x(1), y(1)]$ we obtain $y(2) = 0.2/k'(2)$; the volume of mobile phase corresponding to this migration distance is equal to $x(2) = 0.2 + y(2) = 0.2/(1 - R_{F(2)})$ (see Fig. 3). The total distance (R_F) travelled by the solute spot in zones 1 and 2 is equal to $y(1) + y(2)$.

By analogous reasoning we obtain for the third concentration zone $y(3) = 0.2/k'(3)$ and $x(3) = 0.2 + y(3) = 0.2/(1 - R_{F(3)})$. After migration in three gradient zones, the R_F value is $y(1) + y(2) + y(3)$, which corresponds to absorption by the layer of eluent fractions with a total volume $x(1) + x(2) + x(3) = 0.6 + y(1) + y(2) + y(3)$.

For single development of the layer $x = 1.0$. It can be seen from Fig. 3 that

the intersection of the solute migration line [slope = $R_{F(4)}$] in zone No. 4 with the migration line of the front of zone No. 5 occurs beyond the area of the diagram so that the calculated $y(4)$ value would be fictitious, and could be obtained only by continued elution with the last eluent fraction until $x = x(1) + x(2) + x(3) + x(4) > 1$. This fact requires the introduction of suitable corrections to the equation describing the migration of solute spot under stepwise gradient conditions.

It follows from the discussion of the case under considerations (Fig. 3, five consecutive eluent fractions equal to 0.2 of the total volume of the solvent in the layer) that the total volume of mobile phase corresponding to i migration steps is

$$x = \sum_i x(i) = \sum_i [0.2 + y(i)] = 0.2 \sum_i \frac{1}{1 - R_{F(i)}} \tag{4}$$

and the hypothetical R_F of a solute travelling through i zones is

$$y = R_F = \sum_i y(i) = 0.2 \sum_i 1/k'(i) \tag{5}$$

To find the real R_F value of solute it must be assumed that the last, say the hth, development step is incomplete:

$$\sum_{i=1}^{h-1} x(i) < 1, \text{ but } \sum_{i=1}^{h} x(i) > 1 \text{ and } y = R_F = 0.2 \sum_{i=1}^{h-1} 1/k'(i) + z(h) \tag{6}$$

and the real migration path $z(h)$ of the solute spot in the last (hth) incomplete stage is calculated from the proportion (see Fig. 3 for solute B, $h = 4$):

$$\frac{z(h)}{y(h)} = \frac{1 - \sum_{i=1}^{h-1} x(i)}{x(h)};$$

$$z(h) = \frac{1 - \sum_{i=1}^{h-1} x(i)}{1 + k'(h)} = R_F(h)\left[1 - \sum_{i=1}^{h-1} x(i)\right] \tag{7}$$

It should be mentioned that only the solutes of very high initial $k'(i)$ values [very low $R_{F(1)}$ values] migrate through all five zones of mobile phase concentrations, so that their final R_F is below 0.2 (Fig. 3, solute A). The number of zones through which the solute spot migrates also depends on the eluent volume fractions used, *i.e.*, the step lengths and heights of the concentration program (gradient steepness) and the slope of the log k' *vs.* log c plot (eqn. 2). On the other hand, a solute of high $R_{F(1)}$ value for the first concentration zone [in the case illustrated, when $R_{F(1)} > 0.8$] migrates all the time in the first zone and its R_F value is then not $1/k'$ (fictitious R_F) but $1/(1 + k')$, in accordance with the well known equation (see Fig. 3, solute C).

The equations can be generalized for a solute (j) for any number of steps (i)

and fractional volumes of the eluent, $v(i)$ [$\sum_i v(i) = 1$]. The total volume of mobile phase corresponding to $y(j)$ is

$$x(j) = \sum_{i=1}^{h} x(j,i) = \sum_{i=1}^{h} [v(i) + y(j,i)] = \sum_{i=1}^{h} \frac{v(i)}{1 - R_{F(j,i)}} \tag{8}$$

The actual final R_F value of solute j (considering that the last, hth, development step is incomplete) is

$$R_F = \sum_{i=1}^{h-1} y(j,h) + z(j,h) = \sum_{i=1}^{h-1} \frac{v(i)}{k'(j,i)} + z(j,h) \tag{9}$$

(for $\sum_{i=1}^{h-1} x(j,i) < 1$)

$$z(j,h) = y''(j,h) \frac{1 - \sum_{i=1}^{h-1} x(j,i)}{x''(j,h)} = \frac{1 - \sum_{i=1}^{h-1} x(j,i)}{1 + k'(j,h)}$$

$$= R_{F(j,h)} \left[1 - \sum_{i=1}^{h-1} x(j,i) \right] \tag{10}$$

(the double primes mean that the value is fictitious, *i.e.*, beyond the migration diagram).

The corresponding computer program for eqns. 9 and 10 in BASIC is given in Table I and can be used to analyse the paths of the series of solutes under stepwise gradient conditions for various parameters (slope of log k' *vs.* log c plots, eqn. 1), lengths [$v(i)$] and heights [$c(i)$] of the gradient program (for any number of steps n). In the authors' laboratory a ZX Spectrum + personal computer was used. The paths of the individual solutes from the series are shown on the monitor screen and the numerical values of the final R_F coefficients are printed. The program can be modified for other sets of solutes (other versions of eqn. 1) and retention–modifier concentration equations. For instance, for reversed-phase systems of the type octadecylsilica–water + methanol another type of retention–eluent composition relationship is frequently observed[8–10,21–22]:

$$\log k' = \log a - mc;\ k' = a \cdot 10^{-mc};\ R_F = \frac{1}{1 + a \cdot 10^{-mc}} \tag{11}$$

where c is the concentration of modifier (methanol) in volume fractions, a is the k'_w value for pure water as eluent (for $c = 0$) and the slope m is equal to $\log k'_w - \log k'_{mod} = \log k'_w/k'_{mod}$. For the hypothetical model mixture a similar geometrical progression of $a(j)$ values can be chosen, *e.g.*,

$$a(j) = \frac{2^{18}}{2^j} = \frac{262\,144}{2^j}\ (j = 1, 2, \ldots, 20) \tag{12}$$

TABLE I

COMPUTER PROGRAM FOR NUMERICAL AND GRAPHICAL REPRESENTATION OF STEPWISE GRADIENT ELUTION

The program is written in BASIC for operation on a Sinclair ZX Spectrum + microcomputer making use of all facilities of this version of BASIC. For this reason, adaptation to other microcomputers has not been taken into consideration. The program requires a screen monitor and a printer as peripherals. All data would be read from the keyboard and every reading is proceeded by a suitable explanatory text. An arbitrary number of concentration steps and an arbitrary number of solutes may be used for simulation. The volumes of portions of the eluent and their concentrations may be arbitrary. Only one subroutine for drawing the diagram is used. Coincidence of notations of variables in the program and the text was preserved if it did not lead to misunderstanding. The results of the program are the diagram of gradient concentrations, the diagram of the paths of solutes and the final R_F values for each substance. It should be noted that the program below is for normal-phase systems. To alter it for reversed-phase systems substitute steps 240 and 260 in the program as follows:

240 LPRINT "THE R-P SYSTEM"

260 DEF FN $k(j,i)$ = 262 144/((2↑j) ∗ (10↑($c(i)$ ∗ m)))

```
 10 LPRINT
 20 LPRINT "STEPWISE GRADIENT"
 30 LPRINT
 40 INPUT "THE NUMBER OF STEPS n=";n
 50 LPRINT "THE NUMBER OF STEPS n=";n
 60 LPRINT
 70 INPUT "THE NUMBER OF SOLUTES b=";b
 80 LPRINT "THE NUMBER OF SOLUTES b=";b
 90 LPRINT
100 DIM c(n): DIM v(n): DIM k(b,n): DIM R(b,n)
110 DIM x(b,n): DIM y(b,n): DIM s(b,n): DIM z(b,n)
120 LPRINT
125 LPRINT "THE CONCENTRATION OF MODIFIER ON i-th STEP"
140 LPRINT
150 FOR i=1 TO n
160 INPUT "c=";c(i),"v=";v(i)
170 PRINT "c(";i;")=";c(i),"v(";i;")=";v(i)
180 NEXT i
190 COPY
200 CLS
210 LPRINT
220 GO SUB 1000
230 LPRINT
240 LPRINT "THE S-P SYSTEM"
250 LPRINT
260 DEF FN k(j,i)=25.6/((2↑j)*(c(i)↑m))
270 DEF FN R(j,i)=INT (1000/(1+FN k(j,i)))/1000
280 INPUT "THE SLOPE m=";m
290 LPRINT "THE SLOPE m=";m
300 LPRINT
310 LPRINT "THE DISTANCE TRAVELLED BY SPOTS AFTER n DEVELOPMENT STEPS
320 LPRINT
330 FOR j=1 TO b
340 FOR i=1 TO n
350 LET x(j,i)=v(i)/(1-FN R(j,i))
360 LET y(j,i)=v(i)/FN k(j,i)
370 NEXT i
380 LET s=0
390 FOR i=1 TO n
400 LET s=s+x(j,i)
410 LET s(j,i)=s
420 NEXT i
430 FOR i=1 TO n
440 IF i>=2 AND s(j,i)>=1 THEN  GO TO 520
450 IF s(j,i)=1 THEN  GO TO 480
460 NEXT i
470 GO TO 640
480 LET R=FN R(j,i)
490 PLOT 10,10: DRAW 160,R*160
500 LPRINT "Rf(";j;")=";R
510 GO TO 640
520 LET z(j,i)=(1-s(j,i-1))*FN R(j,1)
530 LET R=0
540 FOR P=1 TO i-1
550 LET R=R+y(j,P)
560 NEXT P
570 LET R(j,i)=INT (1000*(R+z(j,1)))/1000
580 LPRINT "Rf(";j;")=";R(j,i)
590 PLOT 10,10
600 FOR h=1 TO i-1
610 DRAW x(j,h)*160,y(j,h)*160
620 NEXT h
630 DRAW (1-s(j,i-1))*160,z(j,1)*160
640 NEXT j
650 LET v=0
660 FOR i=1 TO n
670 LET v=v+v(i)
680 PRINT AT 21,v*20," ";v
```

(*Continued on p. 70*)

TABLE I (*continued*)

```
 690 PLOT 10+v*160,10: DRAW (1-v)*160,(1-v)*160
 700 CIRCLE 10+i*160/n,10,1
 710 CIRCLE 171,10+i*160/n,1
 720 NEXT i
 730 PRINT AT 10,24;"Rf=0,5"
 740 PRINT AT 0,24;"↑";"Rf"
 750 PRINT AT 21,24;"-->";"V,X,S"
 760 NEXT i
 770 PLOT 10,10: DRAW 160,0: DRAW 0,160: DRAW -160,0: DRAW 0,-160
 780 COPY
 790 CLS
 800 INPUT "REPEAT PROFILE OF GRADIENT a$=1 OR REPEAT ANOTHER SLOPE a$=0
";";"a$=";a$
 810 IF a$="1" THEN  GO TO 10
 820 IF a$="0" THEN  GO TO 240
 830 STOP
 840 RUN 10
1000 LPRINT "THE PROFILE OF STEPWISE GRADIENT"
1010 LPRINT
1020 FOR i=1 TO n
1030 IF i>=2 THEN  GO TO 1070
1040 PLOT 0,c(1)*160: DRAW 160/n,0
1050 PLOT 0,0: DRAW 0,c(1)*160
1060 GO TO 1090
1070 PLOT (i-1)*160/n,c(i)*160: DRAW 160/n,0
1080 PLOT (i-1)*160/n,c(i-1)*160: DRAW 0,(c(i)-c(i-1))*160
1090 NEXT i
1100 PLOT 0,0: DRAW 0,160: DRAW 160,0: DRAW 0,-160: DRAW -160,0
1110 FOR i=1 TO n
1120 PRINT AT 21,(i-1)*20/n;i
1130 PRINT AT (21-30*c(i))/20;"_(";i;")="c(i)
1140 NEXT i
1150 COPY
1160 CLS
1170 LPRINT
1180 LPRINT
1190 RETURN
```

STEPWISE GRADIENT

THE NUMBER OF STEPS n= 5

THE CONCETRATION OF MODIFIER ON i-th STEP AND THE VOLUME OF ELUENT ON i-th STEP

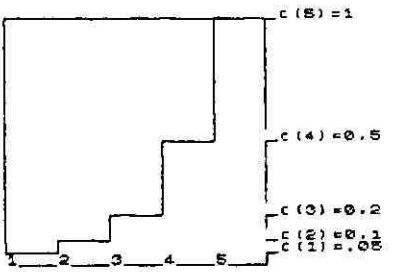
```
c(1) =.05          V(1) =0.2
c(2) =0.1          V(2) =0.2
c(3) =0.2          V(3) =0.2
c(4) =0.5          V(4) =0.2
c(5) =1            V(5) =0.2
```

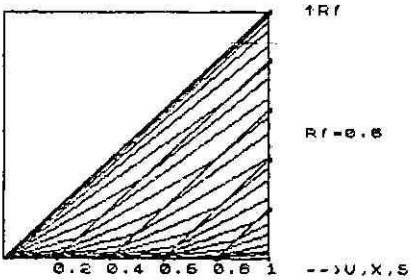
THE S-P SYSTEM

THE SLOPE m=2

THE DISTANCE TRAVELLED BY SPOTS AFTER n DEVELOPMENT STEPS

```
Rf(1)=.018
Rf(2)=.035
Rf(3)=.062
Rf(4)=0.1
Rf(5)=0.144
Rf(6)=0.186
Rf(7)=0.245
Rf(8)=0.316
Rf(9)=0.368
Rf(10)=0.407
Rf(11)=0.504
Rf(12)=0.573
Rf(13)=0.647
Rf(14)=0.735
Rf(15)=0.791
Rf(16)=0.864
Rf(17)=0.927
Rf(18)=0.962
Rf(19)=0.98
Rf(20)=0.99
```





The analysis of stepwise gradient processes in reversed-phase systems requires a suitable modification of the program (included in Table I).

The assumed mathematical model is somewhat simplified; for instance, it does not take into account solvent demixing effects[4,25,26], which are especially significant for the low-concentration region of the gradient profile[4,18] (the effect can be minimized by pre-wetting the layer before spotting the sample[4]). Part of the mobile phase is stagnant in the pores of the adsorbent and the exchange of the stagnant liquid in contact with the new concentration zone may delay the migration of its front and cause some smoothing of the sharp (initial) concentration steps.

CONCLUSION

The computer simulation of stepwise gradient elution can be used for various purposes. The study of migration paths and R_F values of solutes for various gradient programs and retention–modifier concentration relationships is valuable for teaching purposes, as it illustrates the operation of gradients in comparison with isocratic elution and gives general experience in choosing the optimal gradient shape (lengths and heights of the steps). The chromatograms obtained by trial gradient runs can be compared with computer data for model mixtures and modified programs tried by computer simulation to give an improved distribution of the spots.

The examples chosen in this study concern simplified situations (parallel, equidistant log k' *vs.* log c plots). In real systems, more complex situations can be encountered, *e.g.*, the spots may form two groups separated by a wide gap on the chromatogram (ref. 1, Fig. 6c). The situation can be simulated by assuming that the middle solutes (*e.g.*, Nos. 7–13) in the hypothetical series are non-existent and the task is to choose a gradient profile that would secure maximal compression of spots 7–13 and equidistant distribution of the remaining solutes 1–6 and 14–20. Another cause of complications may be differentiation of the slopes (m) of the log k' *vs.* log c plots of the individual solutes, which may lead to crossing of some of the paths and changes in the sequence of the spots. Some more complex situations will be analysed in subsequent papers in this series.

The computer program can also be adapted to stepwise gradient elution in column chromatography (for other programs for optimization of continuous gradients in column chromatography, see ref. 10, p. 485, and ref. 27; several programs for the optimization of TLC are given in ref. 28). The differences are that numerous void volumes of the eluent are used in elution and the process is terminated when the last solute ($j = 1$) leaves the column [*i.e.*, its $\sum_i y(i) = 1$; see refs. 9–17].

LIST OF SYMBOLS

Different symbols are used in the BASIC program for technical reasons (*e.g.*, *Rf* instead of R_F, *s* instead of $\sum$).

j	No. of solute (1–20).
i	No. of elution step (eluent fraction).
$k(j,i)$	capacity factor of solute j in the ith step.
$R(j,i)$	corresponding fraction of solute in the mobile phase.
$Rf(j,i)$	R_F value.

$c(i)$ concentration of modifier (molar or volume fraction) in the ith step.
$v(i)$ volume of eluent introduced in the ith step.
$y(j,i)$ distance (ΔR_F) travelled by solute j in the ith step.
$x(j,i)$ corresponding volume of mobile phase.
$s(j,i)$ total distance (R_F) travelled by solute j after i steps.
$z(j,i)$ fractional distance travelled by solute j in the last (incomplete) step.
$-m$ slope of $\log k$ *vs.* $\log c$ plot.

$$x(j,i) \quad \frac{v(i)}{1 - Rf(j,i)}$$

$$y(j,i) \quad \frac{v(i)}{k(j,i)}$$

$$z(j,i) \quad R(j,i) \cdot [1 - s(j,i-1)]$$

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS AND PEPTIDES ON SILICA COATED WITH AMMONIUM TUNGSTOPHOSPHATE

I. CHARACTERISTICS OF THE STATIONARY PHASE

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SUMMARY

Silica coated with ammonium tungstophosphate is proposed as a stationary phase for high-performance liquid chromatography. The chromatographic characteristics of columns packed with this material were investigated. The retention of compounds containing an NH_4^+ group, such as amino acids and peptides, is determined by an ion-exchange mechanism; the silica coated with ammonium tungstophosphate behaves as a reversed phase in the case of aliphatic and aromatic organic acids when eluting with aqueous buffer solutions. The new stationary phase shows a high selectivity towards glycine and tyrosine oligomers.

INTRODUCTION

Recent progress in high-performance liquid chromatography (HPLC) is due to continuous improvements in the equipment[1,2] and to the use of new stationary phases[3–5]. Today, research into new stationary phases for selective chromatographic separations of organic compounds is one of the primary goals in this field[6]. The production of supports with polar functional groups is of particular interest[7,8]. We propose here the use of ammonium tungstophosphate (AWP), a synthetic inorganic ion exchanger that has already been widely used in the thin-layer chromatography (TLC) of many nitrogen-containing organic compounds[9–16], as a stationary phase for use in HPLC.

EXPERIMENTAL

Apparatus

HPLC separations were performed on a Perkin-Elmer Series 3B liquid chromatograph. The samples were monitored at 210 nm with a Perkin-Elmer LC-75 variable-wavelength detector. The columns were 25 × 0.46 cm I.D. stainless-steel tubes with stainless-steel frits at the ends, packed with a Shandon apparatus. The chro-

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matographic data were stored and processed with a Shimadzu computer system.

Thin layers for TLC (10 × 10 cm, thickness 300 μm) were prepared with a Chemetron automatic apparatus.

Reagents

Amino acids, peptides and organic acids were obtained from Sigma. Standard solutions were prepared by dissolving different amounts of the compounds in phosphate buffer (pH 2.1) to give concentrations in the range 0.5–2 mg/ml, depending on the detector response. The injection volumes varied between 1 and 5 μl of solution. In all separations elution was performed with equimolecular solutions of phosphoric acid and ammonium phosphate at different concentrations, and with solutions of phosphate buffer and acetonitrile.

Preparation of the stationary phase

A 50-g amount of Lichrosorb Si 60 (10 μm) (Merck) was suspended in 150 ml of 2 *M* nitric acid containing different amounts of tungstophosphoric acid (HAWP) to obtain silica with different degrees of coating (see Fig. 1a). A 150-ml volume of 2 *M* ammonium nitrate solution was added dropwise to the suspension with stirring. After 10 min the precipitate was collected in a glass filter funnel (Jena-Duran, porosity 3), washed with acetone and air dried. The resulting product was purified and fractionated according to the particle size using the following method: the material was slurried in isopropyl alcohol and then slowly transferred into a glass column (100 × 2.5 cm I.D.) filled with the same solvent, avoiding turbulence. After 10 min the first sediment was removed, and the solid sedimented after a further 20 min was recovered and the suspended particles were eliminated. The resulting fraction showed a narrow size range and was able to be packed.

HPLC column packing

The material was slurried in isopropyl alcohol and then packed into the column with 200 ml of phosphate buffer solution (pH 2.1) using both "up-flow" and "down-flow" methods successively. The packing pressure was about 8000 p.s.i.

Preparation of thin-layers

A 4-g amount of stationary phase and 1 g of $CaSO_4 \cdot 1/2H_2O$ were suspended in 50 ml of water and stirred for 10 min.

Determination of AWP content on the silica

A spectrophotometric method, based on the formation of yellow complexes of thiocyanate with tungsten, was applied[17]. The transmittance was measured at 420 nm after 20 min.

RESULTS AND DISCUSSION

The ammonium tungstophosphate obtained by precipitation from HAWP solution has a wide size range (1–5 μm), but there are also smaller particles (1 μm). In addition, AWP easily forms large agglomerates. Although the particles of AWP are strong enough to resist high packing pressures, the presence of the smallest particles,

which are difficult to separate and may obstruct the porous frits of the columns, makes this material unsuitable for packing. For this reason, silica coated with ion exchanger was prepared and used to pack the HPLC columns, as with ammonium molybdophosphate[18].

Characteristics of the stationary phase

The degree of coating of the silica depends on the concentration of tungstophosphoric acid in the solution from which AWP is precipitated. The relationship between these two parameters is shown in Fig. 1a, where the percentage of AWP on silica is plotted against the percentage of HAWP (calculated with respect to the weight of silica) in the solution. The resulting graph is similar to an adsorption isotherm. In fact, the degree of coating increases with increasing amount of tungstophosphoric acid in the solution up to the maximum value of 25%. When the percentage of HAWP increases above this value, the content of AWP on the silica remains almost unchanged.

In order to examine, by another method, the correlation of the degree of coating with the amount of HAWP in the solution and to show at the same time the retention capacity of this new stationary phase, thin-layer chromatographic data for

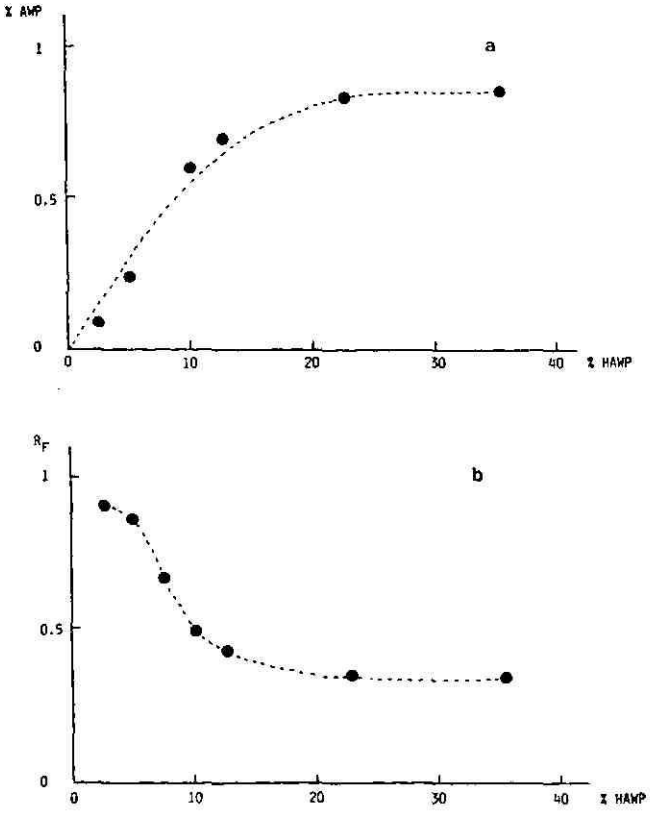

Fig. 1. (a) Percentage of AWP on silica *versus* percentage of HAWP in solution. (b) R_F values of tetraglycine on layers of AWP-coated silica *versus* percentage of HAWP in solution. Eluent, *0.05 M* phosphate buffer.

tetraglycine on layers with different amounts of AWP were obtained. All experiments were carried out with 0.05 *M* phosphate buffer solution (pH 2.1). A plot of R_F values against the percentage of HAWP in the solution is shown in Fig. 1b. In general, this behaviour is in agreement with the data in Fig. 1a; in addition, it shows that the influence of AWP becomes significant only at levels above 0.1%, corresponding to HAWP levels above 2.5%.

Degree of coating and selectivity of the stationary phase

In order to study the retention capacity and the selectivity of the new stationary phase, HPLC columns with silica alone and silica coated with different amounts of AWP were packed. Four columns were tested with aliphatic and aromatic compounds and particularly with oligomers of glycine up to four residues and of tyrosine up to three residues. All the samples were eluted with 0.05 *M* phosphate buffer solution. The choice of these compounds was suggested by their different chromatographic behaviour observed on AWP thin layers[11]. Fig. 2 shows the chromatograms of (a) glycine and (b) tyrosine oligomers on columns of (1) silica, (2) silica + 0.3% AWP, (3) silica + 0.48% AWP and (4) silica + 0.65% AWP, corresponding to percentages of HAWP in solution of 0, 5, 7.5 and 15%, respectively (see Fig. 1a). On silica columns the members of the two series of oligomers are not retained and give rise to one peak only. An increase in the amount of AWP on the silica results in an

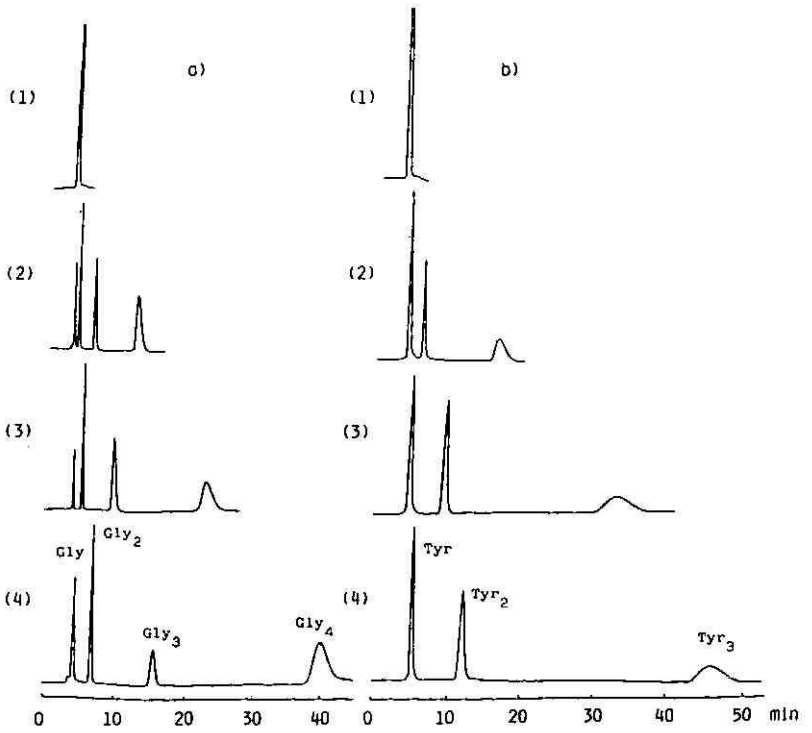

Fig. 2. Chromatograms of (a) glycine and (b) tyrosine oligomers on columns of (1) SiO_2, (2) SiO_2 + 0.3% AWP, (3) SiO_2 + 0.48% AWP and (4) SiO_2 + 0.65% AWP. Mobile phase, 0.05 *M* phosphate buffer (pH 2.1); column, 25 × 0.46 cm, I.D.; flow-rate, 1 ml/min; detection, UV (210 nm).

TABLE I

SELECTIVITIES OF SILICA GEL WITH DIFFERENT DEGREES OF COATING FOR PAIRS OF AMINO ACIDS AND PEPTIDES

Pair	α_2*	α_3*	α_4*
Gly_2–Gly	3.5	4.3	4.1
Gly_3–Gly_2	3.5	3.7	3.7
Gly_4–Gly_3	3.0	3.1	3.1
Tyr_2–Tyr	4.1	5.6	5.4
Tyr_3–Tyr_2	5.1	5.5	5.2
Tyr–Gly	2.4	2.6	2.1

* α_2, α_3 and α_4 refer to columns 2, 3 and 4 in Fig. 2.

increase in retention for all the compounds. This increase is greater for tyrosine than for glycine oligomers and for both series the retention increases considerably with increasing number of amino acid residues. Both glycine and tyrosine oligomers can also be well separated on columns packed with material with a low degree of coating (see column 2). This behaviour demonstrates the unusual retention capacity even of small amounts of this ion exchanger and its great selectivity, as shown in Table I. The increase in the degree of coating of silica does not involve a corresponding change in selectivity for either aliphatic or aromatic compounds. The greatest variations of α values are found for the pairs Gly_2–Gly and Tyr_2–Tyr.

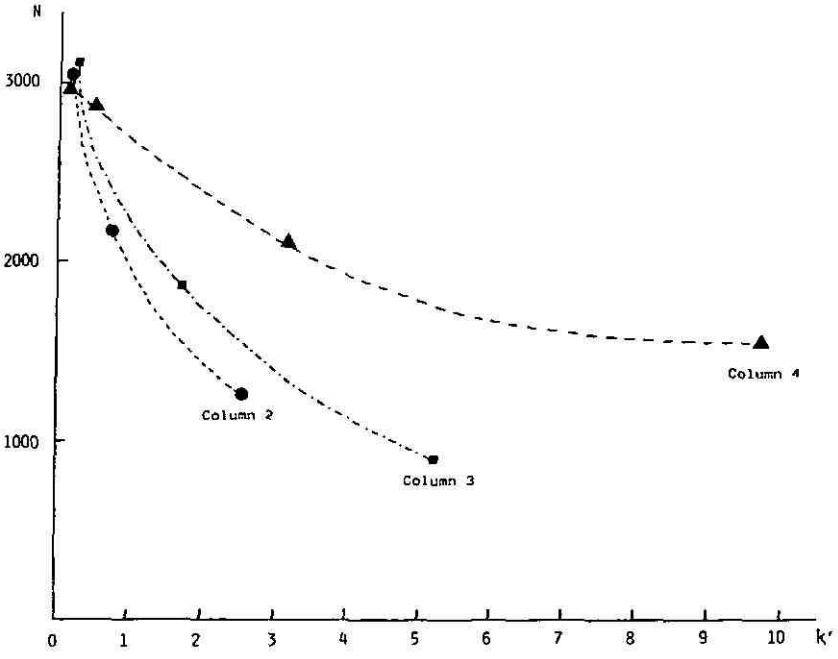

Fig. 3. Plot of theoretical plate number (N) *versus* the capacity factor (k') of glycine oligomers on columns 2, 3 and 4. Mobile phase, 0.05 *M* phosphate buffer (pH 2.1); flow-rate, 1 *ml/min*.

Column efficiency

The theoretical plate numbers (N) were obtained for three different columns using glycine oligomers. In Fig. 3 the capacity factors (k') are plotted against the theoretical plate numbers for columns 2, 3 and 4. Data relative to glycine are reported only for column 4, as this compound is eluted near the void volume on the first two columns and the corresponding k' values are not reliable.

All the curves show a decrease in N with increasing k'. The decrease is greater when the degree of coating of the silica is low. The differences in N for the three columns are very slight for compounds having no appreciable retention. The maximum theoretical plate number, for example, is 3000 (or 12 000 plates/m) on all the columns.

The curves obtained by plotting HETP (height equivalent to a theoretical plate) values against the linear flow-rate (u ml/min) of the eluent are in agreement with the Van Deemter equation and reach their minima at a mobile phase flow-rate of *ca.* 0.3 ml/min. The high selectivity of this stationary phase gives good separations of oligomer mixtures even when using a flow-rate much higher than the optimal value, as shown in the chromatogram in Fig. 4.

Aqueous–organic solutions

The addition to the mobile phase of increasing amounts of acetonitrile results in no appreciable change in the chromatographic behaviour of glycine and tyrosine oligomers. For example, on eluting column 4 with phosphate buffer–acetonitrile (60:40), the retention times of glycine and tyrosine increase from 0.75 to 1.35 min and from 1.48 to 1.59 min, respectively, without any analytical advantage. In order to avoid salt precipitation and consequent obstruction of the column, acetonitrile concentrations above 40% are inadvisable.

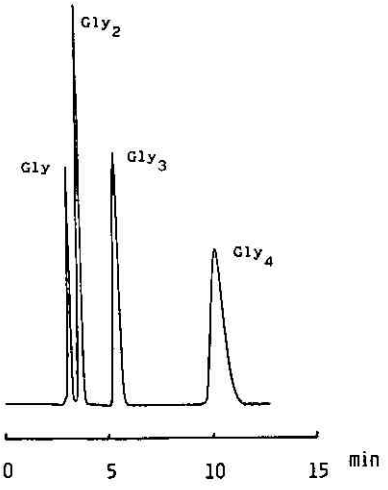

Fig. 4. Chromatogram of glycine oligomers on column 2. Mobile phase, 0.05 *M* phosphate buffer; flow-rate, 2.5 ml/min; detection, UV (210 nm).

Mechanism of retention

As the stationary phase and solutes contain groups able to undergo ion-exchange reactions, we wanted to investigate whether the ion-exchange process was the primary mechanism for the retention of amino acids and peptides. For this purpose we performed retention measurements on column 4 at different concentrations of ammonium ion at constant pH. The results are reported in Fig. 5, where log k' is plotted against $-\log[NH_4^+]$.

Peptides give straight lines with slopes between 0.85 and 1.03, in agreement with a retention process controlled by an ion-exchange mechanism. With amino acids, the mechanism of solute retention is more complex. The log k' *versus* $-\log[NH_4^+]$ trend for glycine is not well characterized, as this compound is eluted with the void volume using 0.1 *M* phosphate buffer. A linear trend can be identified for tyrosine but the slope is much lower (0.57) than that for peptides.

Such a behaviour indicates that no single retention mechanism is operative with amino acids, whose chromatographic characteristics are probably dependent on both an ion-exchange process and non-ionic interactions. The log k' *versus* $-\log[NH_4^+]$ trends for benzene sulphonic and acetic acids confirm such a hypothesis. These compounds, in fact, are lacking an NH_4^+ group and are not affected by changes in the ionic strength of the eluent.

Further information on the behaviour of the new stationary phase with aqueous eluents is derived from the retention data for several aliphatic and aromatic organic acids and for the corresponding amino acids. The results in Table II indicate that silica coated with AWP behaves as a reversed phase under the experimental

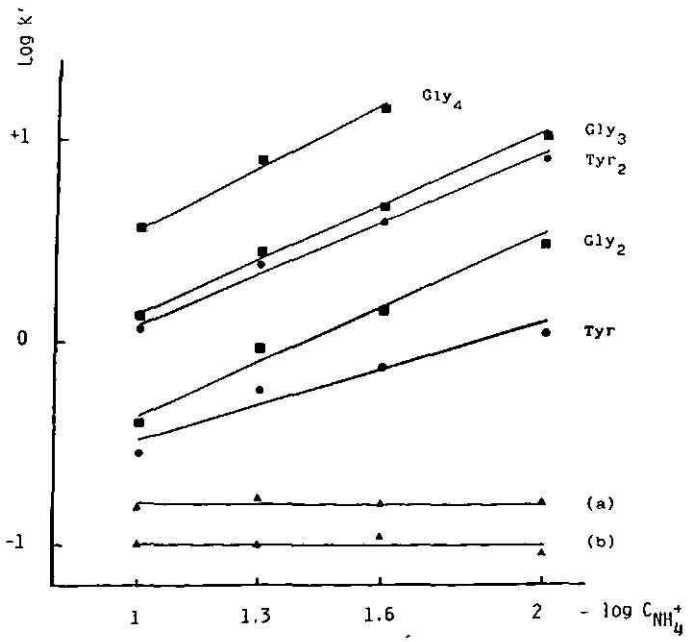

Fig. 5. Plot of log k' *versus* $-\log[NH_4^+]$ for amino acids peptides and organic acids on column 4. (a) Benzene sulphonic acid; (b) acetic acid.

TABLE II

RETENTION TIME (t_R) OF ORGANIC ACIDS AND OF THE CORRESPONDING AMINO ACIDS ON COLUMN 4 (SEE FIG. 2) ON ELUTING WITH 0.05 M PHOSPHATE BUFFER

Organic acid	t_R *(min)*	*Amino acid*	t_R *(min)*
HCOOH	3.74	NH_2CH_2COOH	4.18
CH_3COOH	3.80	$CH_3CH(NH_2)COOH$	4.09
CH_3CH_2COOH	4.16	$HOCHCH(NH_2)COOH$	3.97
$CH_3CH(OH)COOH$	3.62	$CH_3CH_2CH(NH_2)COOH$	4.31
$CH_3(CH_2)_2COOH$	4.74	$CH_3(CH_2)_2CH(NH_2)COOH$	4.87
$CH_3(CH_2)_3COOH$	5.15	$CH_3(CH_2)_3CH(NH_2)COOH$	5.49
C_6H_5COOH	5.42	$C_6H_5CH_2CH(NH_2)COOH$	6.73
$C_6H_5CH_2COOH$	4.83		
$C_6H_5SO_3H$	4.01		

conditions used. In fact, organic acids with more marked hydrophobic characteristics are the most retained. The same behaviour is shown by amino acids, even if their retention is also affected by an ion-exchange process that determines the reversal in retention between glycine and alanine, probably owing to the steric hindrance of the methyl group on the NH_4^+.

Reproducibility of the technique

As regards the lifetime and reproducibility of the columns, we verified that after rinsing with 3000 column volumes of acqueous solutions of different ionic strength (0.01–0.1 *M*) and pH (1–3), no appreciable change in the capacity factors of the test compounds was observed.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF INORGANIC ANIONS ON A SILICA GEL COLUMN MODIFIED WITH A QUATERNARY AMMONIUM SALT

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SUMMARY

Silica gel columns dynamically modified with a quaternary ammonium salt have been investigated for the high-performance liquid chromatographic separation of inorganic anions. The parameters which affect the chromatographic retention behavior have been examined. The mobile phase is composed of an aqueous solution of salicylate, which competes with the analyte anions for active sites and maintains a background signal. The analyte anions are separated based on ion exchange and are detected by indirect photometry or indirect fluorometry. The latter allows detection of 6–15 pmoles (sub-ng level) of anions by means of a microbore column.

INTRODUCTION

Ion chromatography has advanced rapidly for the determination of ions since it was developed by Small *et al.*[1]. Meanwhile, several improvements have been accomplished. Single-column ion chromatography (without the use of a suppressor column) has been demonstrated[2], leading to a simplified system. Packing materials with low ion-exchange capacities are favored for both single-column and dual-column ion chromatography with conductometric detection, because they allow the use of lower eluting ion concentrations. The lower the concentration in the mobile phase, the lower the background noise is obtained in single-column ion chromatography. A low concentration is also favored in dual-column ion chromatography because then the suppressor column requires less frequent regeneration. There have been published several papers which deal with the synthesis of ion exchangers with low capacities[1–3].

Other approaches for the separation of ionic species include ion-pair chromatography[4–6], dynamic ion-exchange chromatography[7–10] and ion-interaction chromatography[11–13], where a hydrophobic counter ion is added to the mobile phase and reversed-phase alkyl-chain bonded silica, polystyrene, or polyacrylate packing materials are used. On the other hand, when the ion-interaction reagent is not added to the mobile phase after the equilibration, the retention mechanism involves ion exchange[14,15]. Ion-exchange columns obtained by dynamic modification[14] of an ODS

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column or static modification[15] of polystyrene resins, in which their ion-exchange capacities can be easily controlled by changing the coating conditions (concentration of the reagent, composition of the matrix solution, etc.) have been investigated.

Indirect photometric or fluorometric detection gives sensitive and universal detection of inorganic anions[16–20]. A visualization UV-absorbing[16–19] or fluorescing[20] reagent was added to the mobile phase. The visualization reagent maintains a background signal and competes (or interacts) with the analyte anions for the active sites. When the analyte anions elute, they are indirectly detected as negative peaks because the concentration of the visualization reagent in the analyte bands decreases. The visualization mechanism has been reported in the literature[12,13,16]. In the indirect detection method, the dynamic reserve (which is defined as the ratio between the background signal and its noise level), the concentration of the visualization reagent, and the displacement ratio (which is defined in this work as the number of visualization ions which are transferred by one analyte ion) all play important roles in the sensitivity that can be achieved. The concentration detectability (C_{lim}) at the detector is given by these parameters as follows.

$$C_{lim} = C_m/RD \tag{1}$$

where C_m is the concentration of the visualization reagent, R is the displacement ratio, and D is the dynamic reserve. The dynamic reserves for fluorescence and for absorption vary differently with the magnitude of the background signal. While the former maintains a $5 \cdot 10^3$ level until the concentration of the visualization reagent falls below 10^{-7} M[19], the latter is proportional to the concentration when the background absorption is below 1.0 a.u. The displacement ratio for monovalent ions is expected to be unity in ion-exchange chromatography (independent of the mobile phase concentration). Ion-exchange columns having very low capacities are required to demonstrate the separation of ions using a mobile phase with low concentrations of visualization reagent.

In contrast to previous work on ODS columns[14] or polystyrene resins[15], this paper describes the potential of silica gel columns, dynamically modified with a quaternary ammonium salt, for the high-performance liquid chromatographic separation of inorganic anions. The analyte anions were detected indirectly.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a reciprocating pump (MiniPump; LDC/Milton Roy, Riviera Beach, FL, U.S.A.) with a dampener or a syringe pump (Model 314; ISCO, Lincoln, NE, U.S.A.), a sample injector (Model 7520; Rheodyne, Cotati, CA, U.S.A.) with a 1-μl injection volume and a conventional silica gel column (HPLC-SI; Alltech, Deerfield, IL, U.S.A., 250 × 4.6 mm, 10 μm) or a microbore silica gel column (Microsphere Silica; Alltech, 250 × 1 mm, 10 μm). A fixed-wavelength (254 nm) UV detector (Model 260; Chromatronix, Berkeley, CA, U.S.A.) and a laser-based double-beam fluorometric detection system were used. The arrangement for the latter detection system was the same as the one reported previously[19,20]. The 325-nm UV beam of a HeCd laser (Model 4240NB; Liconix, Sunnyvale, CA, U.S.A.)

was used as excitation source. The polarization of the beam was modulated at 100 kHz by an electrooptic modulator and the polarization-modulated beam was split into two beams by a calcite beam displacer. Each signal from the sample and the reference cell was collected by separate optical fibers to a common photomultiplier tube after passing several filters. The photomultiplier tube output was directed through an amplifier to a lock-in amplifier with a 1-s time constant. Two parallel quartz tubes were used as the flow cells and were fixed vertically. The same eluent was supplied to a reference flow cell by gravity with a flow-rate of *ca.* 0.3 ml/min. Both eluents flow upwards in the cell and laser beams hit the quartz tubes at right angles. Flow-rates for preparation of the column and the separation of the analytes were 1 ml/min for conventional HPLC and 50 μl/min for microbore HPLC unless otherwise noted.

Reagents

HPLC-grade methanol was supplied by Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water employed in this work was pure water prepared with the Barnstead Nanopure II system (Barnstead, Division of Sybron, Boston, MA, U.S.A.). Cetyltrimethylammonium bromide (cetrimide), phenyltrimethylammonium chloride, tetramethylammonium bromide, and cetylpyridinium chloride monohydrate were supplied by Aldrich (Milwaukee, WI, U.S.A.). The other reagents used in this work were reagent grade and supplied by Fisher Scientific, unless otherwise noted. The mobile phase was degassed in an ultrasonic bath under vacuum. The reference solution for fluorometric detection was degassed the same way and then filtered with a Disposal Nylon 66 filter (Alltech, 0.45 μm). The sample was dissolved in pure water.

Preparation of the column

A quaternary ammonium salt was dissolved in methanol–10 m*M* potassium phosphate buffer (50:50) or 100% 10 m*M* potassium phosphate buffer. The prepared reagent solution was passed through the silica gel column for 4 h, followed by washing with water or methanol for 10 min. When a minipump was employed, the effluent was collected into the reagent reservoir for recycling. After the above dynamic modification, a $5 \cdot 10^{-4}$ *M* sodium salicylate aqueous solution (pH 6.9) or an aqueous solution of a mixture of sodium salicylate and salicylic acid (pH 4.7) was passed through the dynamically modified column, from which the breakthrough volume can be measured. In order to wash out the modified reagent from the column, methanol–0.05 *M* phosphoric acid (50:50) was passed through the column at least for 1 h, followed by washing with methanol for 10 min.

RESULTS AND DISCUSSION

Porous silica gel and zirconia gel dynamically modified with a quaternary ammonium salt were investigated for the HPLC separation of neutral species and ionic species[4,21–26]. The quaternary ammonium salt was added to the mobile phase. Retention of neutral species on the dynamically modified silica gel column is due to hydrophobic interactions between the analyte species and the hydrophobic layer on the silica surface. The retention mechanism for ionic species is more complex. Wall[27] has reported that silica gel and zirconia gel columns dynamically modified with a

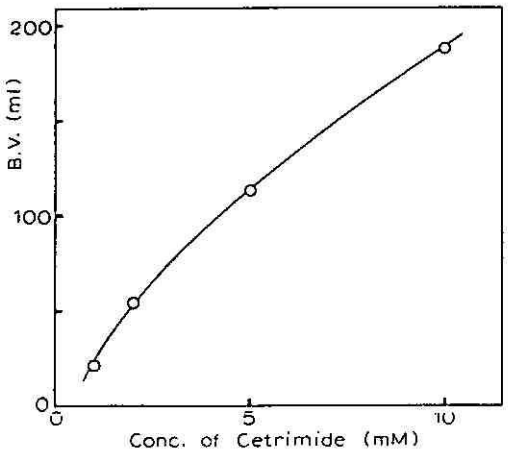

Fig. 1. Effect of the concentration of cetrimide on the breakthrough volume. Column: 250 × 4.6 mm. Eluent: $5 \cdot 10^{-4}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). pH of the reagent buffer: 6.35.

mixture of a non-ionic and an anionic surfactant can separate tyrosinyl peptides in the cation-exchange mode. The initial surface monolayer is formed rapidly by interaction of polar groups of the non-ionic surfactants with gel hydroxyls. Then, a partial bilayer is formed more slowly by hydrophobic interaction of the long alkyl chains of the bound non-ionic surfactant and those of the anionic surfactant dissolved in the mobile phase. On the other hand, it has been suggested that the quaternary ammonium reagent forms a monolayer on the silica gel surface[22,24]. If the quaternary ammonium reagents electrostatically interact one-to-one with silanol groups and form a monolayer on the silica gel surface, the modified surface is expected not to have a net charge. In this work, we found that silica gel columns dynamically modified with quaternary ammonium salts with a long alkyl chain retain anions even if the eluent does not include the quaternary ammonium reagent after the dynamic modification. This observation implies that electrostatic interaction between the analyte anions and the modified surface occurs. Either a double layer of the quaternary ammonium reagent is formed on the silica surface, as suggested by Wall[27], or there exists another structure of the dynamically generated phase.

We have examined the preparation conditions using cetrimide as the quaternary ammonium reagent. Fig. 1 shows the effect of the concentration of cetrimide on the breakthrough volume for salicylate after modification. A volume of 200 ml of each solution was prepared with methanol–10 m*M* phosphate buffer (pH 6.35) (50:50) and the effluent was recycled during the modification. The breakthrough volume increased with increasing concentration of cetrimide. In other words, the amount of cetrimide adsorbed on the silica gel increased with increasing concentration of cetrimide. The amounts of salicylate adsorbed on the modified surface can be calculated from the breakthrough volume, *e.g.*, 94 μmoles of salicylate anions were adsorbed on the column modified with 10 m*M* cetrimide. Strictly speaking, the recycling method does not give precise adsorption data because the concentration of the reagent gradually decreases during the modification. In fact, when the column is modified with 2 m*M* cetrimide in methanol–10 m*M* phosphate buffer (pH 6.35)

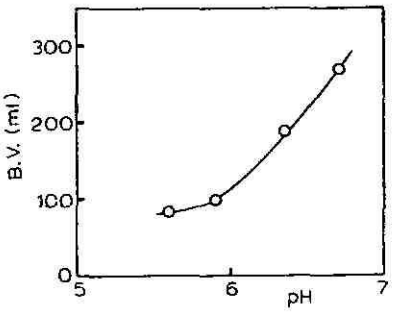

Fig. 2. Effect of pH of the reagent solution on the breakthrough volume. Operating conditions are the same as in Fig. 1 except the concentration of the reagent is 10 m*M*.

(50:50), the breakthrough volumes are 54 ml for the recycling method and 85 ml for the non-recycling method, respectively. Salicylate and the analyte anions were not retained on the bare silica column or on the modified column after washing with methanol–0.05 *M* phosphoric acid (50:50).

The pH of the modifying solution affects the amount of cetrimide adsorbed on the silica surface because the dissociation of silanol groups is affected by the pH of the eluent. Fig. 2 shows the effect of the pH of the reagent solution on the breakthrough volume for salicylate. The breakthrough volume increases with increasing pH of the buffer solution. The apparent pH of the methanol–buffer solution is not considered here. Fig. 3 shows relationships between the retention time of the analyte anions and the breakthrough volume. The larger the breakthrough volume, the longer is the retention time. This is consistent with increasing capacity of the column.

Table I shows breakthrough volumes obtained for columns modified with different reagents. Each reagent, prepared with methanol–10 m*M* phosphate buffer (pH

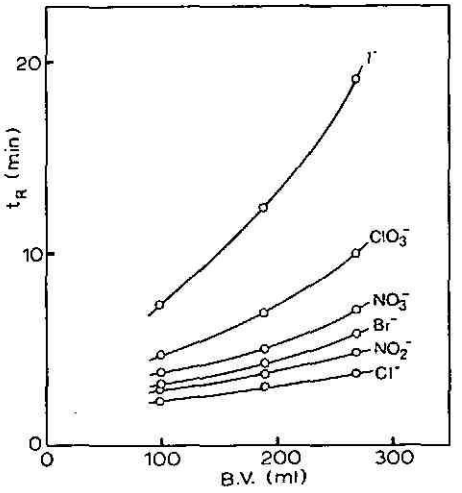

Fig. 3. Relationships between the retention time and the breakthrough volume. Column: 250 × 4.6 mm. Mobile phase: $5 \cdot 10^{-4}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 1.0 ml/min.

TABLE I

BREAKTHROUGH VOLUMES FOR $5 \cdot 10^{-4}$ *M* SALICYLATE

Reagent	*Concentration (mM)*	*Breakthrough volume (ml)*
Cetyltrimethylammonium bromide	2	85
Cetylpyridinium chloride	2	74*
Phenyltrimethylammonium chloride	10	0
Tetramethylammonium bromide	10	0

* After washing with methanol–water (50:50) for 50 min and with methanol for 1 h.

6.35) (50:50), was passed through the conventional silica gel column by the non-recycling method. The breakthrough volumes were measured using $5 \cdot 10^{-4}$*M* sodium salicylate. The columns modified with cetrimide and cetylpyridinium chloride retain salicylate and analyte anions. The selectivity of the columns modified with these two reagents is very similar. On the other hand, the columns modified with phenyltrimethylammonium chloride and tetramethylammonium bromide do not retain salicylate or analyte anions. For tetramethylammonium bromide, a phosphate buffer reagent solution (without methanol) was also examined, but the breakthrough volume for salicylate was again zero. Nevertheless, all four reagents adsorb on the silica surface during modification, which can be deduced from the observation that the modified surfaces are more hydrophobic than the bare silica surface. For the columns modified with phenyltrimethylammonium chloride and cetylpyridinium chloride, adsorption of these reagents on the silica surface can be confirmed by monitoring the effluent with a UV detector when washing with 0.05 *M* phosphoric acid–methanol (50:50) solution.

On the other hand, when pure methanol or acetonitrile was passed through an ODS column which was previously coated with cetylpyridinium chloride, it took a large volume to wash out the reagent. This means that the interaction between cetylpyridinium chloride and the ODS surface is very strong. This was also observed with the silica gel column dynamically modified with the cetylpyridinium reagent. From these observations, it may be concluded that cetrimide and cetylpyridinium chloride form a double layer on the silica surface, while phenyltrimethylammonium chloride and tetramethylammonium bromide form only a monolayer on the silica surface. The first layer of the reagent is formed by electrostatic interaction between nitrogen with a positive charge and a silanol group, while the secondary layer is formed by the hydrophobic interaction between the cetyl groups of the first layer and those of the reagent in the treating solution. It can be assumed that methyl or phenyl groups are not hydrophobic enough to form a secondary layer. The electrostatic interaction between the silica surface and the first layer is much stronger than the hydrophobic interaction between the first and second layer. Cetylpyridinium chloride is convenient for estimating the amount of the reagent adsorbed on the column. When 2 m*M* cetylpyridinium chloride in methanol–phosphate buffer (pH 6.35) (50:50) was passed through a conventional column for 4 h at 1.0 ml/min, followed by washing with methanol–water (50:50) for 50 min and with methanol for 1 h, 228

μmoles of the reagent were adsorbed on the surface. From the breakthrough data, 37 μmoles of salicylate were adsorbed on the modified surface. Assuming that each ammonium ion on the secondary layer interacts with one salicylate anion and hydrophobic adsorption of sodium salicylae on the modified surface is negligible, it can be estimated that 191 μmoles of the reagent form the first layer and 37 μmoles of the reagent (locally adsorbed on the first layer) form the secondary layer. Although we have no information about the surface properties of the silica gel used in this work, the surface coverage in the above case is roughly estimated to be 4%[23]. The retention mechanism of anions on the dynamically modified silica surface involves anion exchange, which is the same as in previous work, in which reversed-phase materials coated with cationic surfactants were used as the stationary phase[14,15].

Other parameters which can affect the chromatographic properties of the prepared columns involve the modification time, the concentration of the phosphate buffer solution, the composition of the treating solution (organic solvent *versus* aqueous solution), properties of the packing material (*e.g.*, surface area), etc. In fact, when the column was modified with 10 m*M* cetrimide dissolved in methanol–water (pH 5.8) (50:50) for *ca.* 17 h at 0.5 ml/min, the breakthrough volume was 3.4 times smaller than that obtained with 10 m*M* cetrimide dissolved in methanol–10 m*M* phosphate buffer (pH 5.9) (50:50) for 4 h at 1.0 ml/min. This difference in the breakthrough volume is mainly due to the effect of potassium phosphate. However, effects of the other parameters mentioned above have not been examined in detail in this work.

Fig. 4 demonstrates the indirect photometric detection of 10 nmoles of each inorganic anion using the silica gel dynamically modified with 10 m*M* cetrimide. It should be noted that the mobile phase does not contain cetrimide but salicylate. The analyte anions which are transparent at 254 nm give negative peaks, while iodide ion gives a positive peak due to its absorption. Iodate ion is not detected because its absorption just cancels the change of the background signal. Nearly the same chromatograms were obtained with the column modified with cetylpyridinium chloride. The retention order of the inorganic monovalent anions obtained with the modified

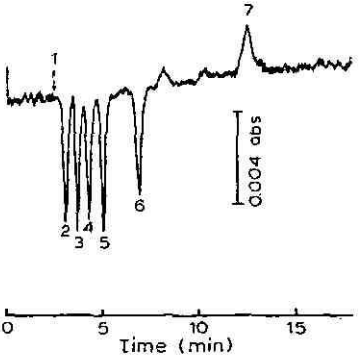

Fig. 4. Indirect photometric detection of inorganic monovalent anions. Column: 250 × 4.6 mm. Mobile phases: $5 \cdot 10^{-4}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 1.0 ml/min. Sample (10 nmoles each): 1 = iodate, 2 = chloride, 3 = nitrite, 4 = bromide, 5 = nitrate, 6 = chlorate, 7 = iodide. Detector: UV.

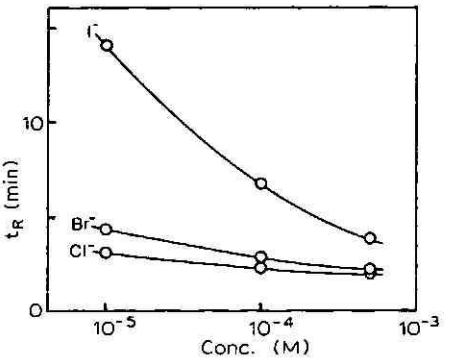

Fig. 5. Retention time *versus* the logarithm of the concentration of salicylate in the eluent. Column: 250 × 4.6 mm, dynamically modified with 2 m*M* cetrimide in methanol–10 m*M* phosphate buffer (pH 6.35) (50:50). Mobile phase: salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 1.0 ml/min.

silica columns is very similar to that observed with the common ion-exchange column[16]. Fluoride anion is not detected in this system, which may be due to its irreversible adsorption on the modified surface. The modified silica columns are very stable because cetrimide (or cetylpyridinium chloride) is not soluble in the mobile phase.

Fig. 5 shows the retention times of the analyte anions as a function of the logarithm of the concentration of salicylate in the eluent. The retention times of the analyte anions increase with decreasing concentration of salicylate. In ion-exchange chromatography, a linear relationship between the logarithm of the adjusted retention time and the logarithm of the eluent concentration was observed[4]. However, the ordinate in Fig. 5 is not plotted as the logarithmic scale of the adjusted retention time because it is difficult to estimate the void volume, and because the pH of the eluent changes depending upon its concentration, *viz.*, 4.7 for $5 \cdot 10^{-4}$ *M*, 5.4 for $1 \cdot 10^{-4}$ *M* and 5.7 for $1 \cdot 10^{-5}$ *M*.

In double-beam laser-based indirect fluorometry, the dynamic reserve maintains a $5 \cdot 10^3$ level until the concentration of the visualization reagent falls below 10^{-7} *M*[21]. Thus, indirect fluorescence provides lower detectabilities at low visualization reagent concentrations than indirect photometry. Also, the ion-exchange column should have quite low ion-exchange capacities so that the analyte ions can be eluted in reasonable time. The ion-exchange capacity of dynamically modified columns can be controlled by the preparation conditions, and is thus well-suited for this detection method.

Mass detectability is improved by using micro HPLC columns and a laser detection system. On the other hand, concentration sensitivity cannot be improved by reducing column dimensions. A laser is naturally suitable for small-volume detection. Fig. 6 demonstrates indirect fluorometric detection of inorganic anions with a microbore silica gel column, dynamically modified with cetrimide, in which 200 pmoles of each analyte is injected. The limit of detection is 6–15 pmoles (sub ng level) at S/N = 2. Many unknown peaks also appear in the chromatogram, which interfere with the separation of the analytes. The sample flow cell is connected to the outlet union of the column via stainless-steel tubing (35 mm × 0.33 mm I.D.), 1/16 in.

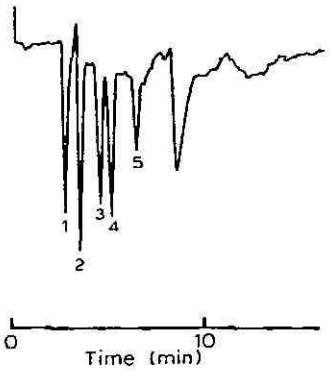

Fig. 6. Indirect fluorometric detection of inorganic anions. Column: 250 × 1 mm, dynamically modified with 1 *mM* cetrimide in methanol–10 *mM* phosphate buffer (pH 6.35) (50:50) for 4 h at 50 μl/min. Mobile phase: $5 \cdot 10^{-5}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 50 μl/min. Sample: (200 pmoles each): 1 = iodate, 2 = chloride, 3 = nitrite, 4 = bromide, 5 = nitrate.

zero-dead-volume union and PTFE tubing (100 mm × 0.19 mm I.D.). Total dead volume is 5.8 μl. The observed peak volume of chloride ion is 20 μl. Band broadening due to the above connecting parts can be estimated to be *ca.* 8% by using the Taylor equation[28]. On the other hand, band broadening due to injection parts is much smaller than the above value.

The displacement ratio between the monovalent anion and the salicylate anion is expected to be unity in ion-exchange chromatography. The displacement ratio can be calculated from the peak height and the peak volume. The change of concentration of the visualization reagent is calculated from the peak height and the background signal, while the concentration of the analyte is calculated from the peak volume using the following equation.

$$C_{max} = \sqrt{\frac{8}{\pi}} \frac{C_0 V_i}{V_w} \tag{2}$$

where C_{max} is the concentration of the analyte at top of the peak, C_0 is the concentration of the analyte in the sample solution, V_i is the injection volume and V_w is the peak volume. The observed displacement ratios are less than unity and decrease with decreasing concentration of salicylate, *e.g.*, 0.29 for $5 \cdot 10^{-4}$ *M*, 0.20 for $1 \cdot 10^{-4}$ *M* and 0.15 for $5 \cdot 10^{-5}$ *M*. This result may be due to the fact that salicylate molecules adsorbed on the hydrophobic groups can compensate for the induced signal derived from ion exchange between salicylate and analyte anions. The unknown peaks that appeared in the chromatogram may be due to complex interactions of salicylate (hydrophobic and electrostatic interactions). We did not use eluents with concentrations of salicylate less than $1 \cdot 10^{-5}$ *M* for fluorometric detection because the displacement ratio became very small (less than 0.1) and unknown peaks severely interfered with the analyte peaks.

CONCLUSIONS

Silica gel columns dynamically modified with quaternary ammonium salts with a long alkyl chain are suitable as stationary phases for the separation of inorganic anions. The retention times can be controlled by changing the preparation conditions. The formation of a double layer of the quaternary ammonium reagent with a long alkyl chain on the silica surface is suggested to explain the chromatographic retention behavior. Laser-based double-beam indirect fluorometry allows detection of sub-ng levels of inorganic anions by means of a microbore column. The advantage of dynamically modified silica gel columns lies in their low price, easy control of the column performance, and easy regeneration (compared to ODS or polystyrene columns). In addition, when a void appears on the top of the column due to hydrolysis of silica gel, fresh silica gel packing material can be manually replaced onto the column with a spatula. Finally, dynamical modification makes it straightforward to scale down to micro LC and open-tubular LC, with improved mass detectability.

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EVALUATION OF CAPILLARY GAS CHROMATOGRAPHIC COLUMNS IN SERIES

ANALYTICAL APPLICATION TO LEMON OIL

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SUMMARY

The advantages of capillary columns connected in series for solving analytical problems are discussed. Two columns of the same length and very different polarity (SE-54 and PEG 20M) were used. Under isothermal conditions, owing to the carrier gas compressibility a noticeable increase in polarity is observed when the sequence of columns SE-54–PEG 20M is reversed. With temperature programming, an increase in the programming rate decreases the influence of the second column. By changing the programming rate during the analysis it is possible to optimize the column polarity to obtain the best separations. An application to the analysis of lemon oil is reported; complete separation of the most important components was achieved.

INTRODUCTION

The analysis of very complex mixtures by gas chromatography is still a problem because, in spite of the high efficiency achieved with capillary columns, it is not always possible to obtain a complete separation in a single column. To solve this problem different approaches have been tried: (1) parallel columns with a single injector and multiple detection; (2) mixed phase columns; and (3) columns connected in series.

For the first method it is necessary to have different detectors and recorders and to carry out a complicated data analysis. The second method is limited to the use of miscible liquid phases and only one polarity is obtained for each column. In the third method it is possible to use a single injector and detector, and the polarity of the coupled columns can also be changed.

Packed gas chromatographic columns have been connected in series to solve some specific problems[1–5] and more recently capillary columns have been used. Pretorius and Smuts[6] and Kaiser and Rieder[7] developed a procedure based on connecting in series columns of the same length, with independent temperature control for each.

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A general theory was developed by Purnell and co-workers on the use of these columns under isothermal conditions[8] and they investigated the resulting efficiency[9]. Kaiser *et al.*[10] presented a method based on the use of a pressure regulator placed between two different columns; by changing their flow-rates it is possible to change the polarity of the system.

In this work a system obtained by connecting in series two columns of very different polarity (SE-54 and PEG 20M) was studied and the effect of changing the temperature programme was investigated. The method has been applied to the analysis of lemon oil and a complete separation of the most important compounds was achieved, whereas this was not possible with any single column[11].

EXPERIMENTAL

Two glass capillary columns of the same length and diameter (15 m × 0.20 mm I.D.) were coated with PEG 20M as described elsewhere[12] and with SE-54 as reported by Grob[13]. The stationary phases were cross-linked and hence could be solvent washed and were more stable during temperature programming.

The two columns were connected in series either with PTFE tubing or with a Supelco butt-connector (Cat. No. 2-3796), taking care to avoid any dead volume. The columns were tested by injections of a standard mixture containing *n*-octanol (Oct), 2,6-dimethylaniline (DMA), methyl decanoate (E_{10}), phenol (Ph), *o*-, *m*- and *p*-cresol (Cres), 2,6-dimethylphenol (DMF) and some *n*-alkanes from C_9 to C_{22}.

The columns were tested separately and also connected in different sequences. The measurements were carried out under various isothermal conditions in the range from 90 to 150°C and with temperature programming from 80 to 180°C at rates of 0.5, 1.0, 2.0, 4.0, 8.0 and 12.0°C/min. The temperature programming rate (TPR) was also changed in the middle of the analysis time by using first a low rate followed by a high rate, or *vice versa*.

The analysis of lemon oil was carried out with the single columns and with the columns in series, optimizing the temperature programming rate.

A Hewlett-Packard Model 5890 gas chromatograph equipped with a flame ionization detector and a Shimadzu Model CR-34 integrator was used with hydrogen as the carried gas.

All chemical used were obtained from Carlo Erba (Milan, Italy) and the lemon oil was a commercial product from Sicily.

RESULTS AND DISCUSSION

The efficiency of the columns alone and connected in series was measured with a standard mixture, at 100°C, of tetradecane, 2,6-dimethylaniline and methyl decanoate. The results, reported in Table I, show that the number of theoretical plates of the two columns connected in series is about 80% of the sum of the values for the individual columns at the same linear gas velocity (44 cm/s).

The best efficiency is obtained for methyl decanoate, which has similar values of the capacity ratio (k') for both columns. The values of k' for the standard mixture *versus* column fraction are shown in Fig. 1. The values for the two columns connected in series are not exactly 0.5 for the effect of the carrier gas compressibility. The

TABLE I

COLUMN EFFICIENCY FOR THE TWO DIFFERENT COLUMNS AND THEIR COMBINATION IN SERIES FOR SOME REFERENCE COMPOUNDS

Compound	*SE-54*		*SE-54–PEG 20M*		*PEG 20M–SE-54*		*PEG 20M*	
	k'	*n*	*k'*	*n*	*k'*	*n*	*k'*	*n*
DMA	4.06	64 800	11.32	100 000	14.15	102 000	21.04	60 500
C_{14}	17.56	69 000	10.80	95 000	8.60	99 000	2.10	65 000
E_{10}	10.96	65 700	8.74	115 000	8.16	120 000	6.13	60 200

contribution of PEG 20M is 0.45 with the columns in the order SE-54–PEG 20M and 0.58 for the order PEG 20M–SE-54.

It is interesting that, with the large difference in polarity between these two columns, the most retained compounds in one column are the least retained in the other, and consequently there are no great increases in the total analysis times when using the two columns connected in series. The retention indices (RI) measured for some test substances on the SE-54 column in the temperature range 90–140°C are reported in Table II. Table II also gives the ΔI values relative to the SE-54 column

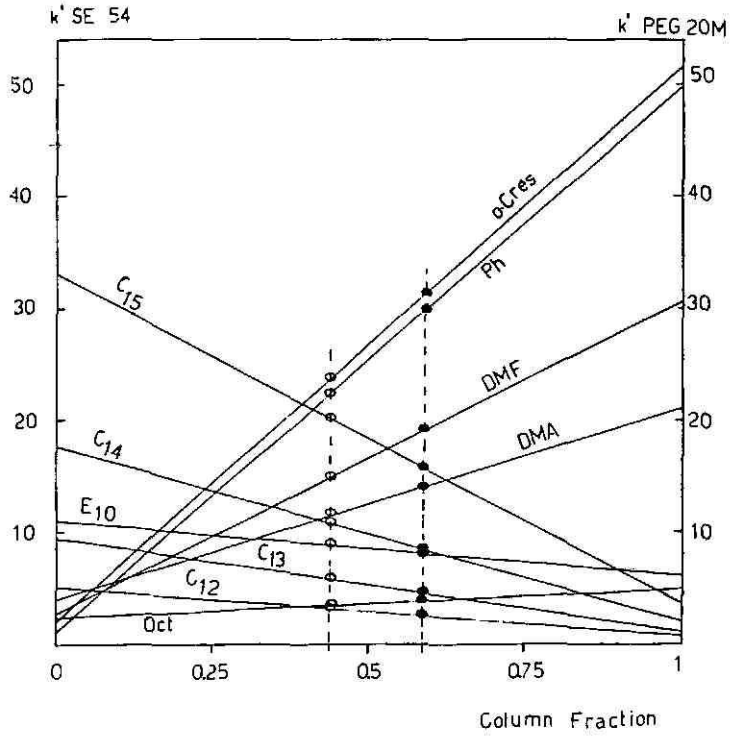

Fig. 1. *k'* values of standard compounds *versus* column fraction with columns in different orders: ○, SE-54–PEG 20M; ●, PEG 20M–SE-54.

TABLE II

RETENTION INDICES (RI) ON AN SE-54 COLUMN AND THEIR INCREMENTS (ΔI) ON THE OTHER COLUMNS

Temperature (°C)	*RI (SE-54)*					*ΔI (SE-54–PEG 20M)*					*ΔI (PEG 20M–SE-54)*					*ΔI (PEG 20M)*				
	Oct	E_{10}	*DMA*	*Ph*	*m-Cres*	*Oct*	E_{10}	*DMA*	*Ph*	*m-Cres*	*Oct*	E_{10}	*DMA*	*Ph*	*m-Cres*	*Oct*	E_{10}	*DMA*	*Ph*	*m-Cres*
90	1070	1327	1167	976	1075	144	36	236	538	510	200	60	305	624	595	478	220	628	878	955
100	1071	1326	1171	975	1072	143	40	236	538	510	200	65	308	625	593	479	235	633	983	962
110	1072	1325	1172	974	1072	143	43	239	536	509	200	70	313	624	599	478	267	642	991	971
120	1072	1324	1170	974	1072	141	46	236	535	508	200	75	312	622	600	478	269	644	999	986
130	1072	1324	1184	975	1072	140	48	236	532	506	201	75	315	620	602	479	276	648	1005	987
140	1073	1323	1190	974	1073	140	51	236	530	504	202	81	318	619	602	478	277	653	1011	992

for the columns connected in series in the two different orders and for the PEG 20M column alone.

The polarity, as shown, increases in the order SE-54, SE-54–PEG 20M, PEG 20M–SE-54, PEG 20M. With temperature programming the SE-54 column does not show a noticeable variation of RI values with the programming rate, and the PEG 20M column shows only small increments. With the columns connected in series there is, as expected, a large effect, as shown in Fig. 2. With the columns in the order SE-54–PEG 20M the RI decreases with TPR, whereas in the order PEG 20M–SE-54 it increases. This opposite behaviour is explained by the fact that the compounds

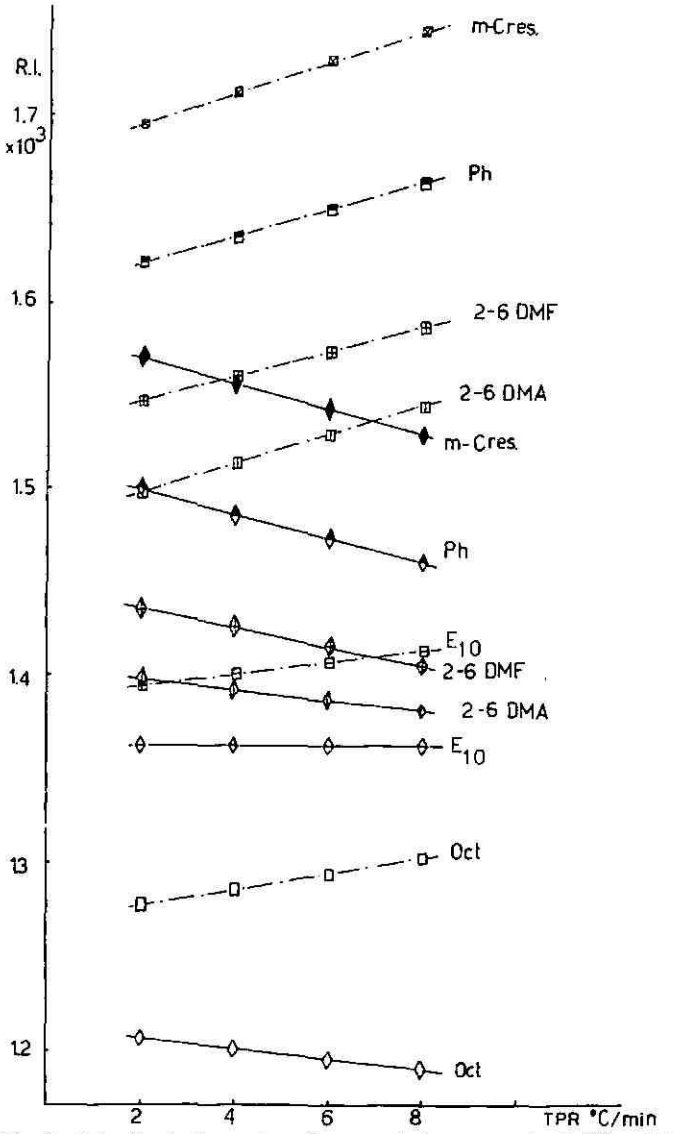

Fig. 2. Retention indices values for some test compounds at different temperature programming rates. (- - - -), PEG 20M–SE-54; (———), SE-54–PEG 20M.

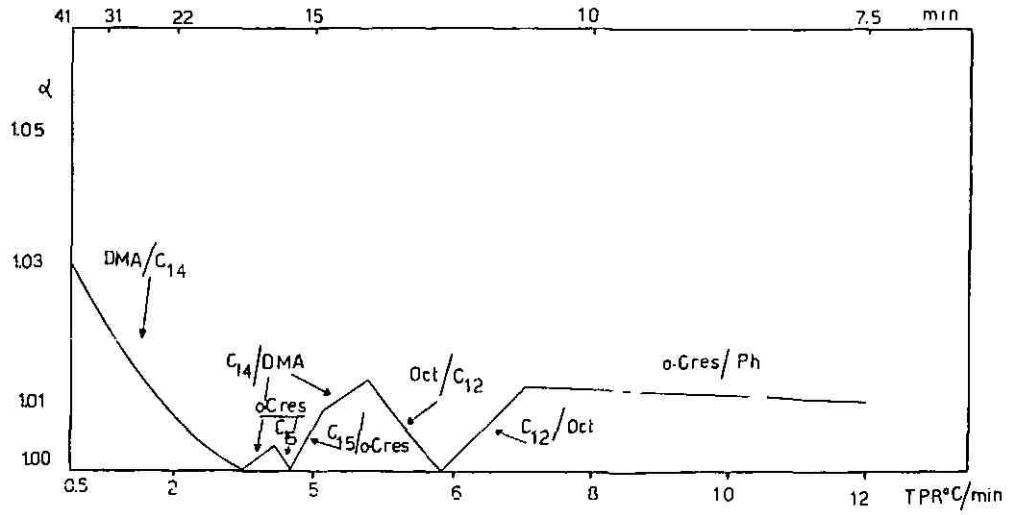

Fig. 3. Plots of separation factor (α) versus temperature programming rate. Column order: SE-54–PEG 20M.

enter the second column at higher temperatures than the first, and consequently the influence of the second column decreases. The system becomes more and more polar on increasing the TPR in the order PEG 20M–SE-54, and less and less polar in the opposite order SE-54–PEG 20M.

Fig. 3 shows a "window" diagram obtained by plotting the separation factor,

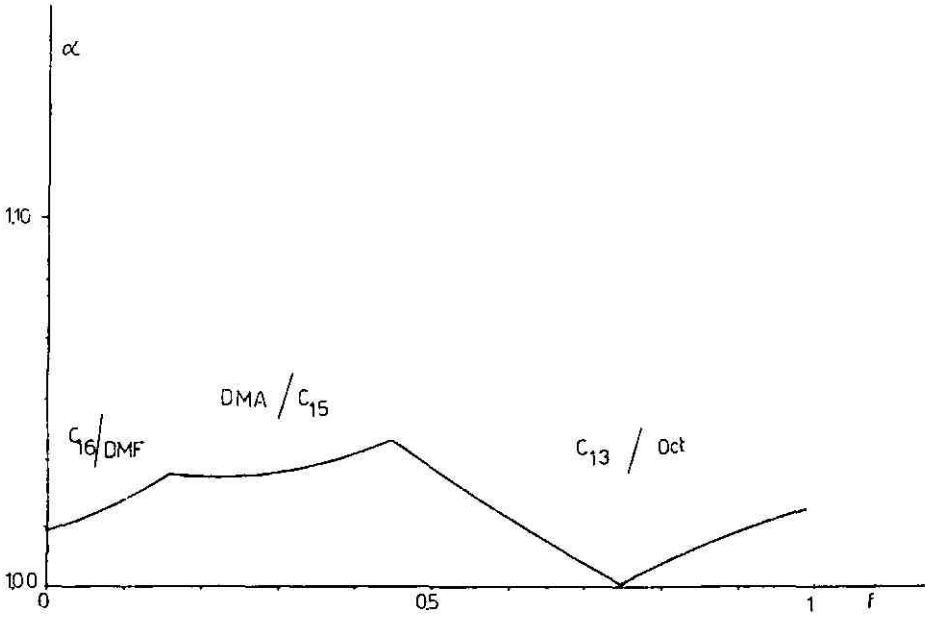

Fig. 4. Plots of separation factor (α) versus temperature increase ratio ($\Delta T_i/\Delta T$). Column order: PEG 20M–SE-54.

α, against log TPR for some pairs that are difficult to separate. As can be seen, there are two different programming rates (at about 5 and 7°C) where good separations are achieved, but as we consider also the analysis time reported in the upper scale, the second TPR is to be preferred, because almost the same separations are obtained but in a shorter time.

The polarity of the system obtained by connecting the columns in series can also be changed if two temperature programming rates are used. In this instance, by using first a high TPR and then a low TPR, or *vice versa*, the polarity changes, but the analysis time remains almost constant. Fig. 4 shows a "window" diagram for the PEG 20M–SE-54 system obtained by changing the TPR, always at the same time (8.25 min), which corresponds to half of the analysis time. The separation factor for some pairs is plotted against the ratio $\Delta T_1/\Delta T$, which is the ratio between the temperature increment from the beginning to the half-time and the temperature increment from the beginning to the end.

In this way the ratio goes from 0 (isothermal conditions for the first part and a rate of 10°C/min for the second part), to 1 (isothermal temperature in the second part after an increase at 10°C/min in the first part). The column polarity changes, and with $\Delta T_1/\Delta T < 0.5$ for compounds eluted before the middle of the chromatogram, the second apolar column (SE-54) is the more important because it is still at

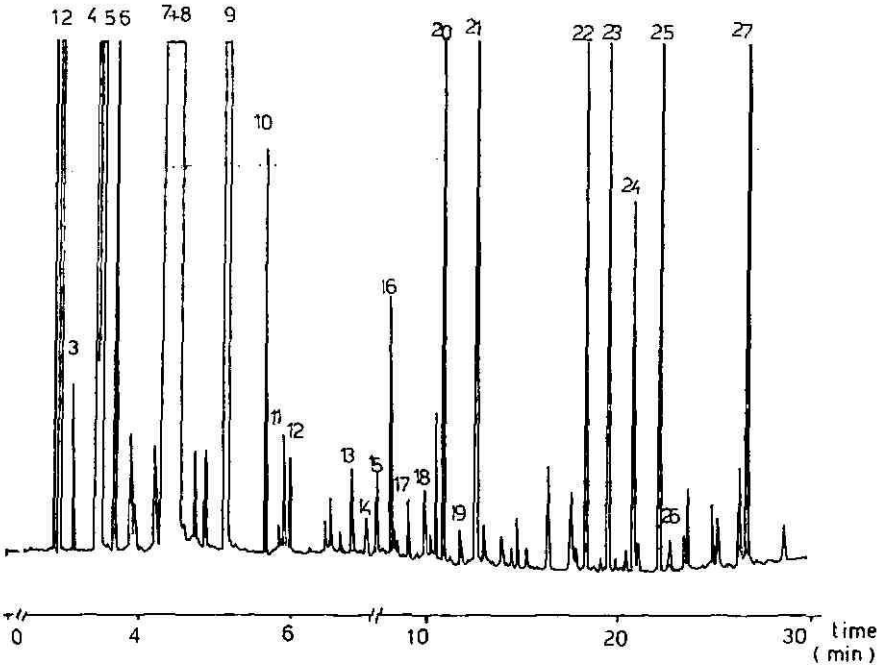

Fig. 5. Chromatogram of a lemon essential oil on an SE-54 column. $\bar{U}$ = 45 cm/s; T = 60°C for 5 min, then programmed at 2°C/min to the end. Compounds: 1 = α-thyjene; 2 = α-pinene; 3 = camphene; 4 = sabinene; 5 = β-pinene; 6 = myrcene; 7 = limonene; 8 = p-cymene; 9 = γ-terpinene; 10 = terpinolene; 11 = linalool; 12 = nonanal; 13 = citronellal; 14 = decanal; 15 = 4-terpinenol; 16 = α-terpineol; 17 = citronellol; 18 = nerol; 19 = geraniol; 20 = neral; 21 = geranial; 22 = neryl acetate; 23 = geranyl acetate; 24 = caryophyllene; 25 = bergamotene; 26 = humulene; 27 = β-bisabolene.

low temperature, whereas for late-eluted compounds the second column becomes less important because its temperature rapidly increases.

An opposite behaviour occurs with $\Delta T_1/\Delta T > 0.5$ and, of course, also on changing the column sequence. In this way many different situations can be obtained and adapted to any particular analysis.

Analysis of an essential oil

Among the many possible applications of the described system, we analysed a lemon oil. Because of the large number of components of different polarity, it is very useful to have the facility to change the column polarity to obtain the best separation.

Figs. 5 and 6 show the best chromatograms obtained on the single columns. With SE-54 the pair limonene–*p*-cimene is unresolved and a very little separation is achieved for sabinene–β-pinene. With PEG 20M alone there is a good separation of many compounds but the pair α-thujene–α-pinene is not resolved at all, and a very poor resolution is obtained for geranial–β-bisabolene.

With the columns connected in series, after some attempts, a complete separation for all the major components was achieved with the columns in the order PEG 20M–SE-54, as shown in Fig. 7. The working conditions reported give a good separation in a short analysis time. By changing the order of the columns to SE 54–PEG 20M no complete separation could be obtained using any temperature programme.

Hence by connecting two capillary columns of different polarity in series, a column system of tunable polarity can be obtained simply by changing the temper-

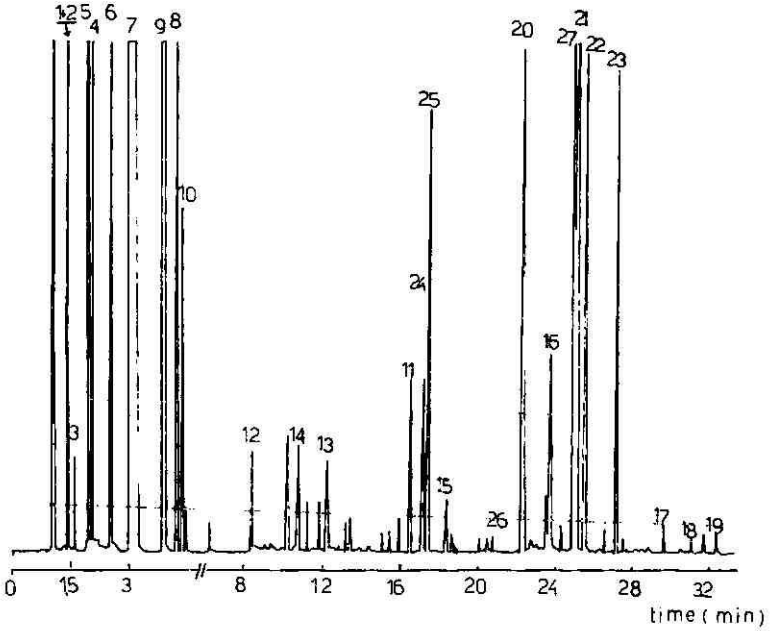

Fig. 6. Chromatogram of the same lemon oil as in Fig. 5 on a PEG 20M column under the same operating conditions. Peaks as in Fig. 5.

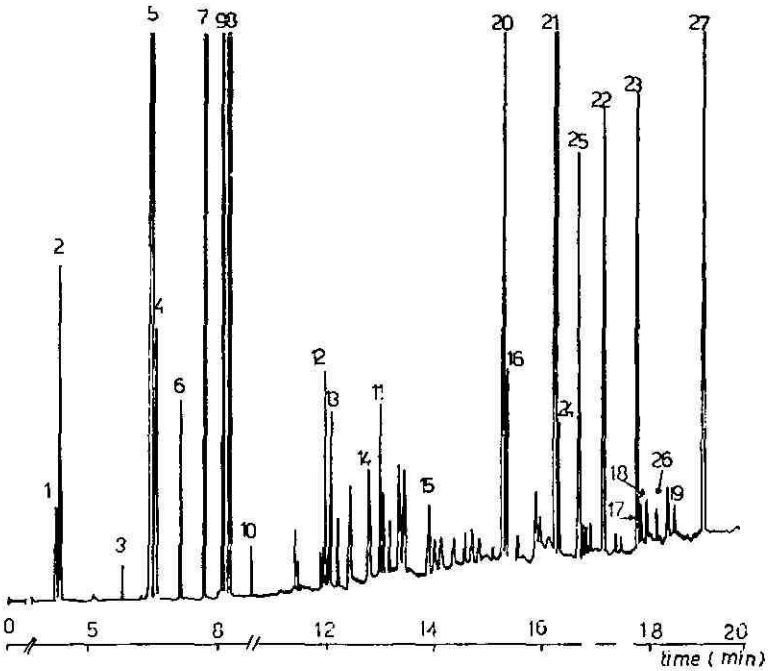

Fig. 7. Chromatogram of the same lemon oil as in Fig. 5 on a PEG 20M–SE-54 column system. Temperature programmed from 35 to 90°C at 5°C/min, then from 90°C to the end at 2°C/min. Peaks as in Fig. 5.

ature programming rates. This column system can easily be installed in any commercial apparatus without any modification, and it gives a very versatile system that can be applied to the analysis of many complex samples.

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QUANTIFICATION OF 2-KETO-3-DEOXYOCTONATE IN (LIPO)POLYSACCHARIDES BY METHANOLYTIC RELEASE, TRIFLUOROACETYLATION AND CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Several conditions of acidic anhydrous methanolysis were examined to optimize the release and minimize the degradation of unphosphorylated 2-keto-3-deoxy-D-*manno*-octonic acid (KDO) from bacterial lipopolysaccharides and polysaccharides. The reaction was monitored by capillary gas chromatography after derivatization by trifluoroacetic anhydride. The best results were obtained by use of 2 *M* hydrochloric acid at 60°C for 2 h. Under these conditions a single KDO component appeared, and KDO was quantitatively released from all model compounds except when glycosidically linked to hexosamines. For quantitative cleavage of this linkage a reaction time of 6 h was required at 60°C, giving rise to 5–10% of secondary KDO products. The KDO detection limit was about 250 pmol (50 ng) and the molar response was the same as for glucose. The KDO derivative gave a mass spectrometric fragmentation pattern consistent with a pyranosidic methylketoside methyl ester structure. Differentiation of KDO linkage types could be obtained by determination of the rates of KDO release by mild methanolysis.

INTRODUCTION

Lipopolysaccharides (LPS) of Gram-negative bacteria consist of a polysaccharide and a lipid A part, usually linked together by 2-keto-3-deoxy-D-*manno*-octonic acid (KDO)[1]. In some capsular polysaccharides (PS) KDO is a major constituent, *e.g.*, in K12[2] and K13[3] of *Escherichia coli*, and in the group-specific PS of *Neisseria meningitidis* 29e[4].

Quantification of the KDO content in bacterial PS or LPS is usually performed by spectrophotometric methods involving a hydrolysis step, as in the most commonly used thiobarbituric acid assay[5–7]. These methods suffer from several limitations. Major difficulties are due to the lability of KDO under the acidic conditions required for its release[8] and to interferences from other deoxy sugars that frequently occur in Gram-negative bacteria[5], *e.g.*, neuraminic acid[9]. Further, substitution in the 4- or 5-position of KDO may give the chromophore in low yield[10].

When the release of KDO is performed by hydrochloric acid in methanol in-

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stead of by acidic hydrolysis, degradation apparently can be reduced[11,12]. Thus, the KDO content of LPS can be determined with reasonable accuracy by methanolysis followed by gas chromatography (GC), although a complex KDO peak pattern is observed[11]. We wanted to overcome this difficulty by establishing more optimal conditions for the release of KDO. In this work we studied the appearance of KDO products during methanolysis at lower temperatures for various periods of time. For most ketosidic and glycosidic linkages complete release and negligible degradation were obtained at 60°C for 2 h, and by use of defined conditions we could discriminate between several types of KDO linkages simultaneously present in the same polymer.

EXPERIMENTAL

Materials

Solvents of analytical-reagent grade (Rathburn Chemicals, Walkerburn, U.K.) were used without further purification. Methanolic hydrochloric acid was prepared by bubbling dry hydrogen chloride gas (Messer Griesheim, Düsseldorf, F.R.G.) into methanol until saturation and dilution to 2 *M* with methanol.

α-Methyl mannoside and *meso*-inositol were obtained from Koch-Light (Colnbrook, U.K.), methyl *n*-heptadecanoate from Sigma (St. Louis, MO, U.S.A.) and C^2H_3OH of Uvasol grade, trifluoroacetic (TFA) anhydride and acetonitrile from Merck (Darmstadt, F.R.G.). Synthetic 3-hydroxymyristic acid (3-OH-14:0) and myristoxymyristic acid was a gift from Ernst Th. Rietschel (Forschungsinstitut Borstel, F.R.G.), synthetic KDO (ammonium salt) from Chris Galanos [Max-Planck-Institut für Immunbiologie (MPI), Freiburg, F.R.G.], authentic L-glycero-D-*manno*-heptose and rough LPS (Rd and Re) of *Salmonella minnesota* from Otto Lüderitz (MPI) and *Escherichia coli* polysaccharides K12, K13 and K82 from Klaus Jann (MPI). LPS of *N. meningitides* was from Carl Frasch (Office of Biologics, FDA, MD, U.S.A.).

Smooth LPS of *Yersinia enterocolitica* O3 and of several salmonellae were prepared in our laboratory by the hot aqueous phenol procedure[13]. Capsular polysaccharide of *N. meningitidis* group 29e was obtained by either hot or cold buffered[14] phenol extraction. Both LPS and polysaccharide were purified by ultracentrifugation twice at 105 000 *g* for 4 h. An extract containing LPS and the polyneuraminic acid PS of serogroup C of *N. meningitidis* was obtained by the hot phenol method.

Methanolysis and trifluoroacetylation

Lyophilized aliquots of polysaccharides and LPS (10–100 μg) were methanolysed (2 *M* hydrochloric acid; 0.1–1 ml) in PTFE-lined screw-capped 2-ml vials. After flushing with nitrogen, the vials were heated as indicated. In kinetic experiments methanolysis was stopped by chilling (−50°C with acetone–dry-ice) and by diluting three-fold with cold methanol. Hydrochloric acid and methanol were removed with nitrogen and the residue was converted into the TFA derivatives[11]. Before injection into the gas chromatograph the TFA anhydride concentration was decreased to 10%.

Gas chromatography and mass spectrometry (MS)

GC analyses were carried out on a fused-silica capillary column (25 m × 0.2 mm I.D.) with SE-30 methylsilicone as the stationary phase (Hewlett-Packard, Avondale, PA, U.S.A.), operated in the splitless mode with helium as carrier gas and

programmed from 90°C (2 min) to 270°C at 8°C min^{-1}. Several samples were also chromatographed on a more polar methylsilicone column (5% phenyl, SE-54) under similar conditions.

GC–MS was carried out with a Hewlett-Packard 5992A apparatus equipped with a glass capillary column (25 m × 0.5 mm I.D.) containing CP-Sil 5 (methylsilicone) (Chrompack, Middelburg, The Netherlands) with helium as carrier gas. Mass selection was performed at 70 eV at 170°C, with scanning of masses from 50 to 700. Additional analyses were performed on a Finnigan 4023 instrument at the Centre for Industrial Research, Oslo.

Primary identification of the GC peaks was made by comparison of retention times with those of methanolysed reference substances[11]. The identities were confirmed by MS and interpretation of the fragmentation patterns was verified by additional MS analyses after methanolysis in C^2H_3OH.

Gas chromatographic quantification of KDO and other components

meso-Inositol, α-methyl mannoside and methyl *n*-heptadecanoate were used as internal standards, added after lyophilization of the samples. Relative molar response factors were determined by methanolysis of polymers of known structures (Tables I and II). The LPS marker 3-hydroxytetradecanoic acid (as the TFA derivative of the methyl ester) was given a response value of 1.00[11].

Spectrophotometric determination of KDO

The thiobarbituric acid assay was applied as described by Brade *et al.*[15], with hydrolysis in 1 *M* hydrochloric acid for 4 h in sealed ampoules, followed by periodate treatment. The calibration graph was based on the ammonium salt of KDO, hydrolysed in four different amounts (32–96 μg), each hydrolysate being processed further in duplicate.

RESULTS

Release of KDO

Various conditions of methanolysis were examined in order to optimize the yield of liberated KDO. Different KDO-containing polymers with known structures were methanolysed (2 *M* hydrochloric acid) at 37, 60 and 85°C for various periods of time, and the reaction products were analysed as TFA derivatives by capillary GC.

When the *E. coli* polysaccharides K12 (Rha-Rha-KDO)n and K13 (Rib-KDO)n[2,3] were methanolysed at 85°C, a time-dependent change in the GC peak pattern was apparent. KDO appeared primarily as a single peak (A in Fig. 1) which slowly decreased, accompanied by the appearance of a new major (heterogeneous) peak (B) and several smaller peaks. The sum of peak areas A and B rapidly rose to a maximum and then fell to *ca.* 70% of the maximum after 12–16 h. At this stage the ratio of the areas of peaks A and B was *ca.* 1.2:1. This characteristic chromatographic development was reasonably reproducible and largely independent of the nature of the KDO-containing polymer, as indicated earlier in studies on LPS[11]. We have now observed this peak pattern for a large number of LPS and polysaccharide preparations. However, owing to its complexity (Fig. 1B) the conditions were clearly not optimal for more accurate KDO determinations.

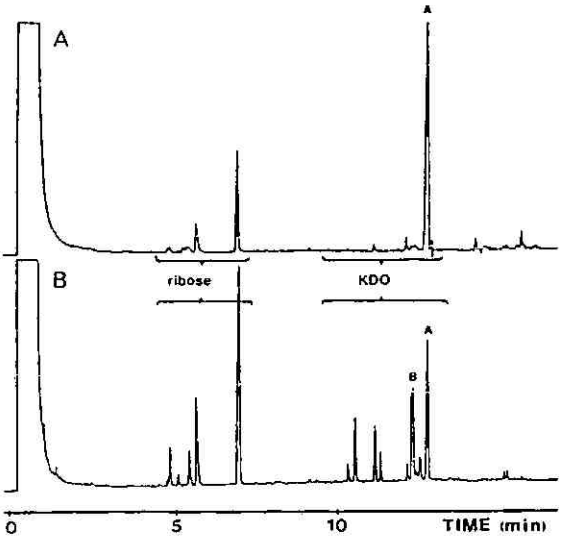

Fig. 1. Gas chromatography of methanolysed *E. coli* K13 polysaccharide (Rib*f*1-7KDO)n on a 25 m OV-1 fused-silica capillary column. Temperature programmed from 90 to 270°C at 8°C/min. Splitless injection. (A) 2 *M* HCl, 60°C, 2 h; (B) 2 *M* HCl, 85°C, 18 h. Peak A, TFA-derivatized methylketoside methyl ester of KDO; peak B, TFA-derivatized degradation product.

On lowering the temperature to 60°C only a single KDO peak (peak A, Fig. 1) was initially recorded, and the two polysaccharides K12 and K13 were completely cleaved in about 1 h (Table I). After 2 h peak B constituted about 5%, increasing to 5–10% after 6 h and 10–15% after 8 h. For *Salmonella* LPS a more complex development appeared during methanolysis. Less than 10 min were sufficient for complete release of KDO from Re LPS, containing solely a KDO disaccharide unit attached to lipid A[16]. For Rd LPS, with a terminal heptose linked to the KDO cluster[16], a biphasic release of KDO was observed (Fig. 2). About 60% was split off in 10 min, and about 90 min were required for completeness of the reaction, indicating a relatively slow cleavage of the heptose–KDO bond.

The use of a temperature of 37°C for methanolysis of K12 and K13 drastically decreased the rate of KDO release (Table I), and only KDO peak A was detected. At this low methanolysis temperature intermittently high amounts of oligosaccharides were formed; for K13 as much as 38% appeared as disaccharides after 15–20 min, which may be of interest for the isolation of KDO-containing oligosaccharides.

Release rates of KDO from various types of linkages

The biphasic methanolytic release of KDO from *Salmonella* Rd LPS (Fig. 2) allowed the selective quantitation of KDO moieties that were glycosidically or ketosidically linked. As seen in Table I, the half-times for release of KDO varied considerably with the type of linkage involved. The following order of increasing stability towards methanolysis was found: ketosidic < ribofuranosidic < rhamnosidic < heptosidic < hexosaminidic. With the exception of the last linkage, all were cleaved

TABLE I

STABILITY OF SOME COMMON (LIPO)POLYSACCHARIDE LINKAGES TOWARDS METHANOLYSIS

Samples were methanolysed (2 M hydrochloric acid) at 37, 60 and 85°C and aliquots were analysed by GC as indicated in the text.

*Linkage**	*Parent structure*	*Time required for 50% release (min)*		
		37°C	*60°C*	*85°C*
KDO2–6GlcN	*Salmonella* LPS; Re; Rd	10	⩽5	–
KDO2–4KDO	*Salmonella* LPS; Re; Rd	10	⩽5	–
Hep1–5KDO	*Salmonella* LPS Rd	400	30	⩽10
Rib*f*1–7KDO	*E. coli* K-13 polysaccharide	40	–	–
Rha1–5KDO	*E. coli* K-12 polysaccharide	120	15	–
GalN1–7KDO	*N. meningitidis* polysaccharide 29e	–	70	–
Abq1–3Man	*Salmonella abortus-equi* LPS	<5	–	–
Man1–4Rha	*Salmonella abortus-equi* LPS	75	–	–
Gal1–4Glc	Lactose; fucosyllactose	75	15	–
Fuc1–2Gal	Fucosyllactose	–	<5	–
GlcN–(3-OH-C_{14}) (amide)	*Salmonella* LPS	–	⩾960	360
GlcN–(3-OH-C_{14}) (ester)	*Salmonella* LPS	–	45	15
(3OH-C_{14})–C_{16} (acyloxy)	*Salmonella* LPS	35	5	–
(3-OH-C_{14})–(2-OH-C_{14}) (acyloxy)	*Salmonella* LPS	35	5	–

* Abbreviations: KDO, 2-keto-3-deoxy-D-*manno*-octonic acid; GlcN, D-glucosamine; Hep, L-glycero-D-*manno*-heptose; Rib*f*, D-ribofuranose; Rha, L-rhamnose; Abq, D-abequose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; Fuc, L-fucose; 3-OH-C_{14}, 3-hydroxymyristic acid; 2-OH-C_{14}, 2-hydroxymyristic acid; C_{16}, palmitic acid. –, Not determined.

quantitatively (>95%) within 2 h at 60°C. The galactosamine–KDO linkage required 6 h at 60°C or 2 h at 85°C for complete cleavage.

For comparison, Table I also shows the stabilities of some linkages not involving KDO, but frequently occurring in LPS or polysaccharides. A very rapid release of 3,6-dideoxyhexoses was apparent (abequose and tyvelose behaved similarly). Amide linkages showed high stability. There was a marked difference between fatty acids ester-linked to glucosamine and those with an acyloxy linkage to the 3-hydroxy group of amide-bound fatty acids. Hence three types of fatty acid linkage may be discriminated by methanolysis.

Molar response

The molar response of KDO and some other sugars (after methanolysis at 60°C for 2 h) was established by the use of structurally well defined polymeric saccharides such as the two *E. coli* polysaccharides K12 and K13. The first of these (Rha-Rha-KDO)n in combination with the disaccharide rutinose (Rha-Glc) revealed a KDO molar response of 0.44. This value was also obtained by use of K13 (Rib-KDO)n in combination with D-ribose. By coincidence this value is the same as for

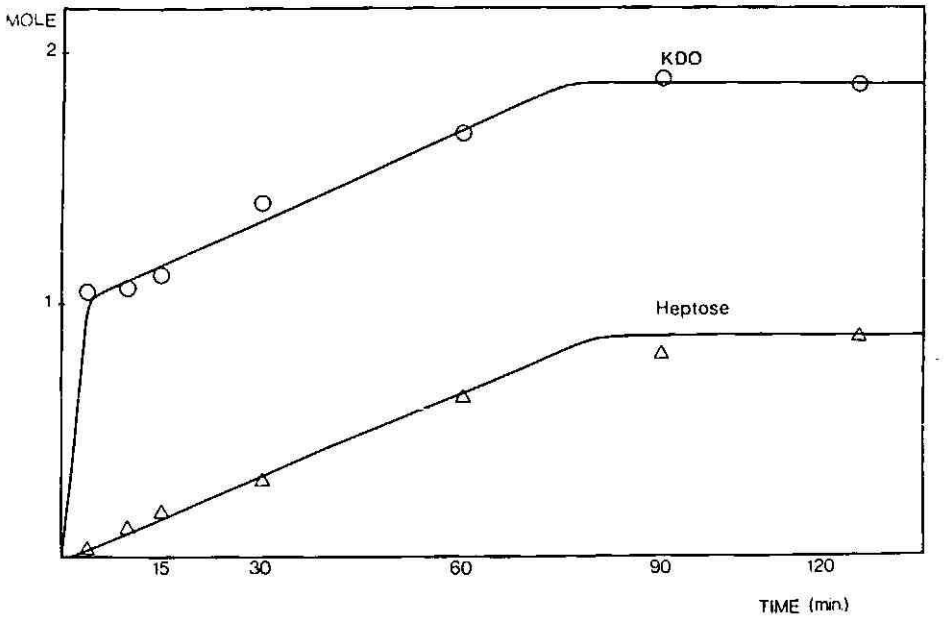

Fig. 2. Release of KDO and L-glycero-D-*manno*-heptose from *Salmonella* Rd LPS at different times of methanolysis (2 *M* HCl, 60°C). Aliquots were withdrawn at the indicated intervals and treated as described in the text.

glucose (Table II), and slightly lower than reported earlier[11]. The detection limit for KDO was about 250 pmol (50 ng) when analysed on a well used GC column.

GC–MS analysis of KDO methanolysis products

The mass spectrum of the primary and main methanolysis product formed under mild conditions (peak A, Fig. 1) is shown in Fig. 3. The fragmentation is consistent with a per(trifluoroacetylated) O-methylketoside of methyl 3-deoxyoctu-

TABLE II

MOLAR RESPONSE FACTORS OF KDO IN RELATION TO SOME OTHER SUGARS

Samples were methanolysed (2 *M* hydrochloric acid; 60°C for 2–6 h); see text and Table I for experimental details and abbreviations. Response of glucose was set as 0.44 (ref. 11).

Sugar	*Parent structure*	*Molar response*
D-Glucose	–	0.44
D-Galactose	Fucosyllactose (Fucl-2Gall-4Glc)	0.44
L-Fucose	Fucosyllactose	0.38
L-Rhamnose	Rutinose (Rha1-6Glc)	0.38
D-Ribose	D-Ribose	0.32
KDO	*E. coli* polysaccharide K12; (-3Rha1-2Rha1-5KDO2-)n	0.44
	E. coli polysaccharide K13; (-3Rib*f*1-7KDO2-)n	0.44

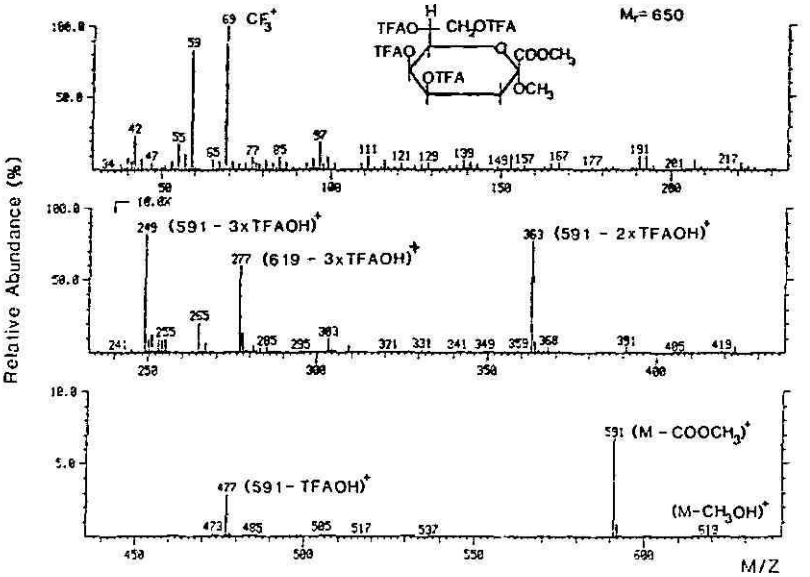

Fig. 3. Electron-impact mass spectrum with suggested fragment composition of TFA-derivatized methylketoside methyl ester of KDO (peak A in Fig. 1).

losonate in a pyranose ring. No molecular ion (*m/z* 650) was seen, but minor fragments of *m/z* 631 ($M^+ - F$) and *m/z* 619 ($M^+ - CH_3OH$) were occasionally observed. A prominent fragment was *m/z* 591 (loss of $COOCH_3$), and the series *m/z* 477, 363 and 249 formed by additional loss of one to three TFAOH groups. Such sequential loss of TFAOH or TFAO appears to be typical of acetylated sugars[17,18]. MS of the secondary peak B was complicated by its heterogeneity. However, constant spectra were obtained throughout the peak, indicating a mixture of chemically closely related substances. These appear to be entirely different from the primary (peak A) compound, as several distinct fragment ions of peak A were completely missing from the mass spectra of peak B. A prominent fragment (*m/z* 59) arising from the carboxyl methyl ester group was present in the spectra of both peaks A and B, indicating an absence of a lactone ring. Hence an anhydro structure is suggested for the conversion product (peak B) of KDO after methanolysis at 85°C.

Comparison of the GC method and spectrophotometry for determination of KDO

The KDO contents of LPS from *N. meningitidis* and of the capsular PS K82, (Rha-Rha-KDO)n from *E. coli* were determined both by the GC procedure (methanolysis in 2 *M* hydrochloric acid at 60°C for 2 h) and by the thiobarbituric acid assay. From Table III it appears that the values obtained by GC vary slightly less, especially when a new column is used. Apparently the amounts of KDO found in the LPS by GC (15.7%) is in good agreement with structural data (see Table III), assuming 3 mol of KDO per mole of LPS. In comparison, the corresponding value obtained by spectrophotometry probably is too high owing to a higher rate of degradation of the standard monomeric KDO than for the KDO bound in LPS (see Discussion). For both methods low KDO values were obtained for polysaccharide

TABLE III

KDO IN (LIPO)POLYSACCHARIDES DETERMINED BY SPECTROPHOTOMETRY AND METHANOLYSIS–GAS CHROMATOGRAPHY

The spectrophotometric method used was the thiobarbituric acid assay, with hydrolysis in 1 *M* hydrochloric acid at 100°C for 4 h[15]. For GC the methanolysis was performed in 2 *M* hydrochloric acid at 60°C for 2 h, followed by trifluoroacetylation and GC (see Fig. 1 and text).

Material	*Method*	*Samples (n)*	*Mean content of KDO (%)*	*S.D.**
LPS of strain M986	GC	5	15.7	0.6
*N. meningitidis***	Spectrophotometry	5 × 2	23.3	1.2
	Calculated***	–	17.0	–
E. coli poly-	GC; old column	5	31.3	1.8
saccharide K-82	GC; new column	5	33.2	1.0
	Spectrophotometry	3 × 2	38.7	2.7
	Spectrophotometry	5 × 2	33.5	2.3

* S.D., standard deviation. For the GC method values were determined from a single GC profile of separately methanolysed samples; for the spectrophotometric method each hydrolysate was processed in duplicate and the means used for calculation of standard deviations.

** Quantification of LPS in the samples was based on the 3-OH-14:0 fatty acid determined by GC[11], and the calculation was based on 6 mol of fatty acids per mol and the published structure of *N. meningitidis* oligosaccharide type L3[19,20].

*** The content of KDO in LPS of *N. meningitidis* is unknown. Assuming 3 mol of KDO per mole of LPS, as for enterobacteria[16], a KDO value of 17% was calculated.

K82 (theoretical value 43%). This may be due to an unknown content of water or other impurities, as the accuracy of the KDO value recorded by GC was supported by the rhamnose:KDO ratio of 2.0:0.93 (2:1 expected).

One important advantage of the GC method for KDO determinations, the lack of interference from other deoxy sugars, is illustrated in Fig. 4. In a phenol–water extract of *N. meningitidis* of serogroup C, with both LPS and the sialic acid-containing group PS, KDO and neuraminic acid could be simultaneously determined, the latter sugar being only partly N-deacetylated under these mild conditions.

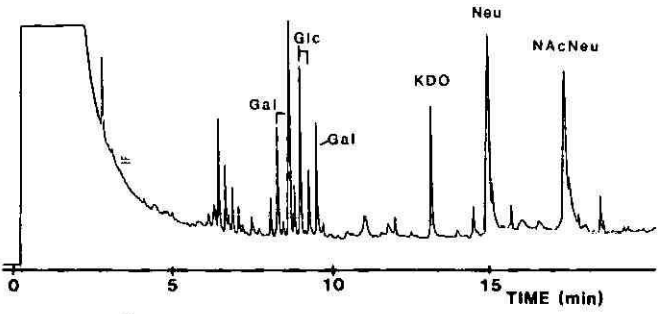

Fig. 4. Gas chromatography of a phenol–water extract of *N. meningitidis* serogroup C, containing LPS and capsular polysaccharide, after methanolysis in 2 *M* HCl at 60°C for 2 h and trifluoroacetylation. Conditions for GC as in Fig. 1. Abbreviations: Gal, galactose; Glc, glucose; Neu, neuraminic acid; NAcNeu, N-acetylneuraminic acid.

DISCUSSION

Release of KDO from (lipo)polysaccharides by acidic hydrolysis may cause a major conversion of the labile sugar to lactone[21,22] or anhydro ring forms[23]. Because as much as 70% of KDO may be converted into products that do not contribute to the colour yield in the thiobarbituric acid assay[15], careful correction for the degradation caused by hydrolysis is required by use of authentic KDO. Brade *et al.*[15] recommended that the conditions for hydrolytic release of KDO should be optimized for each polymer to be analysed.

When hydrolysis is replaced with methanolysis for the release of sugars, these are stabilized as methyl glycosides and ketosides[1,24,25]. However, KDO degradation was not avoided during methanolysis in 2 *M* hydrochloric acid for 18 h at 85°C, our conditions for LPS composite analysis[11]. The major degradation product was probably an anhydro structure rather than a lactone, as judged by the MS analysis. When the release of KDO from LPS or PS was performed by 2 *M* hydrochloric acid in methanol at 60°C, the degradation was almost completely prevented. At this temperature KDO was released quantitatively from most linkages after 2 h (Table I). KDO appeared as a single structure (peak A, Fig. 1) identified by GC–MS as the methyl ester of the methylketoside. Although the glycosidic linkage between KDO and hexosamine required 6 h for quantitative cleavage, the method compares well in speed and simplicity with another GC method for the determination of KDO[12], requiring reaction times of 2 days. Hence acidic methanolysis at 60°C followed by GC of TFA derivatives appears to be a good approach for KDO determination.

Degradation of KDO might also be avoided by lowering the acid strength, but we found no obvious advantages with methanolysis in 0.1–1 *M* hydrochloric acid (data not shown). During methanolysis, loss of hydrochloric acid due to reaction with methanol may occur[26] and we prefer to use 2 *M* hydrochloric acid to ensure a sufficient concentration for consistent cleavage of the more resistant KDO linkages.

The methanolysis–GC method presented shows slightly less variability than the spectrophotometric determination of KDO (Table III). Although the exact amount of KDO in the *N. meningitidis* LPS is unknown, the GC value (15.7%) comes very close to what could be calculated (17%) assuming 3 mol of KDO per mol of LPS. The higher KDO value (23.3%) found by spectrophotometry can probably be explained by a faster degradation of monomeric KDO used to establish the calibration graph, and released from ketosidic linkages, than for the heptosyl-linked KDO in LPS. The latter KDO moiety is released considerably more slowly than ketosidically linked KDO[15], and hence may have been better protected from degradation during hydrolysis. For the methanolysis–GC procedure such biphasic KDO release presents no analytical problem, as degradation of KDO does not occur. The sensitivity (about 250 pmol) is comparable to that obtained by spectrophotometry[15]. A considerably higher sensitivity can probably be obtained by using an electron-capture detector to take advantage of the many fluorine atoms in TFA derivatives, or by utilizing mass spectrometric detection (selected ion monitoring) with *m/z* 591 as a diagnostic ion.

Phosphate esters of KDO may occur in LPS[16], and the phosphate linkages are resistant towards methanolysis and all commonly used conditions of aqueous hydrolysis. Analysis of phosphate-substituted KDO by liquid chromatography may be

an attractive alternative as localization of the phosphate group may also be achieved[27].

The advantage of the methanolysis–GC procedure for KDO determination in cases of an abundance of deoxy sugars that would interfere in spectrophotometric tests is illustrated in Fig. 4, where KDO from LPS and neuraminic acid from capsular PS could be determined simultaneously. Hence our procedure may have certain potential in the determination of KDO in meningococcal capsular PS vaccines of serogroups B, C, Y and W135. We have determined KDO without difficulty in the presence of more than a 100-fold excess of neuraminic acid in such preparations[28,29].

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ANALYSIS OF OLIGOMERIC AND MONOMERIC SACCHARIDES FROM ENZYMATICALLY DEGRADED POLYSACCHARIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The analysis of monomeric and oligomeric reaction products of enzymatically degraded polysaccharides using a cation-exchange, 8% cross-linked resin column in the lead(II) form (HPX-87P) is described. Digests of cellulose, xylan, arabinan and galactan prepared with various pure enzymes were characterized. Saccharides up to tetramers could be separated. With this method, information regarding enzyme purity, specificity and mode of action can rapidly be obtained.

INTRODUCTION

The analysis of the reaction products of enzymatically degraded polysaccharides provides valuable information on the substrate specificity and pattern of action of polysaccharide-degrading enzymes. Paper, thin-layer, high-performance thin-layer, ion-exchange and gel filtration chromatography have been used successfully for this purpose. High-performance liquid chromatography (HPLC) has simplified the difficult task of determining oligomeric and monomeric sugars. Its suitability was increased further by the introduction of fixed-ion resin columns[1], which has led to the development of ion-moderated partition chromatography and the design of specific resin columns for carbohydrate analysis. The resins have a polystyrene–divinylbenzene matrix with a degree of cross-linking ranging from 4 to 8%, and contain sulphonic acid groups which are loaded with a particular cationic counter ion. The resin is used in a non-ion-exchange mode and the cationic counter ion remains on the column, effecting the desired separation. The choice of cation determines the selectivity and resolution. Resins in the calcium(II) form have found general use in the separation of monosaccharides, oligosaccharides up to a degree of polymerization (DP) of 4 and sugar alcohols. Resins in the silver(I) form are more selective for oligosaccharides up to DP 11 and resins in the lead(II) form are more selective for a range of monomeric sugars[1].

In our studies on the enzymatic degradation of plant cell-wall polysaccharides, we were looking for an HPLC system which would enable the determination of the

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sugar composition of polysaccharide digests. Since these polysaccharides comprise various sugar residues, and since preliminary experiments showed that oligosaccharides of low DP could also be separated on a resin in the lead(II) form, an HPLC system with an HPX-87P column was applied to the analysis of polysaccharide digests. The saccharides present in the digests were identified by establishing their sugar composition. For this purpose they were isolated in fairly large quantities by gel permeation chromatography.

MATERIALS AND METHODS

Enzymes

Four types of endoglucanases (1,4-β-D-glucan glucanohydrolase, E.C. 3.2.1.4; Endo I, III, V and VI) and an exoglucanase (1,4-β-D-glucancellobiohydrolase, E.C. 3.2.1.91; Exo 1) were isolated from a commercial cellulase preparation as described by Beldman *et al.*[2]. Endoarabinanase (1,5-α-L-arabinan arabinohydrolase, E.C. 3.2.1.99), arabinofuranosidase (α-L-arabinofuranoside arabinohydrolase, E.C. 3.2.1.55), endogalactanase (1,4-β-D-galactan galactohydrolase, E.C. 3.2.1.89) and β-galactosidase (β-D-galactopyranoside galactohydrolase E.C. 3.2.1.23) were isolated from a fungal pectinase preparation kindly supplied by Gist-Brocades (Delft, The Netherlands)[3].

Substrates

The polysaccharides used as substrates were Avicel cellulose (Type SF; Serva, Heidelberg, F.R.G.) swollen in phosphoric acid as described by Wood[4], xylan (ex-oat spelts; Koch-Light, Haverhill, U.K.), arabinan (ex-sugar beets, Koch-Light) dialysed against distilled water and lyophilized prior to use, haze arabinan isolated from apple juice concentrate as described by Churms *et al.*[5] and galactan isolated from destarched potato fibre as described by Labavitch *et al.*[6] and treated with arabinofuranosidase to remove arabinose containing side chains from the galactan backbone. Table I summarizes the structural features of these polysaccharides.

Enzyme treatments

The substrates were dissolved or suspended (Avicel) in 0.05 *M* sodium acetate buffer pH 5. After the addition of the enzyme, the reaction mixtures were incubated at 30°C for various reaction times. The enzyme units added (expressed in μg protein per ml) and the incubation times are specified in the figure legends. The reactions were terminated by centrifugation of the residual substrate (cellulose, 10 min, 3000 *g*) and the enzymes in the clear reaction liquid were inactivated by immersion in a bath of boiling water for 5 min.

Apparatus

A Spectra Physics liquid chromatograph Model SP8000, equipped with an Aminex HPX-87P column (300 mm × 7.8 mm; Bio-Rad Labs., Richmond, CA, U.S.A.) and a guard column (50 mm × 4.6 mm) packed with a mixture of equivalent amounts of dried AG50W-X4 (H^+, 400 mesh) and AG3-X4A (OH^-, 200–400 mesh; Bio-Rad Labs.), was used. The life of the guard column was about 200 injections and of the analytical column at least 4000 injections. The analytical column was operated

TABLE I

STRUCTURAL FEATURES OF POLYSACCHARIDE SUBSTRATES

Abbreviations: Xyl = *xylose*; Ara = *arabinose*; Gal = *galactose*; p = *pyranose ringform*; f = *furanose ringform*.

Polysaccharide	*Main composite sugars*	*Main glycosyl linkages*		*"Systematic" name*
		Backbone	*Branch point*	
Cellulose	Glcp	1,4		β-1,4-D-Glucan
Xylan	Xylp (90%) Araf (10%)	1,4	1,3,4	β-1,4-D-Xylan with single unit arabinosyl side chains
Arabinan				
ex-sugar beets	Araf (90%) Galp (10%)	1,5	1,2,5/1,3,5 1,2,3,5	Highly branched L-arabinan, with galactose containing side chains
ex-aple juice	Araf (100%)	1,5		α-1,5-L-Arabinan
Galactan	Galp (90%) Araf (10%)	1,4 1,5	1,2,4/1,4,6	β-1,4-D-Galactan with arabinan side chains

at 85°C, the guard column at ambient temperature and a flow-rate of 0.5 ml/min with water (Millipore) as eluent. Sugars were detected with an ERMA-ERC 7510 refractive index detector thermostated at 40°C.

Sample pretreatment

Since cationic resins may cause the precipitation of proteins and uronides, a clean-up of the sample was necessary. A Bio-Rad deashing system as well as a guard column containing a mixed-bed ion exchanger, as described by Brons and Olieman[7] for a cation resin in the calcium form (HPX-87C), was insufficient for the resin in the lead form (HPX-87P) used in this study. An additional pretreatment of the sample with lead nitrate prior to injection was efficacious. For this purpose, aliquots of the enzyme digests were mixed with 0.1 or 1 *M* (depending on the oligomer concentration) lead nitrate solutions to bring the final concentration of lead nitrate in the mixture to 0.05 *M*. The precipitate which formed was removed by centrifugation (3000 *g*) and 20 μl of the clear supernatant were injected.

Identification of saccharides

To identify the saccharide fractions in the digests, these fractions were isolated in fairly large quantities by gel permeation chromatography. An aliquot of the sample was applied on a Bio-Gel P-2 (200–400 mesh, Bio-Rad Labs.) column (100 cm × 2.6 cm, V_0 = 150 ml) thermostatted at 50°C and eluted with degassed distilled water at

50°C. The fractions were analyzed for neutral sugar content by the phenol–sulphuric acid assay[8]. Those exhibiting a peak were pooled and evaporated to dryness, and the composite sugars of the saccharide fractions were analyzed as alditol acetates by gas chromatography[9].

RESULTS

A typical chromatogram of the separation, using our system, of a reference mixture containing five monomeric sugars plus saccharose, raffinose and stachyose is shown in Fig. 1. A good separation of the sugar mixture was obtained within 25 min. Fig. 2 shows the saccharides released from phosphoric acid-swollen cellulose by endoglucanase I and III and by exoglucanase I after incubation for 2 and 21 h. The different patterns of reaction products released by these gucanases, and the changes in the composition of the reaction mixture in the course of the degradation by Endo I and III and Exo I, are clearly demonstrated.

Electrophoretically homogeneous endoglucanase have been found to be able to degrade β-1,4-xylans[10,11]. This activity could also be demonstrated by HPLC. Fig. 3 shows chromatograms of xylan digests produced by endoglucanase V and VI after incubation for 3 and 20 h. Gel permeation chromatography (GPC) of digest samples on Bio-Gel P-2 gave elution profiles comparable to those obtained with HPLC. From their elution volumes and sugar compositions, the peaks were identified as xylose (X_1), xylobiose (X_2), xylotriose (X_3) and xylotetraose (X_4). From the chromatograms in Fig. 3 it is concluded that endoglucanase V and VI have different patterns of action on polymeric and oligomeric xylans.

Fig. 4 shows the chromatograms obtained for digests of linear and highly branched arabinans degraded with endoarabinanase and arabinofuranosidase. Comparable chromatograms were obtained by GPC and the peaks were identified as arabinose (A_1), arabinobiose (A_2) and arabinotriose (A_3). From the chromatograms it is concluded that endoarabinanase is only active towards linear arabinan, producing arabinose oligomers, while arabinofuranosidase prefers highly branched arabinans producing monomeric arabinose.

The compositions of galactan and galactotetraose digests produced by endogalactanase and β-galactosidase are shown in Fig. 5. The peaks were identified as

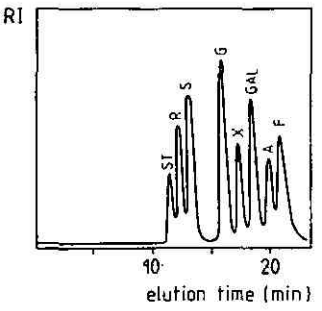

Fig. 1. Separation of stachyose (ST), raffinose (R), saccharose (S), glucose (G), xylose (X), galactose (Gal), arabinose (A) and fructose (F) on an HPX-87P column as specified in Materials and methods. RI = Refractive index.

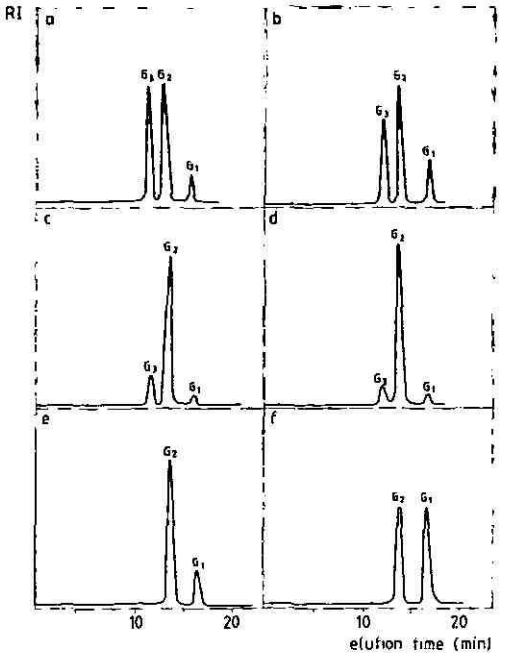

Fig. 2. HPLC analysis of products released from phosphoric acid swollen cellulose (1%) by cellulases (10 μg protein per ml) after incubation for 2 h and 21 h respectively; (a, b) Endo I; (c, d) Endo III and (e, f) Exo I. G_1 = Glucose; G_2 = cellobiose; G_3 = cellotriose.

galactotetraose (Gal_4), galactotriose (Gal_3), galactobiose (Gal_2) and galactose (Gal_1). β-Galactosidase was only active towards galacto-oligomers which could be completely degraded to galactose. The release of galacto-oligomers from galactan by endo-galactanase over an incubation period of 6 h is presented in Fig. 6. The tetramer,

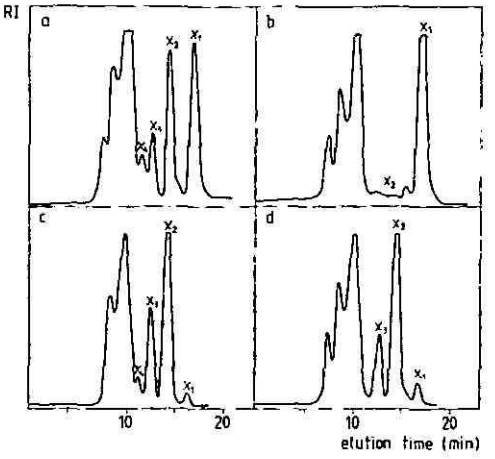

Fig. 3. HPLC analysis of reaction products of xylan (0.1%) with cellulases (60 μg per ml) after incubation for 3 and 20 h respectively: (a, b) Endo VI; (c, d) Endo V. X_1 = Xylose; X_2 = xylobiose; X_3 = xylotriose; X_4 = xylotetraose.

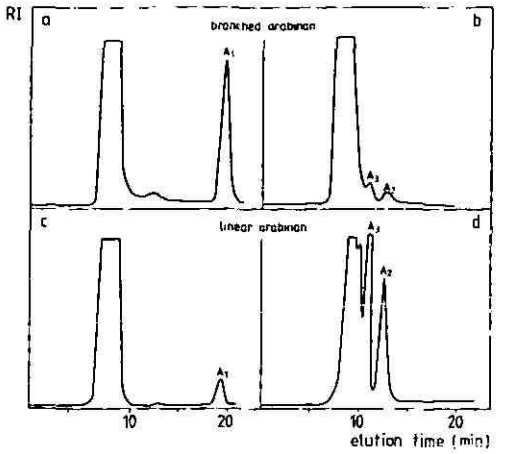

Fig. 4. HPLC analysis of reaction products released from branched and linear arabinans (0.4%) by arabinanases (20 μg protein per ml) after incubation for 1 h. (a, c) Arabinofuranosidase; (b, d) endoarabinanase. A_1 = Arabinose; A_2 = arabinobiose; A_3 = arabinotriose.

and to a lesser extent the dimer and trimer, were found to accumulate rapidly in the initial stage of the degradation. After 1.5 h the amount of the tetramers decreased, and the trimer content decreased after 3.5 h. The accumulation of dimer gradually levelled off; monomeric galactose accumulated at a low rate. The total amount of galactose oligomers reached a constant level, corresponding to 25% hydrolysis, within .3 h.

Table II summarizes the relative retention times of the various homologous series of oligomers and reference sugars.

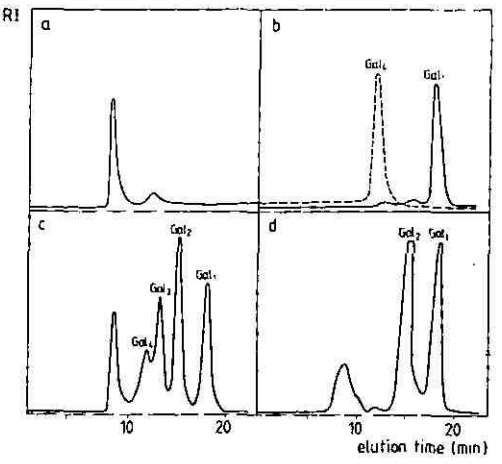

Fig. 5. HPLC analysis of reaction products released by β-galactosidase (1 μg protein per ml) from (a) galactan (0.02%) and (b) galactotetraose (0.02%) (dotted line is Gal_4 control) after incubation for 30 min and by endogalactanase(1 μg protein per ml) from galactan (0.4%) after incubation for 45 min (c) and 24 h (d). Gal_1 = Galactose; Gal_2 = galactobiose; Gal_3 = galactotriose; Gal_4 = galactotetraose.

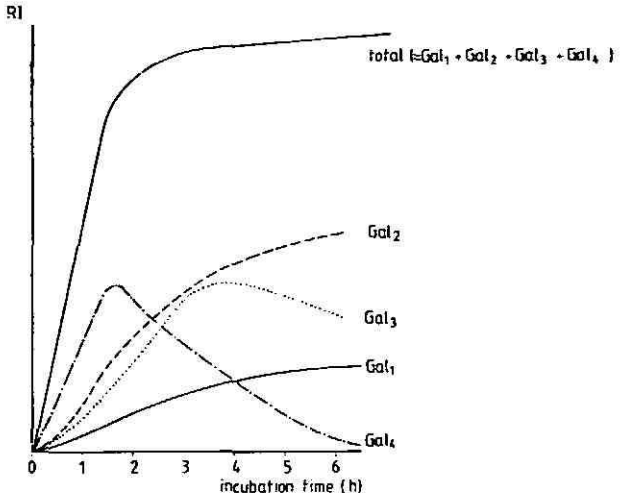

Fig. 6. Release of galacto-oligomers from galactan (0.4%) by endogalactanase (1 μg protein per ml) over an incubation period of 6 h. Abbreviations as in Fig. 5.

TABLE II

RELATIVE RETENTION TIMES OF MONO- AND OLIGOMERIC SUGARS COMPARED TO THOSE OF ARABINOSE

Sugar	*Relative retention time*				
	Mono	*Di*	*Tri*	*Tetra*	*Penta*
Arabinose	1	0.62	0,53	0.49	0.48
Xylose	0.86	0.74	0.66	0.59	–
Galactose	0.92	0.76	0.68	0.62	–
Glucose	0.81	0.67	0.58	0.52	–
Fructose	1.06	–	–	–	–
Saccharose	–	0.67	–	–	–
Raffinose	–	–	0.61	–	–
Stachyose	–	–	–	0.58	–

DISCUSSION

Amino-bonded silica columns (carbohydrate columns) are widely used for the analysis of mono- and oligosaccharides[12,13]. Brons and Olieman[7] have, however, demonstrated that this type of column is not suitable for the quantitative analysis of reducing sugars; it is insensitive, sugars are lost on the column due to interactions with amino groups and the column material is very unstable. A further disadvantage is that the retention times of oligomers increase with increasing DP; oligomers with high DP are strongly retained and can be eluted only by adapting the eluent.

The results presented have demonstrated that the resin column in the lead(II) form (HPX-87P) enables a rapid and sensitive estimation of the composition of poly-

saccharide digests. Oligomers up to DP 4 could be separated; larger oligomers and substrate residues passed through the column unretained. Resins in the silver(I) form exert greater selectivity for higher oligomers than resins in the lead(II) form[1]; good separations were obtained for cellulodextrins[14,15] and maltodextrins[16], up to a DP of 13. However, for the arabinan digest we observed partial hydrolysis of the relatively weak arabinofuranosidic linkages on this type of resin. For optimum performance they are not fully loaded with silver(I) ions, so that some sulphonic acid groups remain in the hydrogen form[16]. At the temperature of 85°C at which this column type is operated these groups will cause acid hydrolysis of weak glycosidic linkages. Leclercq and Hageman[17] have demonstrated this for saccharose. By operating the column at a lower temperature this hydrolysis can be minimized, but at the expense of the resolution of the oligomers. Van Riel and Olieman[18] suppressed acid hydrolysis by partially converting the column into the lead(II) form. By varying the ratio of the counter ions they were able to manipulate the column selectivity. Resins in the calcium(II) form are suitable for oligomers up to DP 4, but show poor selectivity for monomers.

The HPLC system studied here enabled a rapid and sensitive analysis of the reaction products of various enzymatically degraded polysaccharides, especially for the investigation of polysaccharides and their fine structure. The elution order of the reaction products allows conclusions to be drawn regarding enzyme purity, substrate specificity and the pattern of action of the enzyme. Further improvement of this system with regard to the separation of monomers and a broader range of oligomers is desirable.

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ION CHROMATOGRAPHIC DETERMINATION OF NITROGEN DIOXIDE IN THE ATMOSPHERE BY USING A TRIETHANOLAMINE-COATED CARTRIDGE

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SUMMARY

Nitrogen dioxide in air was sampled by the use of Sep-Pak C_{18} cartridge impregnated with triethanolamine. The trapped nitrogen dioxide was determined as nitrite and nitrate ions by ion chromatography. In the active sampling mode, air could be sampled at 0.8–1.2 l/min through the cartridge. Nitrogen dioxide in an air sample was determined with a 2.7% relative standard deviation at a concentration of 84 ppb with an 87–94% recovery. In the passive sampling mode, the average NO_2 concentration was determined as nitrite with a 2.2–9.8% relative standard deviation at atmospheric levels for 7–42 days.

INTRODUCTION

Nitrogen dioxide (NO_2) is one of various air pollutants that is photochemically reactive in the atmosphere. A number of automatic analytical instruments have been developed to monitor continuously the NO_2 concentration in the atmosphere or at emission sources. Atmospheric NO_2 is usually monitored by a chemiluminescence method[1] or the Saltzman method[2] at air monitoring network stations. These instrumental analyses may be inconvenient for personal monitoring or field work not covered by the monitoring stations. The instruments are expensive and should be operated in an air-conditioned room or automobile unit equipped with a stable power supply.

On the other hand, simple and convenient methods have been developed for sampling simultaneously a number of NO_2 samples at various locations difficult to cover by monitoring networks. In the active sampling mode, gaseous NO_2 is usually trapped by bubbling an air sample through an absorbing solution. The collection efficiency is poor, however, and sample preparation involving clean-up and concentration may be tedious and troublesome. Passive sampling, in which NO_2 is collected by diffusion on triethanolamine (TEA)-impregnated screens in badges or capsules exposed to air, may be simple and convenient for long-term monitoring of atmo-

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spheric NO_2[3–11]. Nitrogen dioxide is trapped on the materials through the formation of adducts with TEA[12]. The conventional methods are unusable, however, for monitoring for more than 1 week because the amount of TEA impregnated is limited. The methods encounter problems such as tedious sample preparation and effects of contamination during the analysis.

Recently, convenient cartridges such as Sep-Pak C_{18} (SP) and Sep-Pak Florisil have been used to sample traces of aliphatic aldehydes[13], aliphatic amines[14,15] and alkanethiols[16] in air samples with considerable savings of time and labour. However, no such cartridges have been used to sample NO_2 in air. Recent, ion chromatography (IC) has proved useful for the determination of traces of inorganic substances in air samples with high sensitivity. A number of reports have been published concerning the application of IC to the analysis of acid rain and airborne particulates[17–21].

The purpose of this study was to develop convenient methods for the determination of NO_2 in air samples at locations such as parks, recreation areas, roadside sites, parking lots and construction sites, which are difficult to cover by monitoring networks. In this paper, an SP cartridge impregnated with TEA is described for sampling NO_2 in ambient air by both active sampling and passive sampling. The NO_2 trapped on the cartridge is removed by passing a buffer solution through it and determined by IC. Passive sampling has been used for monitoring atmospheric NO_2 for more than 70 days without any effects due to temperature or humidity. The proposed method provides a simple and easy determination in the field of traces of NO_2 in air.

EXPERIMENTAL

Reagents and materials

All the chemicals were of special grade from Wako (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan). A Sep-Pak C_{18} (SP) cartridge was obtained from Waters Assoc. (Milford, MA, U.S.A.). Standard nitrogen dioxide (NO_2), nitrogen oxide (NO) and sulphur dioxide (SO_2) at levels of 97, 47.5 and 184.4 ppm in nitrogen cylinders were purchased from Seitetsu Kagaku (Osaka, Japan).

Apparatus

A Dionex (Sunnyvale, CA, U.S.A.) 2010i ion chromatograph equipped with a loop injector with a 50-μl sample volume was employed. The analytical column and the ion suppresser were a Dionex AS-4A ion-exchange column and a Dionex P/N 035691 anion fibre suppresser, respectively. The mobile phase was 2 m*M* sodium carbonate (Na_2CO_3)–0.75 m*M* sodium hydrogen carbonate ($NaHCO_3$) solution at a flow-rate of 1.7 ml/min. The ion-suppressing solution was 25 m*M* sulphuric acid at flow-rate of 1.7 ml/min. A Kimoto (Osaka, Japan) NO-A chemiluminescence nitrogen oxides analyser and a Yanagimoto (Kyoto, Japan) APF-510 SO_2 analyser were used to monitor the concentrations of NO_2 and SO_2, respectively, in the test samples.

Preparation of the sampling cartridge

An SP cartridge was washed with 5 ml of methanol and with 10 ml of deionized water. A 3–4-ml volume of 2% (v/v) TEA in methanol was forced through the car-

tridge and the empty part of the cartridge was wiped with filter-paper. The cartridge was dried for 1 h under reduced pressure in a stream of nitrogen and then by passing pure nitrogen at 100 ml/min for 30 min. The cartridge was closed with glass plugs, sealed in a vial and stored in a cool place in the dark until used.

Preparation of test samples (NO_2, NO and SO_2) and sampling test

Fig. 1 shows the system for the preparation and both active and passive sampling of test samples. The calibration gases and other test samples were dynamically produced at 1–3 l/min by diluting the cylinder gases to 20–400 ppb* levels with purified air in the distributor. In the active sampling test, a 35–74-l volume of the NO_2 sample was sampled at 0.8–1.2 l/min through the coated cartridge.

Active sampling

A 100–500-l volume of air sample was sampled at 0.8–1.2 l/min with a coated cartridge after filtration with a Sumitomo (Osaka, Japan) FP-045 Fluoropore filter and a Sartorius (Götingen, F.R.G.) SM 11904 (0.8 μm) polyamide filter.

Passive sampling

A coated cartridge was attached to a holder as shown in Fig. 2 and placed at a sampling site for 7–42 days.

Analytical procedure

The cartridge was wetted with 0.3 ml of methanol and the adsorbed substances were eluted with a 4 m*M* Na_2CO_3–1.5 m*M* $NaHCO_3$ solution. In active sampling, the elution was performed in the direction opposite to that of the sampling flow. An

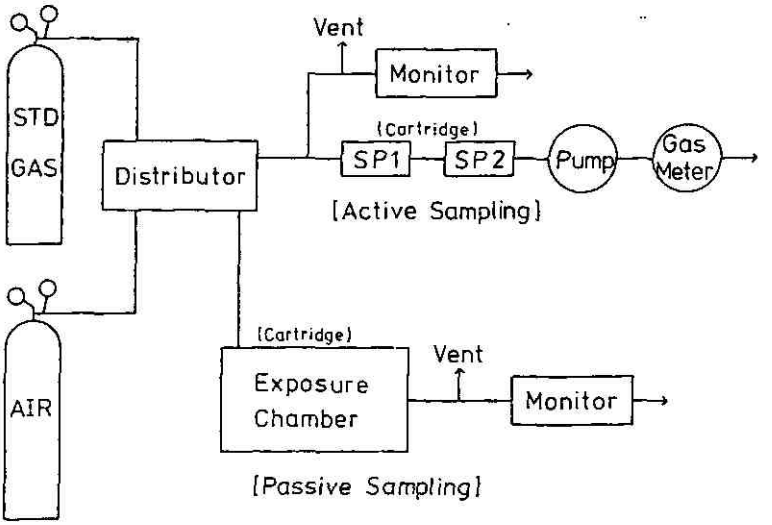

Fig. 1. Preparation and sampling of test gases. The exposure chamber was a 5-l brown-glass bottle. In the passive sampling mode, 30 SP cartridges were placed in the chamber in the calibration test.

* Throughout the article the American billion (10^9) is meant.

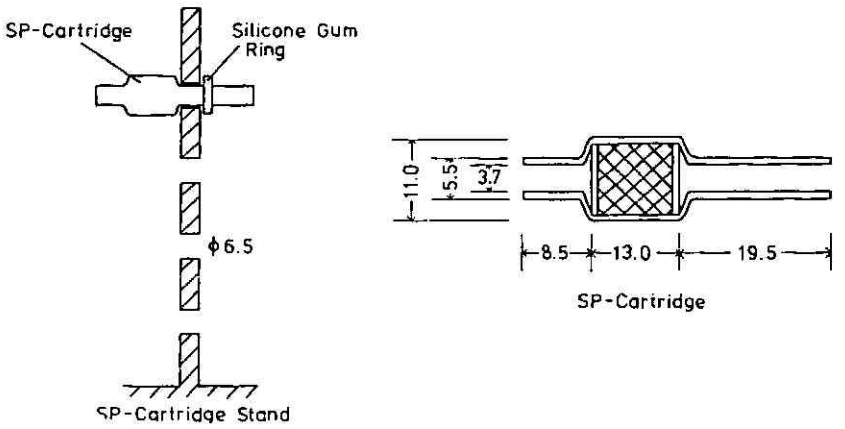

Fig. 2. Passive sampling with the use of an SP cartridge.

initial 5-ml volume of the eluate was collected. The sample solution was filtered through a Millipore (Bedford, MA, U.S.A.) HAWP (0.45 μm) filter and 50 μl of the sample were analysed by IC. The anionic substances were identified by retention time and quantified by peak area. A blank test was carried out with a coated SP cartridge in a similar manner.

Calculation

Active sampling. The concentration of NO_2 was calculated from the total amounts of nitrite and nitrate ions determined by IC.

Passive sampling. A calibration graph was prepared by plotting amount of nitrite ion detected by IC against the time-weighted average (TWA) concentration, defined as the product of NO_2 concentration (ppb) and exposure period (days). The concentration of NO_2 was calculated from the amount of nitrite ion detected by IC by using the calibration graph.

RESULTS AND DISCUSSION

The SP cartridge showed a high permeability of air through it. The cartridge used was packed with 0.4 g of porous packing with a large surface area (300 m^2/g). It was allowed to retain 8–10 mg of TEA, which is far more than the amounts used in conventional devices.

A recovery test was carried out with anionic species, including chloride and sulphate in addition to nitrite and nitrate, placed on a coated SP cartridge. Volumes of 10 μl of aqueous solutions containing 2.5–12.5 μg of the anions were injected on to the cartridge by using a microsyringe and 3 l of nitrogen were passed through the cartridge at 1 l/min. The anionic substances were eluted in the same direction as that of the nitrogen flow. The anions were eluted in the first 5 ml of eluate and no anions were detected in the later eluate, except chloride (observed at 0–3% levels in the second 5 ml of eluate). Table I indicates that 96–99% of the anions were recovered with a 0.4–5.1% relative standard deviation.

TABLE I

RECOVERY OF ANIONS PLACED ON AN SP CARTRIDGE

Anion	*Amount added (μg)*	*Amount found ± S.D.* (μg)*	*R.S.D.** (%)*	*Recovery (%)*
Cl^-	2.5	2.45 ± 0.125	5.1	98.1
NO_2^-	12.5	12.0 ± 0.128	1.1	96.3
NO_3^-	10.0	9.64 ± 0.142	1.5	96.4
SO_4^{2-}	12.5	12.3 ± 0.047	0.4	98.6

* Average of four runs ± standard deviation.

** Relative standard deviation.

Active sampling

The sampling conditions and analytical accuracy were investigated by passing 14–74 l of the test samples at 0.8–1.2 l/min through a Fluoropore filter, a polyamide filter and two coated SP cartridges in series. The concentration of NO_2 in the test samples was 84 ppb. The NO_2 in the samples passed through the filters without any adsorption and was completely trapped on the first cartridge; no NO_2 was detected on the second cartridge. The NO_2 trapped on the cartridge was detected as NO_2^- and NO_3^- by IC. Table II reports the determination of NO_2 in the standard samples. Nitrogen dioxide was determined with a 2.7% relative standard deviation at the 84 ppb level. The concentration obtained from the total of NO_2^- and NO_3^- corresponded to 87–94% of that determined by the NO_2 analyser. The conversion factor of NO_2 to NO_2^- was 0.81–0.85, which is similar to those (0.5–1.0) reported previously by a number of workers[3,12,22].

The Fluoropore filter was used to remove particulate materials more than 0.45 μm in diameter. The polyamide filter effectively excluded nitric acid vapour and trapped part of the SO_2 before the coated SP cartridges. Volatile chlorides seemed to pass substantially through the filters and to be trapped on the cartridges.

Nitrogen dioxide-free samples containing 20–200 ppb of NO or 37–184 ppb of SO_2 were used to investigate the effects of the co-existing substances on the determination. A 14–73-l volume of test gas was passed through the coated cartridge. No NO was trapped and detected on the cartridge, whereas SO_2 was completely trapped on the first cartridge at the above concentration levels and quantitatively detected by IC as sulphite and sulphate ions. These ions and other co-existing ions were separated from NO_2^- and NO_3^- by IC. Fig. 3 shows typical ion chromatograms from a blank sample and from ambient air. The co-existing substances in the atmosphere did not interfere with the determination of atmospheric NO_2. The detection limit of NO_2 in the active sampling mode was 0.4 ppb for a 100-l air sample.

A number of air samples were sampled and analysed both by the proposed method and with the NO_2 analyser. Fig. 4 shows plots of the NO_2 concentration determined by the proposed method against those obtained with the NO_2 analyser. Good agreement was obtained, the average ratio of the former to the latter values being 1.03.

These features suggest that the proposed method in the active sampling mode

TABLE II

DETERMINATION OF NITROGEN DIOXIDE IN THE ACTIVE SAMPLING MODE

Run No.	*Sample volume* (l)*	*Concentration of NO_2 detected (ppb)***			*Total/84 × 100 (%)*	*NO_2^-/NO_2*
		NO_2^-	*NO_3^-*	*Total*		
1	35	71.6	7.4	79.0	94	0.85
2	36	70.7	7.0	77.7	93	0.84
3	72	67.7	5.7	73.4	87	0.81
4	74	71.0	5.8	76.8	91	0.85
Average ± S.D.***				76.7 ± 2.07		0.83 ± 0.016
R.S.D.§ (%)				2.7		1.9

* The concentration of NO_2 in sample used was 84 ppb.

** The concentration was that of NO_2 to which the corresponding ions were converted under the standard conditions (at 25°C and 760 mmHg).

*** Average of four runs ± standard deviation.

§ Relative standard deviation.

is useful for the determination and/or monitoring of NO_2 in the atmospheric environment.

Passive sampling

Conventional passive devices are limited to use for the long-term monitoring

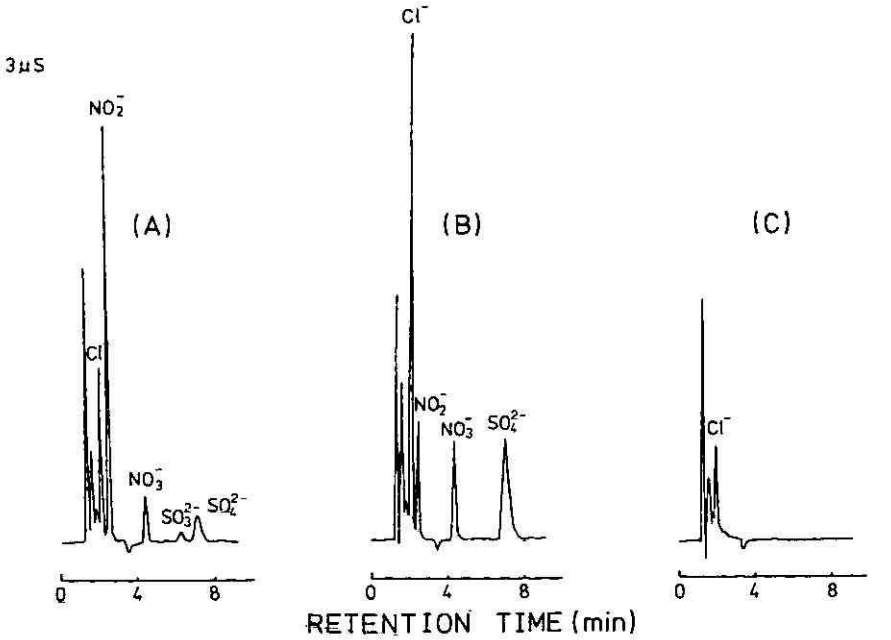

Fig. 3. Typical ion chromatograms for air samples. (A) Active sampling, sample volume 164 l, NO_2^- 71 ng, NO_3^- 16 ng, SO_3^{2-} 8 ng, SO_4^{2-} 15 ng; (B) passive sampling, sampling period 3 weeks, NO_2^- 18 ng, NO_3^- 24 ng, SO_4^{2-} 56 ng; (C) blank.

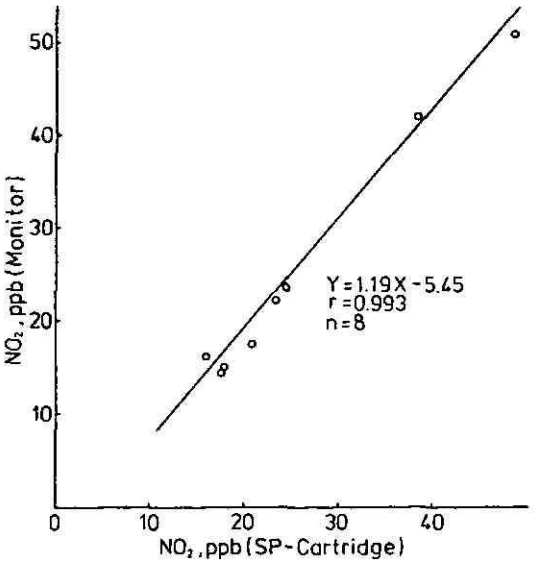

Fig. 4. Nitrogen dioxide concentrations determined by the active sampling mode *vs.* those obtained with the NO_2 analyser. Sampling volume, 120–300 l; sampling date, August 21st and 22nd and September 4th and 5th, 1985; sampling site, Environmental Pollution Control Centre, Osaka, Japan; temperature, 29.8–34.0°C (1-h average); relative humidity, 50–69% (1-h average).

of NO_2 in indoor or ambient air. The allowable exposure period may be less than 1 week. Also, the sampling devices, which depend on physical principles of mass transport across a diffusion layer or permeation through a membrane as the rate-limiting step, are susceptible to effects of temperature and humidity. One of the purposes of this study was to develop a passive sampling mode usable for long-term monitoring of NO_2 in indoor and/or ambient air.

The IC determination of NO_2 in the active sampling mode may be subject to effects of nitrate compounds on particulare matter trapped in the cartridge in the passive sampling mode. Fortunately, the conversion ratio of NO_2 to NO_2^- was constant (0.83 ± 0.016) in trapping with TEA, as shown in Table II. Hence the calibration graph could be constructed by plotting the amount of NO_2^- detected by IC against TWA concentration. In the calibration, coated cartridges were exposed to streams of standard gases at various concentration levels at 20 ± 1°C for 6 days. The practical sampling rate of NO_2, obtained from the slope of the calibration graph, was 2.55 ng/pbb · day in the SP cartridge. The linearity range of the calibration was 100–2000 ppb · day, which was ten times greater than those of conventional methods[3–11]. The detection limit of NO_2 was 50 ppb · day. Nitrite ion could be determined with a 2.2–9.8% relative standrd deviation in the calibration range. The cartridge can be used for monitoring NO_2 at atmospheric levels for more than 70 days. Virtually no effects of temperature and humidity on the determination of NO_2 were observed. This may be due to the high air permeability, the large surface area and the large amount of TEA retained in the SP cartridge.

A number of air samples were sampled for 7–42 days in the passive sampling mode. Fig. 3 shows a typical chromatogram obtained from a coated cartridge after

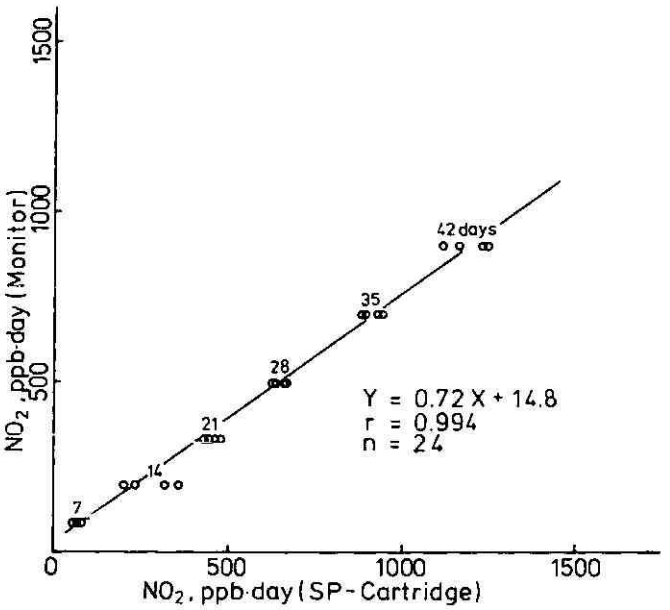

Fig. 5. TWA concentrations of NO_2 determined by the proposed method *vs.* those obtained by a monitoring instrument (Saltzman method). Sampling period, August 9th–September 20th, 1985; sampling site, Environmental Pollution Control Centre, Osaka, Japan; temperature, 22.9–30.7°C (1-day average); relative humidity, 52–81% (1-day average); wind velosity, 1.4–4.0 m/s (1-day average).

a passive sampling. The relative amounts of Cl^-, NO_3^- and SO_4^{2-} to NO_2^- were much higher than those in the active mode. This may be due to the introduction of particulate matter or an aerosol containing the ion species. Fig. 5 shows the relationship between TWA concentrations of NO_2 determined by the proposed method and those obtained by the monitoring instrument. Good linearity was obtained between the two variables, although the former tended to be slightly higher.

CONCLUSIONS

The features described above suggest that the use of the coated SP cartridge is simple and convenient for the determination of NO_2 in indoor and ambient air in both the active and passive sampling modes. The proposed method may have advantages over conventional methods in terms of rapidity and simplicity, high analytical sensitivity and low background effects. The passive sampling mode may be especially suitable for long-term monitoring of NO_2 in ambient air.

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ANALYTICAL CONTROL OF ENZYME-CATALYZED PEPTIDE SYNTHESIS USING CAPILLARY ISOTACHOPHORESIS

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SUMMARY

Analytical capillary isotachophoresis (ITP) was employed to control an enzyme-catalyzed peptide synthesis and the subsequent purification, as well as to evaluate the amino acid composition after acid and enzymatic hydrolysis. Compared with alternative chromatographic techniques, ITP offers certain advantages such as simultaneous detection of the synthetic peptide, amino acids, amino acid derivatives and contaminating inorganic ions in amounts of less than 200 ng. In addition, ITP provides quantitative information about the composition of the untreated samples directly analyzed without the mandatory use of suitable reference substances.

INTRODUCTION

In numerous studies, carrier-free capillary isotachophoresis (ITP) has been found to be a promising analytical tool in peptide chemistry. The basic work of Kopwillem and co-workers[1–4] opened the way for new applications, especially allowing the examination of the purity of chemically synthesized[5–13] and naturally occurring[14–21] peptides.

In the present study, analytical ITP was employed to control carboxypeptidase-Y catalyzed synthesis of a N-protected dipeptide, N-benzoyl-L-alanyl-L-glutamine (N-Bz-Ala-Gln). The peptide N-Bz-Ala-Gln, free amino acids, as well as the side product N-Bz-Ala and contaminating salts could be analyzed in a single experiment. In addition to the qualitative approach, ITP affords information about the composition of the samples without the necessity to concomitantly analyze reference substances, thereby facilitating a simple subsequent quantification. Furthermore, the method allows easy control of the subsequent gel chromatographic purification and, as further benefit, the feasibility directly to evaluate the amino acid composition of the peptide after enzymatic and acid hydrolysis.

EXPERIMENTAL

Materials

All free amino acids and their derivatives were obtained from Sigma (St. Louis,

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MO, U.S.A.). Solvents and reagents (all analytical grade) were provided by E. Merck (Darmstadt, F.R.G.). The enzyme carboxypeptidase-Y (CPD-Y, E.C. 3.4.16.1) was obtained from E. Merck as lyophilized powder (10% enzyme in sodium citrate), exhibiting an activity of 100 U per mg protein. The enzyme was desalted by gel filtration on Sephadex G-25 superfine (25 cm × 1.5 cm, Pharmacia, Uppsala, Sweden) using distilled water as eluent as described by Widmer and Johansen[22]. After lyophilization, an CPD-Y stock solution of 7 mg/ml (100 μM) was prepared and stored at −20°C.

Analytical isotachophoresis

Anionic isotachophoretic analyses were performed by using a 2127 Tachophor (LKB, Bromma, Sweden) with an automatic driving control unit[12]. Separations were carried out in a PTFE capillary (300 mm × 0.55 mm I.D.). Conductivity and UV (254 nm) signals were monitored by employing a two-channel recorder (Kipp & Zonen, Delft, The Netherlands) with a chart speed of 6 cm/min. For detection, the migration current was reduced from 210 to 60 μA. Depending on the chloride concentration of the sample and the chosen terminating electrolyte, the separations required 15–30 min. As leading electrolyte, a 10 mM solution of hydrochloric acid titrated to pH 9.1 with Ammediol was used. The terminating electrolyte buffer contained 10 mM glutamine (electrolyte system I) or β-alanine (electrolyte system II), respectively, adjusted to pH 10 with saturated barium hydroxide solution. These electrolyte solutions were prepared from analytical grade chemicals provided by E. Merck, Sigma and Fluka (Buchs, Switzerland) as described in detail previously[9], and were stored at 4°C.

Enzymatic peptide synthesis

With the aid of a Radiometer (Copenhagen, Denmark) pH-Stat assembly (PHM 84 research pH-meter, TTT/TTA 80 titrator and ABU 80 autoburette), CPD-Y catalyzed peptide synthesis was carried out at pH 9.5 using benzoyl-L-alanine methyl ester (N-Bz-Ala-OMe) as substrate and the free amino acid glutamine (Gln) as nucleophile, as described by Widmer and Johansen[22]. A 0.3 M stock solution of Gln was prepared by dissolving the respective amount in 0.1 M potassium chloride containing 1 mM EDTA. Aliquots (50 μl) of the reaction mixture were taken several times during the reaction period (20 min) and were mixed with 200 μl of 1 M hydrochloric acid to quench the reaction. A 1.5-μl aliquot of each sample was then directly analyzed by isotachophoresis.

The crude peptide material was purified by gel filtration on a glass column (250 cm × 2.5 cm) filled with Sephadex G-10 (Pharmacia) as described earlier[23].

Enzymatic and acid hydrolysis

By adding 0.1 mg aminopeptidase M (E.C. 3.4.11.2, E. Merck), enzymatic hydrolysis of N-Bz-Ala-Gln was performed at pH 7.2 and room temperature in 1 ml sodium phosphate buffer (12.5 mM) containing 1.5 mg of the purified peptide. Isotachophoretic analysis was carried out on an aliquot obtained after 1 h. For acid hydrolysis, 1.0 mg of the synthesized peptide was placed in a glass tube and dissolved in 0.5 ml of 5.7 M hydrochloric acid. Subsequently, the tubes were sealed *in vacuo* and incubated for 24 h at 110°C. The dried hydrolysates were resolved in distilled water and analyzed using β-Ala as terminating electrolyte (electrolyte system II).

RESULTS

Seven specimens of N-Bz-Ala-Gln obtained at intervals during the reaction period were analyzed by applying Gln as the terminating electrolyte. Three of the respective isotachopherograms are compared with an electrolyte run in Fig. 1. Despite barium hydroxide being added to the terminating electrolyte as recommended[24], considerable amounts of hydrogen carbonate formed during the separation were detectable in all analyses performed. The presence of impurities in the electrolyte system is apparent (Fig. 1A). In each sample analyzed at least five zones were detectable, as indicated by the conductivity signal (Fig. 1B–D). Only two zones showed characteristic high UV absorption at 254 nm, suggesting the presence of benzoyl protecting groups.

Four of the solutes present in the reaction mixture could be identified with the aid of reference substances. As illustrated in Fig. 2, the analysis of an equimolar standard mixture of acetate, glutamic acid (Glu), pyroglutamic acid (pGlu) and N-Bz-Ala provided convincing evidence that these substances are identical with those detected in the samples. The remaining UV-absorbing zone by necessity corresponded to the synthesized peptide N-Bz-Ala-Gln, as identified after purification (*vide infra*).

In Fig. 3, an attempt is made to illustrate the time course of the enzyme-catalyzed aminolysis and hydrolysis, respectively. The conductivity zone lengths of both N-Bz-Ala-Gln and N-Bz-Ala rapidly increased during the first 10 min, followed by a steady decrease in the rate of formation of both products. The maximum zone lengths seen after 20 min suggest that the enzymatic reaction is complete. It is also emphasized that the conductivity zone lengths for the three non-UV-absorbing solutes remained essentially unchanged during the reaction period (not depicted in Fig. 3, but indicated in Fig. 1). This finding indicates that the solutes acetate, Glu and pGlu have not participated in the CPD-Y catalyzed reaction.

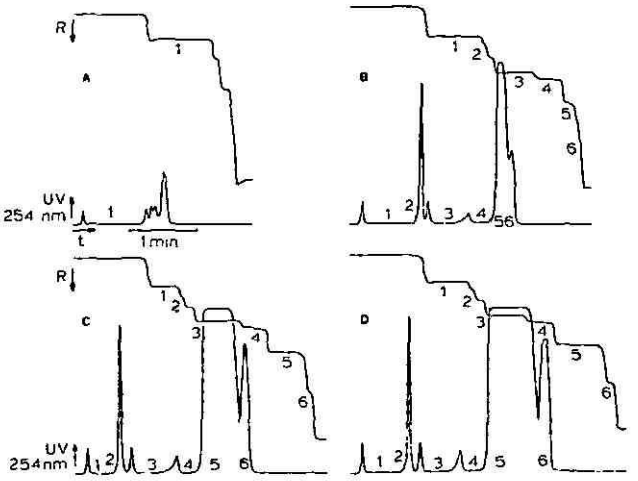

Fig. 1. Isotachophoretic analyses (electrolyte system I) of three different specimens (injection volume 1.5 μl) of N-Bz-Ala-Gln obtained during the reaction period. (A) Electrolyte system; (B) 0.5 min, (C) 5 min and (D) 20 min after addition of CPD-Y. Key: 1 = hydrogen carbonate; 2 = acetate; 3 = Glu; 4 = pGlu; 5 = N-Bz-Ala; 6 = N-Bz-Ala-Gln. R = Increasing resistance.

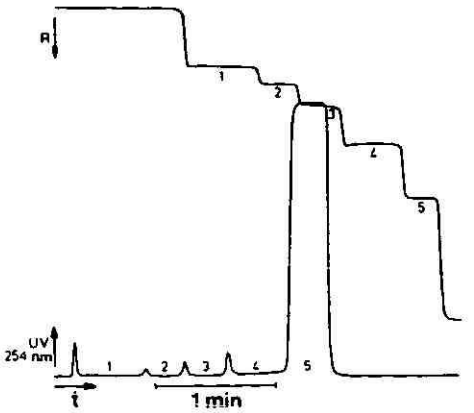

Fig. 2. Isotachophoretic analysis (electrolyte system I) of a reference mixture of acetate, Glu, pGlu and N-Bz-Ala. Injection volume: 10 μl, corresponding to 0.30 μg acetate, 0.74 μg Glu, 0.65 μg pGlu and 0.97 μg N-Bz-Ala. Key as in Fig. 1.

In Fig. 4, isotachopherograms of the crude material (A) and the purified peptide fraction (B) are given. Obviously, a simple gel chromatographic purification step is sufficient to approach 100% enrichment of the peptide N-Bz-Ala-Gln.

Identification of the synthetic product N-Bz-Ala-Gln could be performed after purification by analyzing the acid and enzymatic hydrolysates, respectively. The corresponding isotachopherograms are compared to the isotachophoretic pattern of the electrolyte system in Fig. 5. Although all chemicals used were recrystallized three times, impurities presumable derived from β-Ala were still present in the electrolyte system (Fig. 5A). Analysis of the acid hydrolysate revealed three distinct zones as indicated by the conductivity signal (Fig. 5B), only one of them exhibiting a characteristic UV absorption at 254 nm. As demonstrated in Fig. 5C, enzymatic hydrolysis of N-Bz-Ala-Gln resulted in the formation of one UV-absorbing and one non-UV-absorbing compound, obviously not identical with those determined after acid hydrolysis.

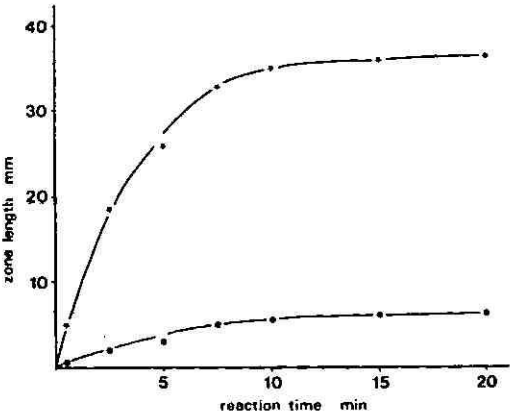

Fig. 3. Time course of CPD-Y catalyzed formation of N-Bz-Ala-Gln (●—●) and N-Bz-Ala (★—★). For synthesis conditions see Experimental.

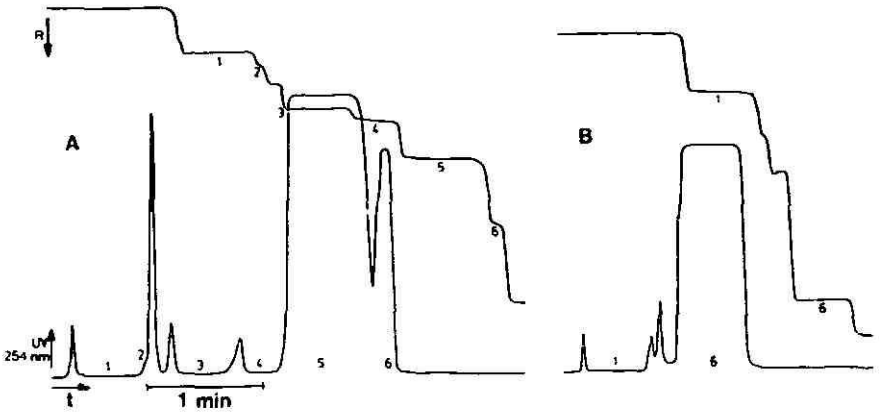

Fig. 4. Isotachophoretic analyses (electrolyte system I) of N-Bz-Ala-Gln before (A) and after (B) gel chromatographic purification. Injection volume: 1.5 μl. Key as in Fig. 1.

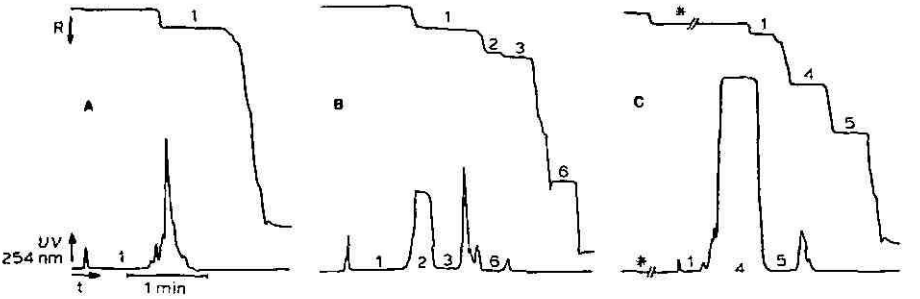

Fig. 5. Isotachophoretic analyses (electrolyte system II) of acid and enzymatic hydrolysates of N-Bz-Ala-Gln. (A) Electrolyte system; (B) acid hydrolysate; (C) enzymatic hydrolysate. Injection volume: 5 μl. Key: 1 = hydrogen carbonate; 2 = benzoate; 3 = Glu; 4 = N-Bz-Ala; 5 = Gln; 6 = Ala; * = phosphate derived from the buffer solution.

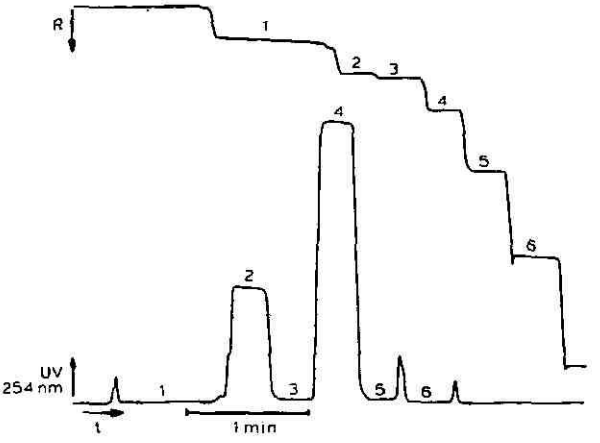

Fig. 6. Isotachophoretic analysis of a reference mixture of acetate, benzoate, Glu, N-Bz-Ala, Gln and Ala. Injection volume: 10 μl, corresponding to 0.30 μg acetate, 0.61 μg benzoate, 0.74 μg Glu, 0.97 μg N-Bz-Ala, 0.73 μg Gln and 0.45 μg Ala. Key as in Fig. 5.

Analysis of an equimolar standard mixture containing benzoate, Glu, N-Bz-Ala, Gln and Ala revealed, as expected, three non-UV-absorbing and two UV-absorbing zones (Fig. 6). These solutes are considered to be possible hydrolysis products of N-Bz-Ala-Gln. A comparison between the characteristic step heights and UV absorptions determined for the reference substances (Fig. 6) and those from the acid and enzymatic hydrolysate (Fig. 5) enabled identification of the respective cleavage products. Both acid and enzymatic hydrolysis fully confirmed the proposed structure of the synthesized peptide.

DISCUSSION

The numerous previous applications of ITP in controlling peptide synthesis suggest the use of this method as tool in controlling the efficacy of enzyme-catalyzed peptide synthesis. Apparently, by this method it is feasible simultaneously to analyze the resulting synthetic peptide as well as free amino acids, amino acid derivatives and contaminating ions present in the reaction mixture. This quality approach, exemplified in Figs. 1–3, allows a direct consideration of the reaction conditions chosen and facilitates the optimization of enzymatic peptide synthesis.

By applying ITP, minor quantities of the synthetic peptide could be directly analyzed in the reaction mixture despite the presence of an extremely high concentration of free Gln. Such an excess might seriously disturb, if not jeopardize, recovery of the peptide when analyzing with alternative electrophoretic[25] or chromatographic[26,27] methods. In the present work, the amino acid Gln itself was chosen as the terminating electrolyte, thus free Gln present in the sample remains in the terminating electrolyte zone and does not disturb the separation of other sample ions migrating in front of the terminating zone. Under these analytical conditions a quantitation of free Gln is not feasible. On the other hand, since only inconsequential amounts of the nucleophile will be consumed during the reaction, one surely may abstain from assessing the concentration of this solute in the reaction mixture.

As outlined under Results, the successful extension of ITP to the determination of the composition of the synthesized peptide N-Bz-Ala-Gln after hydrolysis facilitates its final identification (Figs. 5 and 6). It is emphasized that simultaneous detection of the cleavage products by applying alternative methods based on ion-exchange chromatography and/or reversed-phase chromatography is not possible.

According to the basic principles of ITP[24], there is a linear relationship between the absolute amount of a sample ion injected and the corresponding zone length measured. This means that, under standardized analytical conditions, ITP provides not only qualitative, but quantitative information about the composition of the sample. Generally, an absolute quantitative evaluation requires a calibration for all ionic species present in a sample. On the other hand, a relative quantification of sample ions with similar molecular weights and net charge is appropriate without the necessity to establish calibration curves or calibration constants. This is possible since the conductivity zone length is not influenced by substance-specific properties, *e.g.*, absorption coefficients. Based on these considerations and guided by the time/zone relationship given in Fig. 3, the ratio of the hydrolysis product/peptide formed can be estimated to be 5.8:1.0, which indicates that only about 17% of the substrate N-Bz-Ala-OMe has reacted to give N-Bz-Ala-Gln.

A possible explanation for this low yield may lie in the instability and/or low solubility of Gln. Evidently, a successful proteinase-catalyzed synthesis of peptides requires that the ester substrate used will be subject to aminolysis instead of hydrolysis[28–30]. Since the relative rate of aminolysis to hydrolysis is mainly influenced by the amino acid/water ratio, an high concentration of the amino component (in general 0.5–1 *M*) must be employed[28–30]. Owing to the low solubility of free Gln, a maximum concentration of only 0.3 *M* is applicable. Another factor contributing to the low concentration of Gln in the reaction mixture is certainly the instability of this amino acid, resulting in the formation of pGlu and Glu as indicated in Fig. 1.

It is currently suggested that synthetic short chain peptides are suitable components of future intravenous solutions[31,32]. A prerequisite for a commercial supply of such preparations, however, is the rationale production of short chain peptides on an industrial scale. Since the commonly used chemical syntheses are extremely uneconomic, laborious and inefficient, a biotechnological enzyme-catalyzed production of short chain peptides might be an appreciated alternative method.

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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY IN ACCELERATED STABILITY STUDIES FOR PREDICTION OF INHERENT SENSITIVITY OF DRUGS TOWARD OXYGEN

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SUMMARY

This paper discusses a novel technique for studying the inherent sensitivity of materials toward oxygen and the utility of quantitative thin-layer chromatography, as a tool in such studies. The degradation generally followed first order kinetics up to about 60% decomposition, indicating that the usual kinetic treatment applied to homogeneous systems can be used. The method can also detect degradation products, in many cases adding a considerable diagnostic element to its predictive value.

Among the model compounds tested testosterone was the most stable with *ca.* 95% recovery following a 190-h exposure to air on standard silica gel plates. The half-life time of the other model substances under similar experimental conditions was estimated by means of direct measurements or by extrapolation, and found to range from approximately 300 h to 1 h 10 min with cortisone marking the upper value and cholesta-3,5-diene the lower one.

INTRODUCTION

Accelerated stability studies are one of the main tools used in drug development. They allow making key decisions in terms of selecting conditions for processing, handling, storing, assaying and formulating any given new product, in an early stage of development. The most frequently investigated stress conditions are temperature, air, light, moisture, pH and various additives or excipients.

The oxidative degradation of many compounds on thin-layer plates has been known for a long time. As a matter of fact, assay procedures are occasionally frustrated by it, so that various methods have been developed to protect samples susceptible to oxidation. For vitamin D_1 it was found that samples on silica gel layers wet with an organic solvent are sufficiently stable, but quickly decompose if the sorbent becomes dry. Hence, speedy work and the elimination of the drying steps were recommended to obtain reproducible data. Other methods for stabilizing vitamins A and D and related derivatives included neutralization of the acid surface of silica gel with weakly alkaline excipients (*e.g.* magnesium oxide, triethanolamine), or preparing the vitamin D_3 adduct with cholesterol[2]. For protection of corticosteroids

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exposed to air during prolonged autoradiography on silica gel plates, the inclusion of ascorbic acid into the sorbent layer was recommended[3]. In this case ascorbic acid, itself prone to decomposition on silica gel carrier[4], probably serves as an oxygen scavanger. For the purpose of protecting oxyphenbutazone against oxidation, butylated hydroxytoluene was also recommended[5,6]. The latter serves to complex trace metals that could act as catalysts of oxidation reactions. According to a survey of DeRitter[7], edetic acid is the chelating agent most commonly used for the prevention of oxidative degradation catalyzed by trace metal ions.

Although correlations between the time of exposure to air and the extent of degradation have previously been described[1,6,8], these observations were mostly qualitative aimed at eliminating losses and errors during analytical work, and have never been utilized for the quantitative characterization of the oxidative sensitivity of materials.

EXPERIMENTAL

Pre-coated thin-layer chromatographic (TLC) plates (silica gel 60 F-254 from E. Merck) were first purified for the experiments. They were developed in methanol to their entire length, then reactivated in a drying oven at 110°C for 1 h. The plates were kept in a closed cabinet protected from moisture until used.

The test substances were dissolved in ethyl acetate at a concentration of 2.00 g/l. Aliquots (5 μl) of the stock solutions were applied onto the TLC plates by means of a micropipettor. The first sample applied had the longest exposure time. The time intervals between each subsequent sample constituted a series of exposure times. The final sample used as a reference was applied and dried in a nitrogen atmosphere. Every step of the experiment was conducted at ambient temperature. Up to ten samples were spotted on a standard size (20 × 20 cm) plate. The distance between the samples was 18 mm in accord with the requirements of the Shimadzu CS-920 scanner for automatic scanning. The integral of the reference sample was defined as 100%, and all the recoveries for the other samples were based on that.

Ethyl acetate was used for the development of the chromatogram in all but one case; in the experiment with estradiol, ethyl acetate was replaced with chloroform–dioxane (9:1, v/v). The development of the chromatograms was carried out in equilibrated chambers in the ascending mode. After development, the plates were transferred into a closed chamber for drying in a stream of nitrogen, under complete exclusion of oxygen.

The dry plates were quickly transferred into the Shimadzu CS-920 high-speed TLC scanner, the plate compartment of which was continuously purged with nitrogen. The quantitative evaluation of the chromatograms was carried out at the wavelength corresponding to the absorption maximum for the compound being tested.

Plots of the recoveries against the respective exposure times allowed the construction of kinetic curves describing the accelerated oxidative decomposition process.

RESULTS AND DISCUSSION

The model compounds selected cover a wide range of oxidative sensitivity. The

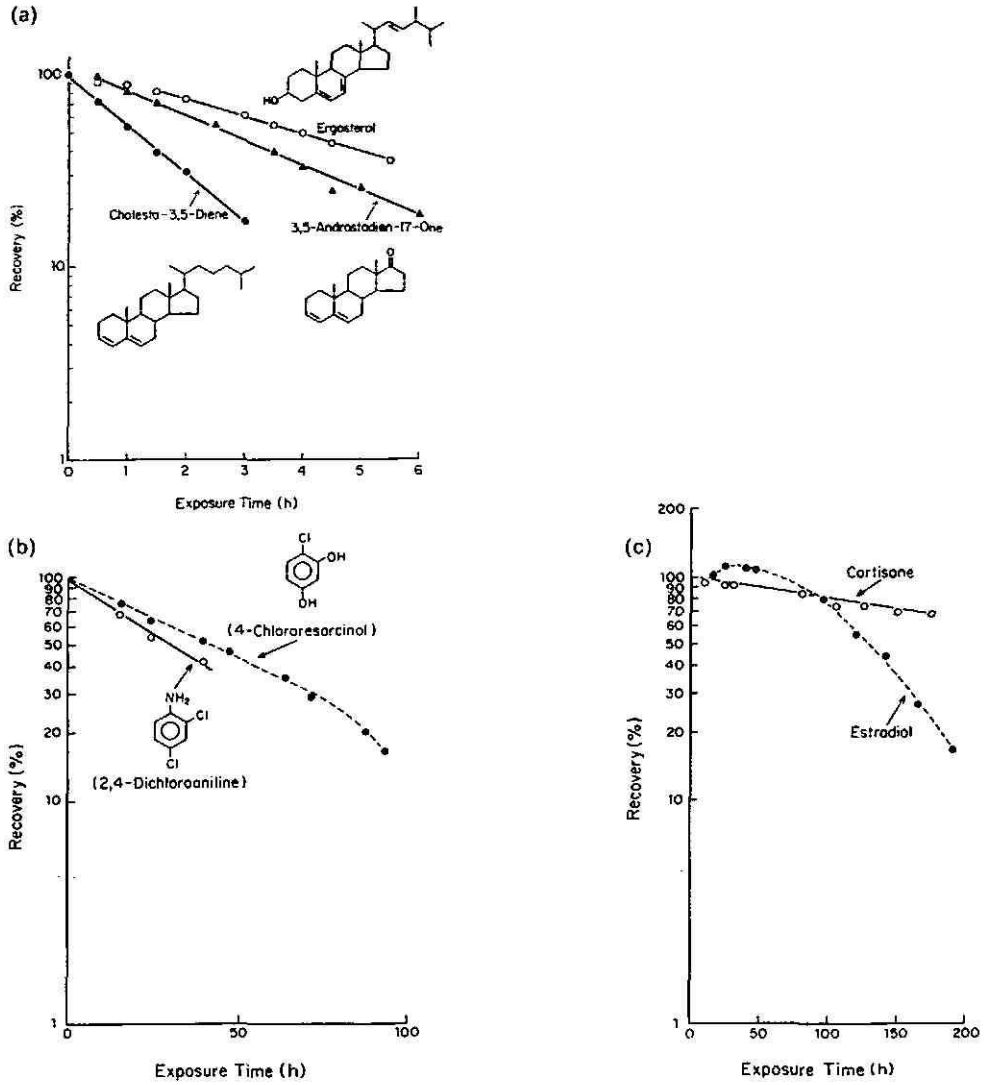

Fig. 1. First order plots of accelerated oxidative degradation as a function of exposure time for (a) cholesta-3,5-diene, 3,5-androstadien-17-one and ergosterol (scanning wavelengths: 235, 235 and 280 nm, respectively); (b) 4-chlororesorcinol and 2,4-dichloroaniline (scanning wavelengths: 280 and 235 nm, respectively); (c) cortisone and estradiol as a function of exposure time, (scanning wavelengths: 245 and 280 nm, respectively).

kinetic plots were constructed from the experimental data as the recoveries on the logarithmic scale against exposure time (Fig. 1). The recovery for testosterone after 190 h of exposure to air was over 95%. Much longer exposure time would be necessary to obtain a meaningful kinetic plot for this particular compound. The other extreme of the series was cholesta-3,5-diene with a half-life time of about 1 h 10 min (Fig. 1a). 3,5-Androstadien-17-one and ergosterol, both structurally related to cho-

lesta-3,5-diene, were more stable with estimated half-life times of 2 h and almost 4 h respectively (Fig. 1a).

2,4-Dichloraniline and 4-chlororesorcinol (Fig. 1b) were found to have half-life times of *ca.* 30 and 40 h, respectively. The half-life of cortisone (Fig. 1c) was estimated by extrapolation to be about 300 h. Estradiol shows a kinetic curve (Fig. 1c) quite different from the other examples. This deviation will be discussed in detail later on.

The decomposition products of cholesta-3,5-diene, 3,5-androstadien-17-one and ergosterol are very poorly detectable on the TLC plate at the respective scanning wavelength, suggesting that the probable site of the oxidative attack is the conjugated diene systems. Attempts to isolate and identify a stable oxidative degradation product were unsuccessful. Various methods indicated that the decomposition involves a very complicated cascade of reactions without the accumulation of any well-defined, detectable degradation product. Although under different experimental conditions, nevertheless similar sensitivity to oxidative degradation of other conjugated double-bond systems was reported earlier[9,10].

In contrast to the above examples, the oxidative degradation products of the substituted benzene derivatives, 2,4-dichloroaniline (Figs. 2 and 3) and 4-chlororesorcinol (Figs. 4 and 5) are readily detectable as distinct chromatographic zones. Since the results are plotted as percentage of the area counts based on the 0-time sample as 100%, the apparent recovery can be over 100% for any given fraction that has an absorption coefficient greater than that of the parent compound. This is the most probable explanation for the seemingly increasing recovery of estradiol in the early phase of the kinetic curve. Obviously, a recovery greater than 100% cannot be real (Fig. 1c). A degradation product with a higher absorption coefficient than estradiol, and not separated chromatographically, is the most likely explanation for the anomaly experienced here. Due to its sensitivity toward oxygen it, too, decomposes, giving rise to a number of more stable subsequent degradation products (Figs. 6 and 7), and, of course, further contributing to the distortion of the kinetic curve.

In case of cortisone the site of oxidative degradation appears to be the 1,3-dihydroxyacetone side-chain. Three well defined fractions increase in intensity con-

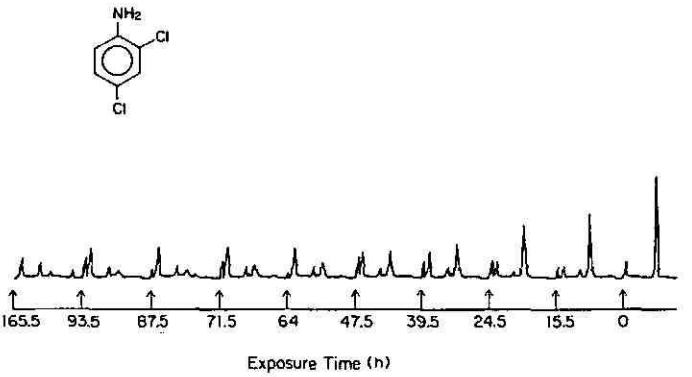

Fig. 2. 2,4-Dichloroaniline: densitometric scan of a series of time samples. Note the poor separation between the parent compound and a third degradation product.

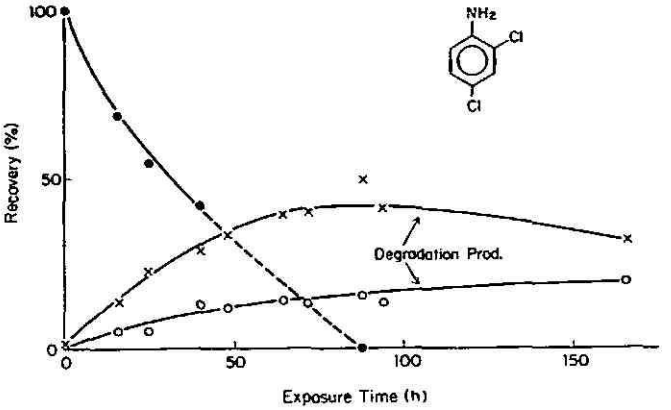

Fig. 3. 2,4-Dichloroaniline: plot of the parent compound and two degradation products. Note that due to poor separation between the parent compound and a third degradation product the data are incomplete.

currently with the decrease of the cortisone concentration (Figs. 8 and 9). The oxidative attack at the α,β-unsaturated keto group would probably lead to derivatives detectable poorly, if at all, at 240 nm. Similar observations were reported in conjunction with stability studies on prednisolone solutions[11].

In the majority of cases the degradation curves followed first order kinetics (Fig. 1), which is quite remarkable for a heterogeneous system. The explanation offered at the present time is as follows: when applied on a sorbent in small quantities, two key conditions are met. First, the solid state form is most likely amorphous, since materials in crystalline form generally are more resistant to most stress conditions[12]. Second, distributed on a large surface as a thin film, the material is uniformly exposed to the gas phase so that the diffusion of oxygen through the film is not a rate limiting factor. As a result, although the reaction formally is heterogeneous, this morphology creates conditions characteristic of a homogeneous system. The kinetic curves are indeed quite linear for a wide range of decomposition. Deviation from linearity is not evident until about 60% of the material is decomposed (Fig. 1a and b), except for an initial lag in case of 3,5-androstadien-17-one and ergosterol.

It is interesting to note that the oxygen-sensitive sites in cholesta-3,5-diene and

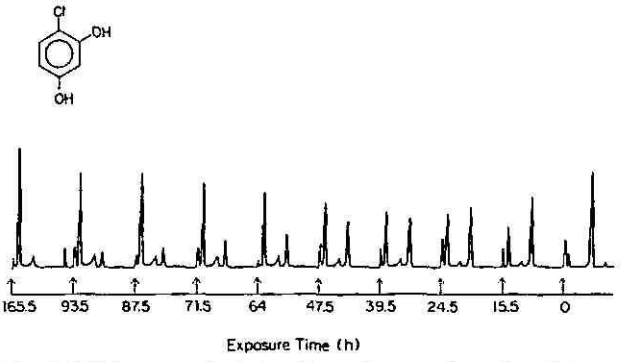

Fig. 4. 4-Chlororesorcinol: densitometric scan of a series of time samples.

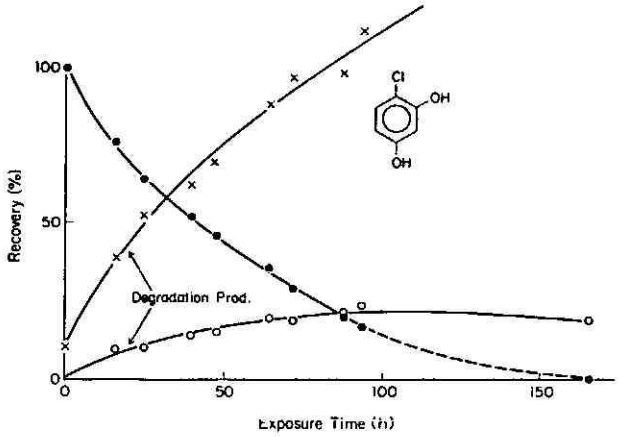

Fig. 5. 4-Chlororesorcinol: plot of the parent compound and two degradation products.

3,5-androstadien-17-one are identical. Yet, the difference in stability is significant. It seems that compounds with identical oxygen-sensitive sites may display greater stability toward oxygen if the molecule contains a polar substituent. This polar moiety can be situated in a location remote from the sensitive site. A possible explanation is that non-polar surfaces adsorb molecular oxygen more readily than polar surfaces thus providing a greater oxygen concentration. A more elaborate interpretation would require studying the three-dimensional structure of the molecules, and their orientation in the film deposited on the adsorbent. Similar observations were made

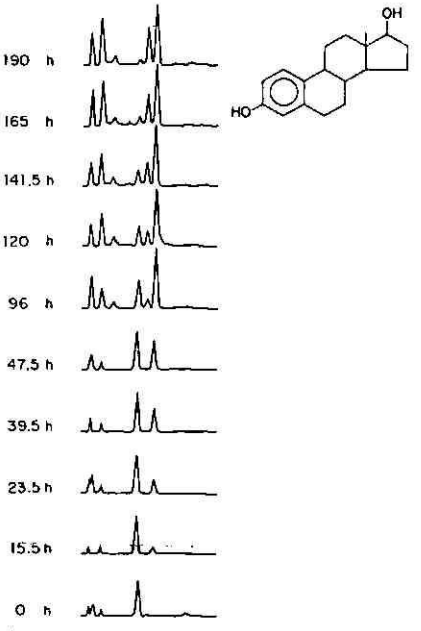

Fig. 6. Estradiol: densitometric scan of a series of time samples. Note the modified arrangement of the scans rendering a better illustration.

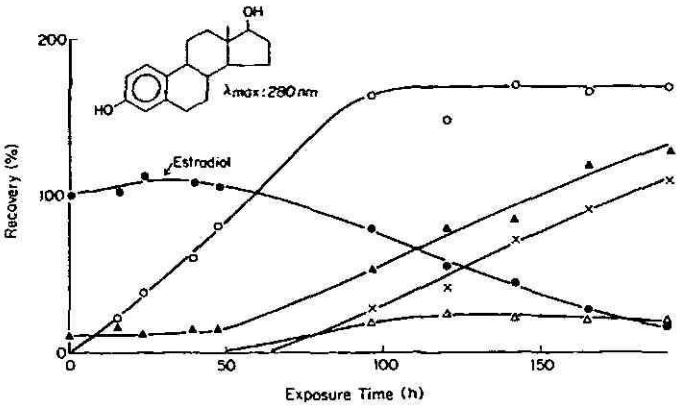

Fig. 7. Estradiol: plot of the parent compound and four degradation products. R_F values: (▲) 0.00, (△) 0.11, (●) 0.34, (×) 0.44, (O) 0.52.

on other classes of compounds currently under investigation. Experimental details will be made available in future publications; however, the examples presented here already demonstrate a relationship between chemical structure and oxidative stability. Future studies will include investigations on how to utilize the above finding in drugs design and formulation.

It should be pointed out that this method is primarily recommended for the rapid evaluation of inherent sensitivity to oxygen, and its use will probably be limited when batch-to-batch variations of stability are studied. Cholesta-3,5-diene, 3,5-androstadien-17-one and ergosterol are known to be sensitive to oxygen in crystalline bulk form. Although the information available from manufacturers is only qualitative, the correlation with the data presented here is convincing; these three compounds were the least stable in this test. Long-time stability studies on the crystalline bulk materials will be necessary to obtain quantitative correlation.

Several of the model compounds investigated are considered practically stable, when the well-developed crystals of the pure materials are exposed to air. However, the comparison of the model compounds is still valid in terms of their inherent sen-

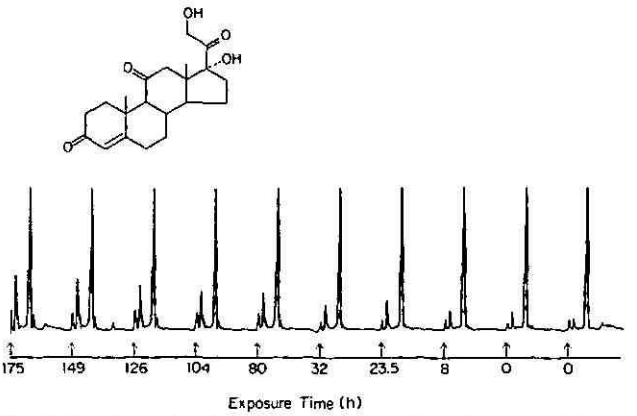

Fig. 8. Cortisone: densitometric scan of a series of time samples.

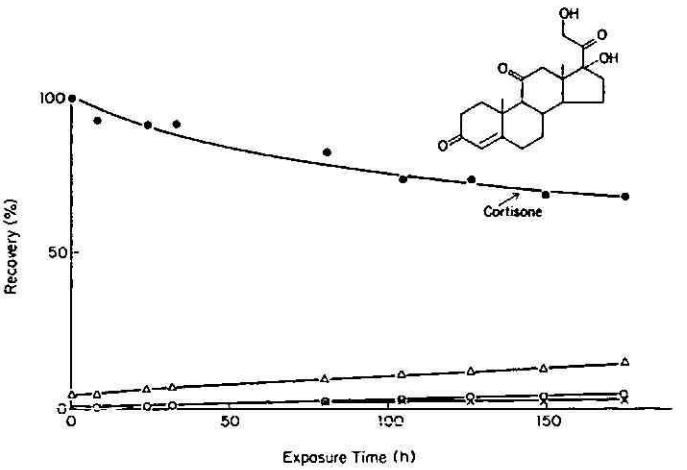

Fig. 9. Cortisone: plot of the parent compound and three degradation products. R_F values: (△) 0.00, (×) 0.05, (●) 0.28, (○) 0.37.

sitivity toward oxygen. A similar objective was previously achieved electrochemically by measuring the standard oxidation potential of an appropriate half-cell[13]. However, this method determines instability in solution. The method described here is much more widely applicable and technically simpler than the electrochemical procedure. Its potential importance lies in the possibility of predicting the behavior of materials under various conditions of manufacturing, storage and formulation. For instance, the method could be used to study the effects of various excipients and formulation methods. Silica gel is occasionally used as additive[2,4,7,12]. If the formulation calls for depositing a compound on silica gel from a volatile solvent, this method could predict possible problems associated with oxidative degradation. Of course, test conditions could be modified as to accommodate virtually any additive considered. Silica gel was selected here only for practical reasons: after exposure, the chromatogram could be developed on the same plate, and in addition to the predictive value of the method, the detection of the degradation products allowed the demonstration of certain diagnostic values. Obviously, silica gel as a carrier for the exposure can be replaced by any other excipient candidate (magnesium oxide, starch, cellulose, etc.), while the chromatography can be performed on any sorbent found suitable for the purpose. TLC plates with pre-adsorbent layers are commercially available. The pre-adsorbent could be designed according to the needs of the screening program. This laboratory is already involved with preliminary experiments including modified silica gel used as carrier for exposure, and TLC as well as high-performance liquid chromatography are employed for the evaluation of the stressed materials.

This method can also serve the evaluation of potential problems associated with manufacturing operations. If a compound is found to be unstable in this test, crystallization conditions will have to be such that the amount of amorphous material formed on the crystal surface, *e.g.*, due to drying of mother liquor residues, be reduced to a minimum. Crystal imperfection can also occur in presence of certain contaminants the effects of which could also be predicted by means of this test.

Finally, appropriate test results might call for processing and storage all together in an inert atmosphere.

The advantages of this method are as follows. (1) Small amounts (<2 mg) of the material are needed per experiment. Even precious chemicals can be tested with minimum quantities sacrificed. (2) The degradation reaction is truly accelarated: even at ambient temperature, the results are obtained within a very short time. (3) As a consequence of (1) and (2), predictive, and to some extent, diagnostic information can be generated in a very early phase of product development. (4) The simplicity and speed of the technique allows its use in screening for excipients and dosage forms.

These and other related topics are currently under investigation, and will be reported in due course.

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SEPARATION OF AMPICILLIN ESTERS AND THEIR DIASTEREOISOMERS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

Bacampicillin, talampicillin and pivampicillin can be separated by thin-layer chromatography on silanized silica gel with ethanol–water–2 *M* ammonium acetate (50:40:10) as the mobile phase. The diastereoisomers of bacampicillin and talampicillin can be separated by use of high-performance liquid chromatography. Good results are obtained with a Zorbax C_8 column (25 cm × 0.46 cm) and ethanol–water–0.2 *M* phosphate buffer pH 7.0 (40:55:5) as the mobile phase. The *R*:*S* ratio of bacampicillin samples varies between about 1:2 and 1:3, and for talampicillin samples *R*:*S* is always about 1:1.

INTRODUCTION

Several esters of ampicillin have been described as pro-drugs to improve oral absorption. Pivampicillin (PIV) was the first ester to be introduced clinically[1,2]. Other similar esters, talampicillin (TAL)[3,4] and bacampicillin (BAC)[5], were subsequently described. Their structures are shown in Fig. 1. The ester groups of TAL and BAC contain an asymmetric carbon atom and therefore the commercial products may consist of a mixture of epimers.

In this paper the separation of TAL, BAC and PIV by thin-layer chromatography (TLC) is described. The epimers of TAL and BAC were separated by high-performance liquid chromatography (HPLC) and the epimer ratio was determined. The separation of BAC diastereoisomers by HPLC has recently been described but no details about the quality of the separation were given[6]. The separation of the two diastereoisomers of ampicillin and of other penicillins, epimeric in the side chain, has been reported[7].

EXPERIMENTAL

Ampicillin (AMP), hydrochloride salts of ampicillin esters (TAL, BAC, PIV) and talampicillin napsylate were obtained from Astra (Södertalje, Sweden), Beecham (Heppignies, Belgium), Belphar (Brussels, Belgium), Continental Pharma (Brussels,

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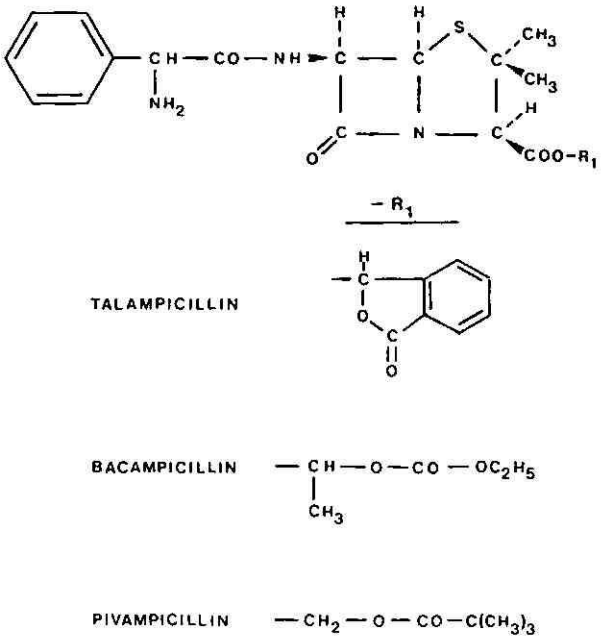

Fig. 1. Structures of the ampicillin esters.

Belgium), Leo Pharmaceutical Products (Hälsingborg, Denmark), Pfizer (Brussels, Belgium) and Upjohn (Puurs, Belgium).

Reagents and solvents

Chemicals for the preparation of buffer solutions were of *pro analysi* quality (E. Merck, Darmstadt, F.R.G.). Ethanol (96%, v/v) was of commercial grade. Acetonitrile for HPLC was obtained from Rathburn Chemicals (Walkerburn, U.K.). Water was distilled from glass apparatus. Other solvents were of > 99% purity (Janssen Chimica, Beerse, Belgium) and were used as such except for tetrahydrofuran, which was distilled to remove the stabilizer, after confirmation of the absence of peroxides.

TLC

Precoated silanized silica gel plates, Fertigplatten Kieselgel 60 F_{254} silanisiert, were obtained from E. Merck. Laboratory-made silanized silica gel plates were prepared using a suspension of 35 g of Kieselgel 60 HF_{254} silanisiert (E. Merck) in 60 ml of water–methanol (2:1). After initial drying at room temperature for several hours, the layers were dried further at 50°C overnight. All layers used were 0.25 mm thick.

The mobile phases used are given in Table I. They comprised one or two organic modifiers, water and buffer solution. The organic modifiers used were acetone, acetonitrile, ethanol, *tert.*-butanol, dimethylformamide, dimethyl sulphoxide, methanol and tetrahydrofuran. A 2 *M* solution of ammonium acetate was used as the buffer solution; when a pH value is indicated, it was achieved with glacial acetic acid.

Sample solutions (10 mg/ml) were prepared in methanol–water (1:1) (AMP, TAL · HCl, BAC · HCl) or in methanol–water (3:1) (PIV · HCl) and 1-μl aliquots were applied to the plates. The plates were developed over a distance of *ca.* 15 cm in filter-paper-lined chromatographic tanks, which had been saturated for at least 1 h. They were dried in a stream of hot air and placed in a tank saturated with iodine vapour for detection.

HPLC

The pump unit consisted of a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) equipped with a pulse dampener and a manometer as described[8]. The HPLC apparatus further comprised a Valco injector, Model CV-6-UHPa-N60, equipped with a 10-μl loop (Valco, Houston, TX, U.S.A.), an Altex UV detector, Model 153 (254 nm), equipped with an 8-μl flow cell (Altex, Berkeley, CA, U.S.A.), a Kipp recorder, Model BD40 (Kipp & Zonen, Delft, The Netherlands) and a Pye Unicam integrator Model DP88 (Pye Unicam, Cambridge, U.K.).

The columns (25 cm × 0.46 cm I.D.) were packed as described[7] with μBondapak C_{18}, 10 μm (Waters Assoc., Milford, MA, U.S.A.), Hypersil ODS, 5 μm (Shandon, Cheshire, U.K.), Spherisorb S5 C_8, 5 μm (Phase Sep, Norwalk, CT, U.S.A.) and Zorbax C_8, 7 μm (Du Pont, Wilmington, DE, U.S.A.). The columns were kept at 25°C by means of a glass jacket, as described[9].

All the mobile phases contained one organic modifier and 5% (v/v) 0.2 *M* phosphate buffer pH 7.0, the volume being made up with water. The amount of organic modifier varied with the column used and is specified in the tables. The organic modifiers were acetonitrile, ethanol, *tert.*-butanol, methanol, 2-propanol and tetrahydrofuran. The mobile phases were degassed by sonication. The flow-rate was set at 1.0 ml/min and the chart speed at 5 mm/min. Sample solutions were prepared as described for TLC and 100-μg aliquots were injected. The detector was set at 0.16 a.u.f.s.

RESULTS AND DISCUSSION

In Table I the R_F values obtained on silanized silica gel are reported. The values are the means of the results obtained from several chromatograms. Values obtained with laboratory-made plates are given in parentheses. The mobile phases, previously described for TLC of penicillins[10] or cephalosporins[11], are not suitable since chromatography of the less polar esters requires an increased content of organic modifier. Although the R_F values obtained with mobile phases A, B and C suggest separation of PIV, BAC, TAL and AMP, the separation is insufficient due to tailing. This could be reduced by the addition of acetonitrile (mobile phases D and E) but the separation of BAC and TAL was still not complete. The use of mobile phase F, containing ethanol, allows complete separation of the three esters. The addition of acetone to the mobile phase (G) does not improve the separation. In order to examine the influence of the buffer pH, the 2 *M* ammonium acetate solution was adjusted to pH 4.2, 5.2 and 6.0 (mobile phases H, I and J). No improvement is observed at these pH values and the spots show tailing.

Although the separation obtained with mobile phase F is very satisfactory, the analysis time is rather long, *i.e.*, about 4.5 h with precoated plates and about 8 h with

TABLE I

$R_F \cdot 100$ VALUES FOR AMPICILLIN AND ESTERS CHROMATOGRAPHED ON SILANIZED SILICA GEL

PIV = Pivampicillin; BAC = bacampicillin; TAL = talampicillin; AMP = ampicillin. Values obtained with laboratory plates are given in parentheses. A 2 *M* solution of ammonium acetate was used as the buffer solution; when a pH value is indicated this was achieved with glacial acetic acid.

Mobile phase	*Sample*			
	PIV	*BAC*	*TAL*	*AMP*
A Methanol–water–buffer (60:30:10)	36 (33)	40 (36)	44 (41)	73 (65)
B Methanol–water–buffer (60:35:5)	35 (39)	38 (44)	44 (49)	75 (65)
C Methanol–water–buffer (65:30:5)	42 (41)	46 (44)	50 (47)	76 (66)
D Methanol–acetonitrile–water–buffer (40:15:35:10)	34 (29)	41 (34)	45 (37)	78 (70)
E Methanol–acetonitrile–water–buffer (45:15:30:10)	42 (37)	45 (44)	49 (47)	78 (75)
F Ethanol–water–buffer (50:40:10)	33 (29)	38 (33)	44 (39)	75 (65)
G Ethanol–acetone–water–buffer (50:5:35:10)	44 (31)	48 (36)	53 (40)	78 (63)
H Ethanol–water–buffer pH 4.2 (50:40:10)	48 (45)	53 (48)	58 (54)	74 (67)
I Ethanol–water–buffer pH 5.2 (50:40:10)	39 (48)	43 (51)	48 (59)	76 (67)
J Ethanol–water–buffer pH 6.0 (50:40:10)	57 (51)	61 (56)	64 (63)	77 (66)
K Acetonitrile–water–buffer (30:60:10)	6	8	7	77
L Acetonitrile–water–buffer (40:50:10)	15	25	19	63
M Tetrahydrofuran–water–buffer (30:60:10)	6	11	9	91
N Tetrahydrofuran–water–buffer (40:50:10)	18	28	27	92
O *tert.*-Butanol–water–buffer (35:55:10)	37	40	43	79
P Acetone–water–buffer (40:50:10)	5	8	8	32
Q Acetone–water–buffer (45:45:10)	13	18	21	89
R Dimethyl sulphoxide–water–buffer (40:50:10)	*	*	*	*
S Dimethylformamide–water–buffer (40:50:10)	*	*	*	*

* Streaking from the start point.

laboratory-made plates. In order to reduce the analysis time a number of other organic modifiers was examined (mobile phases K–S). All these separations are very poor except that with *tert.*-butanol (mobile phase O), but even this separation is inferior to that obtained with mobile phase F and the development time is even longer

(about 6.5 h). Therefore mobile phase F is considered as the best choice. Generally the separation pattern obtained with the laboratory-made plates is comparable with that obtained with the precoated plates, but differences in R_F values are observed.

None of the TLC systems used (Table I) shows any separation of the diastereoisomers of BAC and TAL. Therefore, the separation of these diastereoisomers was attempted with a system similar to that formerly described for the separation of side-chain diastereoisomers of penicillins[7]. This involves the use of a Zorbax C_8 column and methanol–water–0.2 *M* phosphate buffer pH 7.0 as the mobile phase. The separation of BAC epimers is easily achieved but the TAL epimers, although separated, appear superposed on a background peak of strongly absorbing impurities and therefore cannot be determined quantitatively. Other organic modifiers were examined and the results are shown in Table II. For completeness the results for PIV are also mentioned. Acetonitrile and tetrahydrofuran only allow a poor separation of TAL epimers and no separation of BAC epimers. Ethanol gives a nice separation of both TAL and BAC epimers. Typical chromatograms are shown in Fig. 2. Other alcohols such as 2-propanol and *tert.*-butanol also separate all the epimers, but the results are inferior. Therefore the use of ethanol is preferred.

TABLE II

RETENTION TIMES OF AMPICILLIN ESTERS ON ZORBAX C_8

The mobile phases comprised organic modifier–water–0.2 *M* phosphate buffer pH 7.0 [*x*:(95 − *x*):5].

Organic modifier	*x*	*Retention time (min)*				
		TAL		*BAC*		*PIV*
Methanol	60	14.7*	16.3*	23.6	25.4	41.4
Methanol	65	8.5*	9.2*	12.7	13.4	20.5
Acetonitrile	40	17.3	18.5	20.8	20.8	32.6
Tetrahydrofuran	35	18.0	19.6	20.0	20.0	33.4
Ethanol	40	18.7	21.4	29.9	32.2	61.7
2-Propanol	28	18.2	21.0	27.6	30.6	62.0
tert.-Butanol	23	15.6	18.2	23.8	25.8	55.2

* Product peaks are superposed on a background peak of strongly absorbing impurities.

The separation of BAC epimers with 2-propanol at pH 4.0 has been described[6]. By carrying out this method with a μBondapak C_{18} column, as described, the BAC epimers were separated but the peaks were much broader than with the system described here. A similar picture was obtained with the TAL epimers. On Zorbax C_8 the epimers were not separated at all with this mobile phase.

The ampicillin esters were analysed on several stationary phases with two mobile phases containing different amounts of ethanol. The results are reported in Table III. The three esters can easily be separated on all the columns. The diastereoisomers can also be separated on all the columns but the resolution on Zorbax C_8 is better. For some separations the resolution is not good enough to calculate a resolution value following the instructions of the European Pharmacopoeia[12].

TABLE III

COMPARISON OF THE SEPARATION OF AMPICILLIN ESTERS ON DIFFERENT REVERSED-PHASE COLUMNS

The mobile phases comprised ethanol–water–0.2 *M* phosphate buffer pH [*x*:(95 − *x*):5]. ND = Not determined due to insufficient separation.

Column	*Peak*	*Mobile phase with 40% ethanol*							*Mobile phase with 42.5% ethanol*						
		Retention time (min)			*Resolution*		*Symmetry factor*		*Retention time (min)*			*Resolution*		*Symmetry factor*	
		TAL	*BAC*	*PIV*	*TAL*	*BAC*	*TAL*	*BAC*	*TAL*	*BAC*	*PIV*	*TAL*	*BAC*	*TAL*	*BAC*
μBondapak C_{18},	1	12.5	16.7	31.7	1.06	ND	1.00	ND	9.9	12.9	22.9	0.89	ND	1.00	ND
10 μm	2	13.9	18.2				1.04	1.05	10.9	13.9				1.00	1.00
Hypersil C_{18},	1	11.9	19.3	41.2	1.37	ND	1.22	ND	10.1	15.7	30.9	1.24	ND	1.27	ND
5 μm	2	13.7	21.0				1.25	1.20	11.5	16.9				1.31	1.13
Spherisorb C_8,	1	14.7	19.6	38.9	1.21	1.86	1.13	1.06	12.8	17.1	31.1	0.97	1.63	1.21	1.00
5 μm	2	15.9	21.7				1.06	1.05	13.7	18.8				1.14	1.10
Zorbax C_8,	1	18.7	29.9	61.7	1.98	1.48	1.10	1.15	12.4	23.3	43.7	1.56	1.25	1.13	1.13
7 μm	2	21.4	32.2				1.09	1.07	13.9	25.2				1.11	1.08

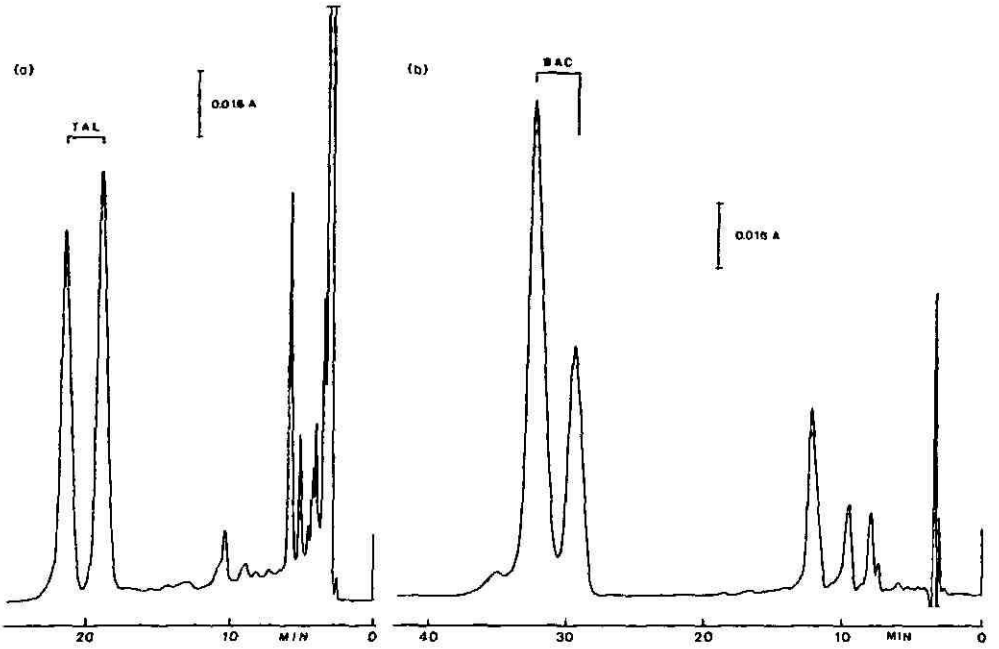

Fig. 2. High-performance liquid chromatograms of (a) talampicillin hydrochloride and (b) bacampicillin hydrochloride on Zorbax C_8 (25 cm × 0.46 cm) with ethanol–water–0.2 *M* phosphate buffer pH 7.0 (40:55:5) as the mobile phase. Detection: 254 nm, 0.16 a.u.f.s. Amount injected: 100 μg. Old samples were chosen to demonstrate the selectivity of the chromatographic system.

TABLE IV

EPIMER RATIO IN COMMERCIAL SAMPLES OF AMPICILLIN ESTERS

X = Relative amount of the epimer eluted first; *n* = number of experiments; S.D. = standard deviation.

Ester salt	*Manufacturer and sample no.*	*Isomer eluted first*	*X*	*n*	*S.D.*
Bacampicillin hydrochloride	A_1	*R*	31.7	4	0.6
	A_2		26.5	2	0.5
	B		30.3	2	0.6
	C		36.5	2	0.2
Talampicillin hydrochloride	D_1	Unknown	51.5	2	0.2
	D_2		53.1	2	0.8
	D_3		51.9	2	0.2
	D_4		54.4	2	0.1
	E_1		52.7	3	1.1
	E_2		52.1	4	2.4
	E_3		51.9	2	0.2
	E_4		52.4	3	1.5
	F		48.9	3	0.4
Talampicillin napsylate	G		51.8	5	1.4

The Zorbax column and ethanol as the organic modifier were used to analyse a number of samples. The results are shown in Table IV. Since the pure *S* epimer of BAC was kindly provided by Astra the first peak eluted could be identified as the *R* epimer. The peaks of TAL were not identified since a pure isomer was not available. For TAL the *R*:*S* ratio was always about 1:1 for the samples examined. These results confirm the data obtained by NMR spectroscopy[4]. It should be noted that the rates of hydrolysis of the TAL epimers by blood are very similar[4]. For BAC the *R*:*S* ratio varies between 1:3 and 1:2. No information is available concerning the ratio of the contents of the two diastereoisomers in commercial samples of this penicillin, although some physical properties have been discussed[5,6,13]. The oral absorption of TAL and BAC by humans has been compared with that of ampicillin and amoxicillin[14,15].

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DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN NATURAL WATERS BY THIN-LAYER CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure is reported for the determination in natural waters of the six polycyclic aromatic hydrocarbons (PAHs) listed by WHO as indicators of pollution. The method consists in continuous liquid–liquid extraction, separation of the PAH fraction by thin-layer chromatography and reversed-phase high-performance liquid chromatography with a spectrofluorimetric detector. The method has been applied to unfiltered samples of river and sea waters with PAH levels below 0.1 ng/l.

INTRODUCTION

Of the many pollutants in surface waters, polycyclic aromatic hydrocarbons (PAHs), some of which are carcinogenic, are of great importance. The World Health Organization (WHO) has set a standard for the amount of PAHs in drinking water derived from surface waters, recommending a maximum level of 200 ng/l for "total PAHs"[1]. The term "total PAHs" refers to the sum of six specified PAHs: fluoranthene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, benzo(*ghi*)perylene and indeno(1,2,3-*cd*)pyrene. A level of more than 200 ng/l indicates unacceptable contamination. This same limit has been established in Directives of the Commission of the European Economic Communities (EEC)[2]. The U.S. Environmental Protection Agency (EPA) has issued a list of organic compounds to be monitored in municipal and industrial discharges in the U.S.A.[3]; the list contains 129 compounds, 16 of which are PAHs. These 16 PAHs include the six listed by the WHO.

In recent years much work has been carried out to develop methods for the determination of PAHs in the aqueous environment. These methods differ in detail, but almost all include extraction, clean-up and analysis.

The extraction techniques used for concentrating PAHs from water include extraction with organic solvents[4–7] or adsorption on columns of macroreticular resins[8–10] and other materials [11–15]. Clean-up is normally carried out by thin-layer (TLC) or column chromatography[16–18]. For the determination of PAHs gas chromatography[19–21], gas chromatography–mass spectrometry[22,23], high-performance

* This research is part of the doctorate post lauream thesis of M.R.

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liquid chromatography (HPLC)[24–27] and fluorescence spectrometry[28–30] have been used.

At the present there is no official method for the determination of PAHs in natural waters; the WHO recommends the two-dimensional TLC method developed by Borneff[18] and Borneff and Kunte[31]. However, this method has the disadvantages that it is laborious and lacking in accuracy, so alternative methods are desirable[32].

In this paper we report a procedure for the determination in natural waters of the six PAHs listed by the WHO. The method, consisting in continuous liquid–liquid extraction, separation of the PAH fraction by TLC and reversed-phase HPLC with a spectrofluorimetric detector, is applicable to unfiltered samples. We do not use preventive filtration, consequently our results refer exactly to the total aqueous medium at the time of sampling.

The applicability of this technique was tested on real samples.

EXPERIMENTAL

Materials

All solvents were obtained from Carlo Erba (Milan, Italy). *n*-Hexane, benzene (analytical-reagent grade), methanol, acetonitrile and tetrahydrofuran (HPLC grade) were used as received. Cyclohexane (HPLC grade) was doubly distilled before use. The water used as the mobile phase in the HPLC system was doubly distilled and filtered through a Norganic Trace Organic Removal Cartridge (Millipore, Bedford, MA, U.S.A.). The purity of the solvents was checked before use.

Silica gel TLC plates (20 × 5 × 0.025 cm) were obtained from Merck (Darmstadt, F.R.G.).

Standard PAH samples were commercial products (Fluka, Buchs, Switzerland; Aldrich, Milwaukee, WI, U.S.A.: Eastman Kodak, Rochester, NY, U.S.A.) or were obtained from the Community Bureau of Reference, BCR (Commission of European Communities, Brussels, Belgium).

A standard solutions was prepared that contained the following amount of PAH per microlitre of methanol: 40 pg of fluoranthene, 20 pg of benzo(*b*)fluoranthene, 5 pg of benzo(*k*)fluoranthene, 20 pg of benzo(*a*)pyrene, 10 pg of benzo(*ghi*)-perylene and 100 pg of indeno(1,2,3-*cd*)pyrene. This solution was obtained by appropriate dilution from individual solutions of the standards of concentration 0.1 μg/μl. The standard solution was stored at 4°C in the dark.

Apparatus

The HPLC analysis was performed using a Perkin-Elmer Series 2 liquid chromatograph equipped with a Series LS-5 spectrofluorimetric detector and a column (16.5 × 0.46 cm I.D.) packed with 5 μm silica, chemically modified with octadecylsilane (ODS). The packing materials were obtained from Carlo Erba and Phase Separations (Norwalk, CT, U.S.A.).

Sampling

Conventional sampling practices were followed. The water samples were collected in duplicate in glass containers. The volume drawn was 5 l.

Mercury(II) chloride (50 mg/l) was added to inhibit microbial activity and the samples were stored at 4°C.

Analytical procedure

The water samples (2 l) were subjected to continuous liquid–liquid extraction with cyclohexane for 50 h at a distillation rate of 5 ml/min. Approximately 150 ml of solvent were used; at the end of the extraction about 100 ml were stratified on the water and the remainder constituted the extract. This was concentrated to 1–2 ml by a rotary evaporator at room temperature and then reduced to dryness under a stream of nitrogen. The residue was dissolved in tetrahydrofuran (100 μl) and applied as a streak to a silica gel plate and developed with *n*-hexane–benzene (1:1) in the dark. After removal of the mobile phase, the plate was examined under ultraviolet light (254 nm) to establish the position of the PAH spot. The spot was scraped off, powdered and transferred to a filtration system consisting of a fritted glass Buchner funnel (porosity 16–40 μm) on a vacuum flask in which was placed a test-tube to collect the filtrate. The PAHs were eluted from the silica gel with 5 ml of tetrahydrofuran. Traces of silica gel, which could damage the column in the subsequent HPLC analysis, were removed from the tetrahydrofuran solution by passage through a Millipore filter (0.5 μm). The filtrate was evaporated to dryness using a stream of nitrogen and the residue dissolved in 50–100 μl of methanol for HPLC analysis (the methanol volume depends on the expected concentration of PAHs in the sample).

The HPLC analysis was performed on a column packed with Erbasil C_{18}. The PAHs were separated isocratically with methanol–water (85:15, v/v) as the mobile phase at a flow-rate of 1 ml/min. The compounds were detected under the optimum conditions by selecting the appropriate excitation and emission wavelengths. The excitation wavelength was fixed and the emission wavelength was varied during the analysis.

The PAH identification was accomplished by comparison of the retention times and the stop-flow excitation and emission spectra of the sample with those of standard compounds.

The determination of individual PAHs was carried out by comparison of the sample peak areas with those of standards. The samples were analysed in parallel with blanks to ensure that the glassware and reagents were interferences free.

RESULTS AND DISCUSSION

Most of the PAHs present in waters are found to be associated with suspended matter and this particulate fraction mostly conditions the choice of the extraction technique. Given the characteristics of the type of water to which we intend to apply the method (waters with relatively low levels of suspended matter), it is not necessary to apply preventive filtration, and consequently the determined PAH concentrations are relative to the total aqueous sample as such. Moreover, a filtration step would necessitate analyses of both the filtered material and the particulate matter, with a consequent probability of losses of the compounds of interest, expecially when working at the ng/l concentration level. Even if a suitable filter is chosen, some of the suspended matter could pass into the filtrate and make the sample non-homogeneous. As the adsorbed PAHs require a different extraction technique to the dissolved PAHs, in which the time of contact with the extraction solvent is longer, there is a risk of incomplete recovery of the PAHs. This has been observed by other workers[33] who have examined the filtration problem.

Preliminary studies were carried out to evaluate more suitable extraction techniques for use with unfiltered samples. Continuous and discontinuous liquid–liquid extraction and adsorption on a microcolumn packed with C_{18} reversed-phase material or with graphitized carbon black were examined. Continuous liquid–liquid extraction gave the highest PAH recovery. To obtain a good recovery, an extraction time of 50 h was sufficient.

The use of TLC to separate the PAHs from the total organic fraction was studied in our laboratory previously[34–37] at the μg/g concentration level in different matrices. Tests carried out with standard solutions showed that tetrahydrofuran is the most suitable solvent for elution from thin layers. Studies to evaluate the recovery of PAHs at concentrations comparable to those in the samples were carried out and some results are reported in Table I.

Coextracted interferents varied considerably with the type of water sample examined. In samples of tap water and sea water the TLC step is not necessary because these samples did not contain impurities at the retention times of the compounds to be determined.

The separation and detection of PAHs by HPLC was carried out using a fluorescence detector, which imparts both selectivity and sensitivity to the system. We compared the performances of some commercial C_{18} packing materials. These materials, although chemically similar, provided different separation efficiences and different retention characteristics for PAHs. We focused attention on pairs of PAH isomers that are difficult to separate, *viz.*, benzo(*b*)fluoranthene–benzo(*k*)fluoranthene (BbF–BkF) and benzo(*ghi*)perylene–indeno(1,2,3-*cd*)pyrene (BghiPe–IP). We found that Erbasil C_{18} gave the best resolution of these PAHs. Fig. 1 illustrates the HPLC separation obtained with columns packed with Erbasil C_{18} and Spherisorb ODS_2. Although the latter column has a higher number of theoretical plates it has a lower resolution for the PAH isomers examined. The resolutions for BbF–BkF were 1.6 and 2.0 and those for BghiPe–IP were 0.9 and 1.5, respectively.

The separation characteristics of the column also depend on the mobile phase. We examined the resolution obtained with methanol–water and acetonitrile–water, eluent systems generally used for this type of separation. Methanol–water is better because the BghiPe–IP isomers are unresolved using acetonitrile–water whatever the percentage of acetonitrile used.

The described procedure has been applied to the evaluation of the degree of PAH pollution of the River Tiber[38] (Fig. 2).

The PAH concentrations were corrected for a blank; samples of water, purified by distillation and passage through cartridges suitable for eliminating organic sub-

TABLE I

MEAN RECOVERY (%) OF PAHs FROM THIN LAYER

Compound	*0.25 ng*	*0.5 ng*	*1 ng*
Fluoranthene	93	100	90
Benzo(*b*)fluoranthene	76	89	90
Benzo(*k*)fluoranthene	61	85	86
Benzo(*a*)pyrene	68	82	83

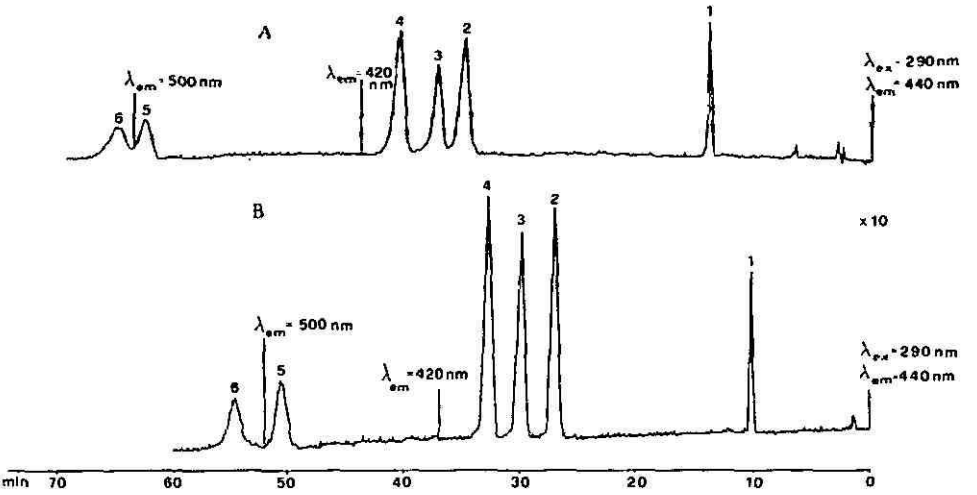

Fig. 1. HPLC traces of a standard mixture obtained on columns packed with different material: (A) 34 × 0.46 cm I.D. column of 5 μm Spherisorb ODS_2; (B) 16.5 × 0.46 cm I.D. column of 5 μm Erbasil C_{18}. Mobile phase: methanol–water (80:20). Flow-rate: 1 ml/min. Spectrofluorimetric detection. Peaks: 1 = fluoranthene; 2 = benzo(b)fluoranthene; 3 = benzo(*k*)fluoranthene; 4 = benzo(*a*)pyrene; 5 = benzo(*ghi*)perylene; 6 = indeno(1,2,3-*cd*)pyrene.

stances, were prepared. These samples, when extracted and analysed under the same conditions as for the real samples, showed the presence of some PAHs. An accurate study to establish the origin of these compounds was therefore undertaken. We first verified the absence of PAHs from the materials and reagents used, then a water

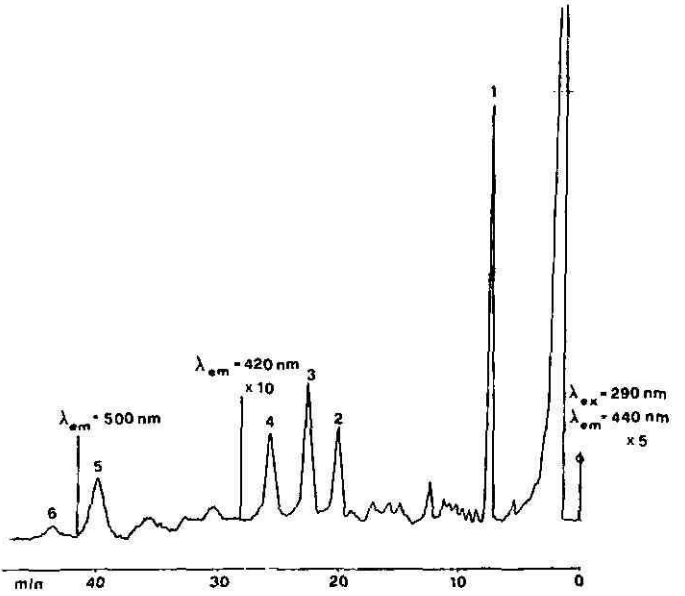

Fig. 2. HPLC trace of extract of River Tiber water. Column: 16.5 × 0.46 cm I.D. of 5 μm Erbasil C_{18}. Mobile phase: methanol–water (85:15). Flow-rate: 1 ml/min. Spectrofluorimetric detection. Peaks as in Fig. 1.

TABLE II

MEAN CONCENTRATIONS AND RELATIVE STANDARD DEVIATIONS FOR SOME PAHs DETERMINED IN WATER FROM THE RIVER TIBER

Compound	*Mean concentration* (ng/l)*	*Standard deviation* (ng/l)*
Fluoranthene	7.22	1.69
Benzo(*b*)fluoranthene	0.46	0.07
Benzo(*k*)fluoranthene	0.17	0.02
Benzo(*a*)pyrene	0.56	0.03
Benzo(*ghi*)perylene	0.11	0.02

* On 5 determinations.

sample of the described type was subjected to extraction and the extract was analysed at different times. After 50 h the concentration of the individual PAHs unexpectly remains almost constant. The probable explanation was that the small amount of PAHs present in the blank could have been formed during the process of boiling of cyclohexane in the extractor. In fact, PAH-free cyclohexane samples after boiling showed levels of some PAHs comparable to those of the blanks of distilled water.

The reproducibility of the method was determined by taking five sub-samples of the same sample of River Tiber water and executing three HPLC analyses on each sub-sample. Table II reports the mean values and the relative standard deviations obtained.

The recovery was determined by spiking samples of previously extracted distilled water with a solution of PAHs at a concentration of 5 ng/l each and values of $85 \pm 10\%$ were obtained.

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RAPID PURIFICATION OF THE MAIN ALLERGEN OF *LOLIUM PERENNE* BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The main allergen from rye grass (*Lolium perenne*) pollen was purified by size-exclusion high-performance liquid chromatography. The purified allergen had a molecular weight of 32 000 daltons and was significantly more active in solid phase radioimmunoassay than the whole extract. The highly purified antigen can be obtained very rapidly and with a recovery of 30%.

INTRODUCTION

The purification of allergens is a prerequisite for up-to-date diagnosis and therapy in allergic diseases, and the need for removal of irritant, toxic or irrelevant components is well recognized[1]. Pollen of Graminiae is the most common cause of atopy throughout the world and *Lolium perenne* (rye grass) is one of the most aggressive species of this family.

High-performance liquid chromatography (HPLC) has been used by several workers for the purification of allergens from pollens[2–4]. Some excelent work has been published on the purification and characterization of the major allergen of rye grass, which binds to specific IgE from sensitive allergic patients[5–10]. Nevertheless, classical biochemical techniques such as ammonium sulphate precipitation. CM- and DEAE-cellulose ion-exchange chromatography and gel filtration using Sephadex have been used, often resulting in large losses of allergenic material and complicated purification schemes.

HPLC is a powerful and rapid method for the purification of the components of complex mixtures. Here we report the purification of the main allergen of *Lolium perenne* by using only two 15-min runs in a size-exclusion HPLC column. The pure allergen is biologically active and its recovery from crude extract is about 30%.

EXPERIMENTAL

Pollen extraction

A 1-g amount of pollen (Allergon, Sweden) was extracted with 10 ml of 0.15

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M phosphate-buffered saline (PBS) (pH 7.4) for 1 h in an ice-bath. After centrifugation at 12 100 *g* for 15 min at 0°C, the extract was filtered on a 0.45 μm Millipore membrane. The extractions were carried out in the presence or absence of phenylmethylsulphonyl fluoride (PMSF) (Sigma, St. Louis, MO, U.S.A.). All protein determinations were performed by the method of Lowry *et al.*[11].

Size-exclusion HPLC

HPLC analyses were performed on a Waters Assoc. chromatography system with Protein Pak 125 columns. The system includes a Model 510 pump, a Model 440 UV detector operated at 254 nm, a loop-type U6K injector and a Waters data module.

The eluent was PBS and the injection volumes varied from 10 to 100 μl.

Solid-phase radioimmunoassay

HPLC-purified rye grass allergen was dialysed against 0.05 *M* carbonate buffer (pH 9.6). A 50-μl volume of the antigen solution was allowed to become attached to the wells of PVC plates (Flow) overnight at 37°C. Residual binding sites were further covered with 5% bovine serum albumin (BSA) for 1 h at 37°C.

Sera from allergic patients having a type 4 RAST to *Lolium perenne* were added to the wells (50 μl) and after incubation for 1 h at 37°C were washed three times with PBS containing 0.5% of Triton X-100.

The radioimmunoassay (RIA) was developed with 50 μl of rabbit anti-human IgE labelled with ^{125}I (Pharmacia, Uppsala, Sweden). Washed and cut wells were counted in a Packard Autogamma 500 C gamma counter.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

All electrophoresis materials was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Molecular-weight markers were obtained from Pharmacia.

Slab gels containing 0.1% of SDS were prepared and run in a system according to Laemmli[12]. The separating gel consisted of 12.5% of polyacrylamide and the stacking gel contained 4.5% of polyacrylamide. The gels were stained with 0.25% Coomassie Brilliant Blue G-250 in 45% methanol–10% acetic acid for 1 h and destained with methanol–acetic acid–water.

Immunoblotting

Immunoblotting was carried out as described by Towbin *et al.*[13]. Proteins were transferred electrophoretically to nitrocellulose strips (0.45 μm) (Schleicher & Schüll, Dassel, F.R.G.) in a Trans Blot Cell (Bio-Rad Labs.) for 2 h at 250 mA. After transfer, the strips were soaked in 3% human serum albumin (HSA) in PBS for 1 h to saturate additional protein binding sites and then incubated overnight with a pool of atopic sera diluted four times. The strips were washed in PBS containing 1% of Triton X-100 and incubated again with anti-IgE ^{125}I ($1 \cdot 10^6$ cpm) in PBS containing 0.3% of HSA. After extensive washing, the nitrocellulose was air-dried and exposed to Kodak X-OMAT R film for 4 days.

RESULTS

Mobile phase

As distilled water and aqueous buffers are the usual solvents used for the extraction of allergens from pollens, water, Tris and phosphate buffers were used as mobile phases in the size-exclusion HPLC purification.

Fig. 1A shows that the use of distilled water led to a very poor resolution, with the presence of two peaks in the void volume of the columns, probably aggregates. An ionic strength similar to the normal physiological conditions (0.15 *M* NaCl) was checked at pH 8 (Tris buffer) and pH 7.2 (PBS). Both systems (Fig. 1B and C) gave good resolutions of the components of the crude extract and PBS (both for extraction and as the mobile phase) was used throughout the work because of its similarity with the natural medium in the nose and throat, target organs in allergy.

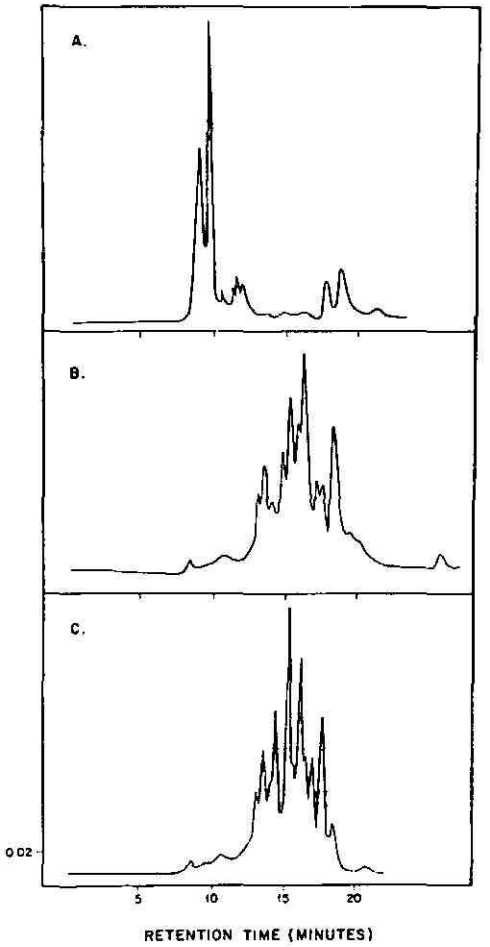


Fig. 1. HPLC of crude extract of *Lolium perenne* pollen on a Protein Pak 125 column. Eluent: (A) distilled water; (B) 0.15 *M* NaCl–0.01 *M* Tris (pH 8); (C) phosphate-buffered saline (pH 7.2).

Flow-rate

As low flow-rates usually lead to the best resolution, three different flow-rates, 1 ml/min (the highest recommended by the manufacturer of the column), 0.75 ml/min and 0.5 ml/min, were tested.

Low flow-rates produce the broadest peaks and therefore a flow-rate of 1 ml/min, which gave rapid resolution and sharp peaks, was adopted in further experiments.

Use of protease inhibitors

During the extraction procedure some enzymes are expected to be released from the pollen membranes[14,15]. This was demonstrated for *Lolium perenne*. Fig. 2B shows that the peak with a retention time of 14.44 min (downward arrow) is digested and the product(s) appears in the peak with a retention time of 16.78 (upward arrow). This digestion is prevented if phenylmethylsulphonyl fluoride (PMSF) was added at a level of 50 μg/ml to the extraction buffer (Fig. 2A).

Our extraction conditions are drastic (1 h at 0°C) but longer periods at 4°C led to increased proteolytic digestion (data not shown).

Other small changes such as the appearance of shoulders were detected but the more dramatic effect in the absence of PMSF during the extraction was the conversion of the material of retention time 14.44 min into the product with a retention time of 16.78 min.

By using integration in the data module we observed a mean decrease of 22.6 ± 1.5% peak area to 13.8 ± 0.8% in the case of the 14.44 min peak. A parallel increase in the 16.78 min peak from 5.4 + 0.3% to 16.8 ± 0.9% was observed.

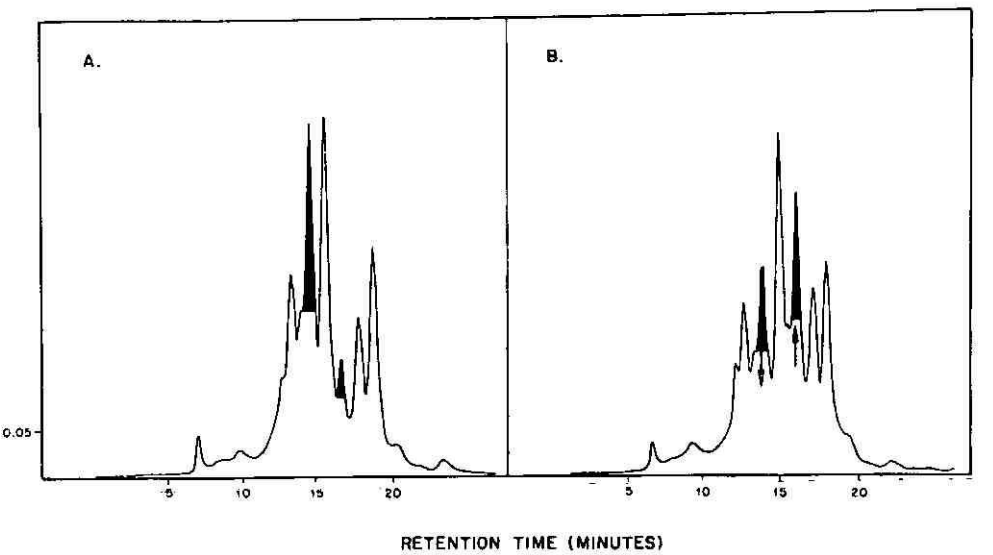


Fig. 2. Effect of protease inhibitor PMSF on the separation of *Lolium perenne* extract. Extractions and runs on the HPLC columns were performed (A) with or (B) without the presence of 50 μg/ml of PMSF in PBS. Shaded peaks indicate material digested (downward arrow) and digestion products (upward arrow).

As we subsequently demonstrated, the main allergen does not appear at this retention time (14.44 min) but, as a rule, we included PMSF during all extractions.

Final purification schedule

Two purification cycles in the Protein Pak 125 column were effective in obtaining the main allergen from *Lolium perenne* (Fig. 3). The position of the allergenic material was determined by performing SPRIA analysis of each fraction. Material from the shaded area in Fig. 3A was dialysed overnight against distilled water at 4°C, lyophilized and run again in the same column. The concentration of the sample after freeze-drying was about 10-fold.

SPRIA analysis of the fractions from the second run demonstrate that the shaded area in Fig. 3B contained the main allergen concentration in a pure form, as demonstrated in Fig. 3C. The molecular weight of this peak, as determined by plotting log (molecular weight) *versus* elution time for some pure protein markers was 32000, in very good agreement with results obtained by other workers[9]. The yield of purified allergen was about 300 mg/g, around twelve times higher that previously reported in purification by conventional biochemical methods[9].

SDS polyacrylamide gel electrophoresis further demonstrated the purity of the allergen, with an apparent molecular weight of 33000 (Fig. 4, lane C). When the crude extract from lane B was blotted to nitrocellulose paper and incubated with a pool of allergic sera, all the IgE-binding activity was associated with the pure allergen band (Fig. 4, lane D).

SPRIA analysis

In order to characterize further the purified allergen from an immunological point of view, we performed allergen titration in the solid-phase radioimmunoassay. As can be seen in Fig. 5, a dose-dependent rabbit anti-human IgE ^{125}I binding is obtained with both crude pollen extract and HPLC-purified allergen. Pure allergen needs about 0.3 μg and the extract 0.9 μg per PVC well to reach 50% radioactivity

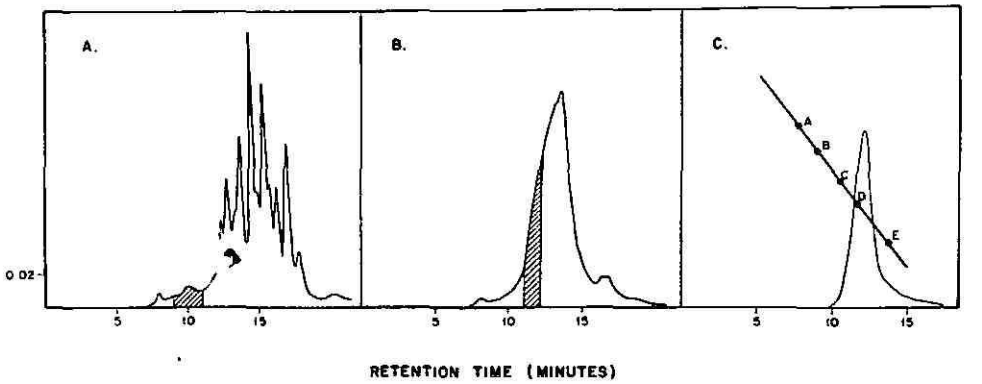
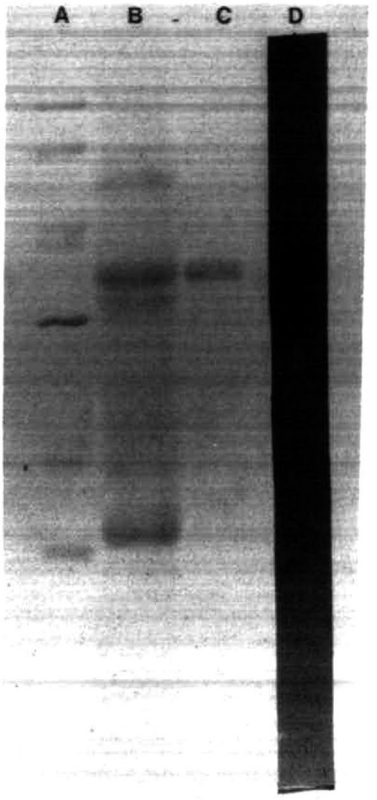


Fig. 3. Final allergen purification scheme. Shaded area in A was lyophilized and recycled in the run shown in B. Shaded area in B contains the allergen the purity of which was checked in C. The column was calibrated with (A) aldolase (MW 161000), (B) BSA (67000), (C) OVA (43000), (D) bovine pancreas DNase (31000) and (E) cytochrome *c* (12500).



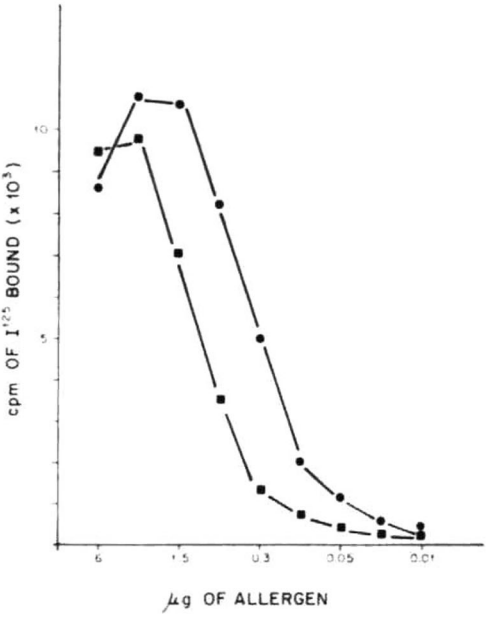


Fig. 4. SDS-PAGE of *Lolium perenne* extract and HPLC-purified main allergen. Lane A, molecular weight markers: phosphorylase *b* (MW 94000), BSA (67000), OVA (43000), carbonic anhydrase (30000), STI (20100) and α-lactalbumin (14400). Lane B, *Lolium perenne* crude extract. Lane C, HPLC-purified main allergen. Lane D, identification of IgE-binding band by immunoblotting.

Fig. 5. Titration of crude extract (■) and pure rye grass allergen (●) in solid-phase radioimmunoassay. A class 4 RAST serum and rabbit anti-IgE labelled with ^{125}I were used to detect the antigenic material.

binding. This indicates a 3-fold purification, an expected rate taking into consideration that the main allergen is a major component of the extract (Fig. 4).

When 1 μg of pure allergen is bound per well and titration of two selected RAST class 4 allergic sera was performed, the results in Fig. 6 were obtained. Serum from a subject allergic to egg white (class 3 RAST) showed a baseline under the same test conditions.

The above findings demonstrate that our SPRIA acts a dose-dependent test and that our allergen retains its specific IgE binding properties after purification. This was also demonstrated by *in vivo* skin-prick tests in which the pure allergen acted with the same specificity as crude *Lolium perenne* extracts.

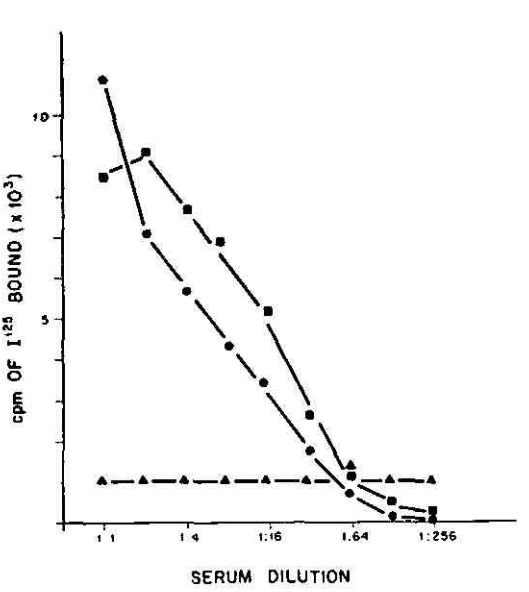


Fig. 6. Allergic sera titration on PVC wells each covered with 1 μg of HPLC-purified rye grass allergen. Two class 4 RAST sera against *Lolium perenne* (●, ■) and a class 3 RAST serum against egg white (▲) were employed.

DISCUSSION

The advantages of purification of the main allergen of *Lolium perenne* by using Protein Pak 125 HPLC columns are that there is no need to defat the pollen or remove pigments from the crude extract, coupled with high speed and good yields of the purified allergen. In addition, the evidence of common allergens in phylogenetically related species of grasses opens the field for the rapid obtaining of purified allergens than can be used for a wide range of therapeutic allergic treatment.

We must stress the use of buffers with an ionic strength equivalent to 0.15 *M* NaCl in order to avoid the formation of aggregates and to minimize the ionic interactions between the proteins and the silica gel stationary phase of the column. We advise the use of shorter periods of extraction than those employed by other workers because 1 h is sufficient for the release of most allergenic material from pollen. Careful control of the temperature of extraction at 0°C in an ice-bath avoids the production of low-molecular-weight products of protease digestion.

The allergenic activity of the purified protein is fully retained, as expected with the mild conditions of separation used.

We conclude that HPLC is a valuable technique for the purification of allergens from crude extracts of Graminiae pollen. The speed of the separation and the high yield obtained suggest applications in industrial-scale processes.

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DETERMINATION OF THE HEROIN METABOLITE 6-ACETYLMORPHINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED PRE-COLUMN DERIVATIZATION AND FLUORESCENCE DETECTION

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SUMMARY

An improved method for the determination of 6-acetylmorphine in the urine of drug addicts receiving morphine was developed. A newly introduced reversed-phase high-performance liquid chromatographic system proved to be more sensitive than a normal-phase system used previously. By replacing the earlier manual derivatization procedure with an automated on-line pre-column method, both the reproducibility and efficiency were considerably improved. Coefficients of variation for repeated analyses typically ranged from 6 to 10% in the 1–100 μg/l concentration range. The detection limit was 1 μg/l and the correction for recovery by calibration with blank urine samples spiked with 6-acetylmorphine was satisfactory. The analytical improvements achieved, however, did not increase the chance of detecting heroin use by drug addicts.

INTRODUCTION

For more than 2 years the Municipal Health Department of Amsterdam has been supplying a limited number of extremely problematic drug addicts with daily rations of injectable morphine. This treatment programme is closely monitored with, among other methods regular urine analyses to detect possible continuation of heroin use by the drug addicts.

Heroin use is most conveniently detected by determining its principal metabolites morphine and morphine-3-glucuronide in urine[1], but this technique is obviously useless in this instance. The only specific marker for heroin use is 6-acetylmorphine, which, unfortunately, is only a minor urinary metabolite as it is further metabolized to morphine to a large extent[2]. To have a reasonable chance of detecting heroin abuse under these conditions, the method used should be extremely sensitive. Previously, high-performance liquid chromatographic (HPLC) method with fluorescence detection was developed[3], based on mild oxidation of 6-acetylmorphine in the

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presence of excess of morphine to yield a highly fluorescent condensation product. The reaction products were separated by normal-phase HPLC, which allowed the detection of approximately 5 μg/l of 6-acetylmorphine in urine. In this paper, an improved method consisting of reversed-phase HPLC and automated pre-column derivatization is presented. The main incentives to improve the method were, in order of importance, (i) the notion that lowering the detection limit could possibly improve the chance of detecting heroin use; (ii) the limited reproducibility of the method, which was mostly due to the instability of the 6-acetylmorphine/morphine condensation product causing the samples to deteriorate slowly while standing in the autosampler; and (iii) the detrimental effect of the solvent used on the piston seal of the HPLC pump.

The new method was compared with radioimmunochemical methods for both total and free morphine to establish the relative efficiencies of these methods in detecting heroin use.

EXPERIMENTAL

Materials and equipment

All solvents and reagents were of analytical-reagent grade. Water was distilled twice in Pyrex glass. 6-Acetylmorphine was prepared from morphine as described previously[3]. HPLC columns (150 × 4.6 mm I.D.) were packed with Hypersil ODS 5 μm (Shandon, Runcorn, U.K.) by means of a Shandon slurry packer and according to the manufacturer's instructions. The chromatograph consisted of a Kipp Analytica (Emmen, The Netherlands) Model 9208 pump, an LKB (Bromma, Sweden) 2153 Autoinjector, a laboratory-built column thermostat and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 650-10LC fluorescence spectrometer.

Methods

Urine samples were extracted as described previously[3]. Briefly, the method consisted of extraction with 15% (v/v) 2-propanol in dichloromethane on an Extrelut

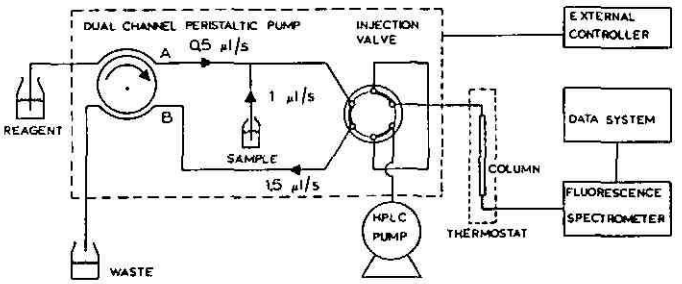

Fig. 1. Automatic pre-column derivatization device of the LKB 2153 Autoinjector as used in 6-acetylmorphine derivatization. The derivatization–injection procedure is initiated by switching the injection valve into the "load" position and starting the peristaltic pump. Channel B of the pump draws the reagent–sample mixture through the sample loop at a rate of 1.5 μl/s. Channel A delivers reagent at a rate of only 0.5 μl/s so that sample is drawn at a rate of 1 μl/s. After 150 s the pump is stopped and the reaction mixture is left in the sample loop for 120 s. Then the valve is switched into the "inject" position and the contents of the sample loop are transferred to the column.

column, followed by back-extraction of the organic solvent with dilute sulphuric acid. The latter was rendered alkaline and extracted with 2-propanol–dichloromethane as described above.

After evaporation of the organic solvent, the residue was dissolved in 200 μl of a 125 mg/l solution of morphine hydrochloride in 0.015 mol/l hydrochloric acid, then 10 μl of 0.3 mol/l Tris buffer (pH 8.5) were added to the samples, which were subsequently placed in the autosampler. The latter was equipped with a pre-column derivatization device as depicted in Fig. 1. The reagent delivered by pump A was a 0.015 mol/l solution of potassium hexacyanoferrate(III) in water. The mixing ratio of sample and reagent was 2:1. The injection cycle was initiated by flushing the 50-μl sample loop with sample–reagent mixture for 150 s. The reaction was then allowed to proceed for 120 s, after which the contents of the sample loop were injected immediately. Separation was accomplished using the solvent system 0.1% (v/v) triethylamine in acetonitrile–water (16:84, v/v) at a flow-rate of 1.5 ml/min. The column temperature was maintained at 30°C. Under these conditions, the back-pressure was 150 bar and the retention time of the 6-acetylmorphine/morphine dimer was approximately 10 min. The column eluate was fed into the fluorescence spectrometer, the excitation and emission monochromators of which had been adjusted to 320 and 436 nm, respectively, with a 10 nm bandwidth. Between the chromatographic runs three consecutive injections were made from a vial containing 0.1% of triethylamine in acetonitrile–water (40:60, v/v) to prevent carry-over between samples. Quantitation was based on peak-height measurement and corrections for recovery were made using the external standard method.

In one experiment both free and total morphine were determined in urine samples by radioimmunochemical methods. For total morphine the Abuscreen kit from Hoffman-LaRoche (Nutley, NJ, U.S.A.) and for free morphine the Coat-a-count kit from Diagnostic Products (Los Angeles, CA, U.S.A.) were used.

RESULTS AND DISCUSSION

The first aspect of the revised method to be investigated was the possible occurrence of compounds in urine samples that would coelute with the 6-acetylmorphine/morphine condensation product. This had been the sensitivity-limiting factor in the normal-phase HPLC system used previously. Therefore, six blank urine samples were obtained from laboratory staff members and were subjected to the new method. In the chromatograms obtained no peaks at or near the retention time of the 6-acetylmorphine/morphine condensation product could be observed and it was concluded that the new method might offer the desired higher sensitivity.

It was felt that the new automatic pre-column derivatization device might give rise to considerable carry-over between samples. As the 6-acetylmorphine concentration of the urine samples to be analysed varied over four orders of magnitude, any carry-over would be unacceptable. By analysing highly concentrated and blank urine samples in turn it was found indeed that considerable carry-over occurred. The LKB Autoinjector used did not offer convenient facilities for flushing between samples but by connecting it to an external programmable timer, any number of flushing cycles could be applied. I was found that the flushing conditions mentioned above reduced carry-over to undetectable levels.

TABLE I

REPRODUCIBILITY OF 6-ACETYLMORPHINE DETERMINATIONS IN ENRICHED URINE SAMPLES

6-Acetylmorphine concentration (μg/l)					*Mean squares*		*F*	*p*
Calculated	*Found*			*Mean*	*Between results*	*Among results*		
	Week 1	*Week 2*	*Week 3*					
5	4.35	4.29	3.93	3.91	0.31	0.05	6.0	<0.05
	3.87	3.93	3.75					
	4.03	3.93	3.40					
	4.03	4.11	3.40					
	3.55	4.29	3.57					
	3.87	4.11	3.93					
25	18.40	19.23	18.58	18.27	1.58	0.35	4.5	<0.05
	19.45	18.00	18.23					
	18.40	18.53	16.78					
	18.40	17.83	17.85					
	18.40	18.88	16.78					
	18.40	18.88	17.85					

To validate the method further, its linearity, reproducibility, recovery and sensitivity were investigated. Solutions of 20, 40, 100, 200, 500, 1000 and 2000 μg/l 6-acetylmorphine in methanol were prepared. As the concentration factor of the extraction procedure was 20, these concentrations corresponded to urine concentrations ranging from 1 to 100 μg/l. From each of these, 33.3 μl (sample loop volume 50 μl, dilution factor 1.5) were injected on the column twice. Linear regression of the 6-acetylmorphine peak heights obtained to the corresponding equivalent urine concentrations gave the regression equation $Y = 4.30X - 6.35$, where Y = peak height (mm) and X = concentration (μg/l). The correlation coefficient was 0.9941, the residual standard deviation was 16.99 and the standard deviations of the regression coefficient and intercept were 0.13 and 5.87, respectively.

To investigate both reproducibility and recovery, a blank urine pool was divided into two portions, which were enriched with 5 and 25 μg of 6-acetylmorphine per litre of urine, respectively. Both pools were analysed six times on each of three occasions with intervals of approximately 1 week. The results from these experiments were used to calculate the reproducibility and, by comparison with concurrently analysed standard solutions, the analytical recovery for both concentration levels. The results are presented in Table I. There was a significant variation between analytical series, as could be shown by an analysis of variance ($p < 0.05$ for both levels). The recoveries calculated from these data averaged 78 and 73% for the 5 and 25 μg/l samples, respectively. As the recoveries were obviously subject to the same between-run variation as mentioned above, it was found useful to include recovery checks in each run. Because the reproducibility data in Table I did not apply to such conditions, another experiment was devised. In this experiment a urine pool was prepared by combining urine samples from heroin addicts and dividing the mixture into eight portions, which were diluted with varying amounts of a blank urine pool. The 6-

TABLE II

REPRODUCIBILITY OF 6-ACETYLMORPHINE DETERMINATIONS USING ENRICHED BLANK URINE SAMPLES AS STANDARDS

Urine pool No.	*6-Acetylmorphine concentration (μg/l)*		
	*Calculated**	*Average found***	*Coefficient of variation (%)*
1	95.3	94.1	7.0
2	76.2	63.3	6.0
3	63.5	57.5	8.3
4	42.3	40.9	7.8
5	21.2	19.4	9.3
6	10.6	10.1	13.9
7	5.3	5.2	9.6
8	2.1	2.0	10.0

* Calculated from the 6-acetylmorphine concentration of the original urine pool that was used to prepare pools 1–8.

** Average of six determinations equally distributed over three analytical runs.

acetylmorphine concentrations in the urine pools so obtained ranged from 2 to 94 μg/l. Urine samples taken from these pools were analysed in duplicate on three different occasions at 1 week intervals. In this instance the peak heights were compared with those obtained from two blank urine samples enriched with 25 μg/l of 6-acetylmorphine. Table II shows the mean concentrations found and those calculated from the concentration as determined in the original urine pool. With one exception, which was probably due to a dilution error, the concentrations found agreed well with those calculated. The coefficients of variation generally were between 6 and 10% with a slight but statistically significant (linear regression: hypothesis $\rho = 0$; $p < 0.05$) increase with decreasing concentration. However, even at the lower end of its dynamic range the method seemed to offer good reproducibility.

The absolute detection limit was 0.25 ng injected on the column (signal-to-noise ratio = 2). When two blank urine samples were enriched with 2 and 1 μg/l of 6-acetylmorphine, easily recognizable peaks were obtained with signal-to-noise ratios of 4 and 2, respectively.

The results mentioned above showed that the analytical aims set had been achieved, but it remained to be demonstrated that the improvements did indeed increase the chance of detecting heroin use. For obvious reasons, it was impossible to test this under experimentally satisfactory conditions. Therefore, it was decided to repeat an experiment carried out previously[3] with the use of a normal-phase HPLC system. In this experiment urine samples had been collected at random from 50 heroin addicts who did not receive morphine and these samples had been shown to contain at least 0.4 mg/l of total morphine by a standard enzyme mediated immunochemical (EMIT) method. In 72% of the samples 6-acetylmorphine had been detectable with reasonable confidence.

Again, 50 urine samples were collected from heroin addicts but in this instance both the free and total morphine concentrations were determined by radioimmu-

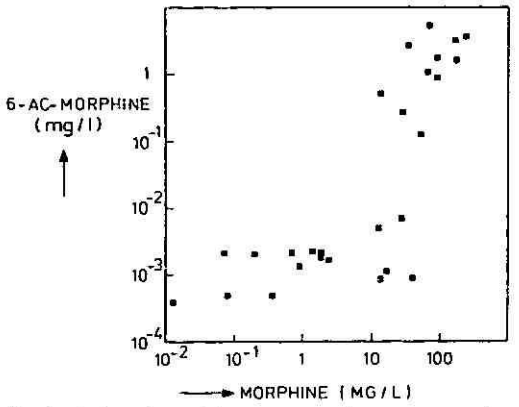

Fig. 2. 6-Acetylmorphine concentrations *versus* total morphine concentration in 28 urine samples collected from heroin addicts.

noassay. The 6-acetylmorphine concentrations were determined subsequently, and the results compared with the total morphine concentrations. In Fig. 2 the 6-acetylmorphine concentrations are plotted against the total morphine concentrations for all 28 samples in which both compounds were detectable. These samples amounted to 56% of those analysed, but this figure cannot be compared directly with the 72% found using the normal-phase HPLC method because, as was shown by radioimmunoassay, only 36 samples contained at least 0.4 mg/l of total morphine. When only the latter samples were taken into consideration, 64% of them were found to contain detectable levels of 6-acetylmorphine, a number fairly close to the 72% found previously. From Fig. 2 it is clear that no simple relationship exists between the concentrations of the two compounds and that the 6-acetylmorphine concentration tends to decrease much faster than that of total morphine. A similar picture emerged when the 6-acetylmorphine concentration was related to that of free morphine. In contrast, the concentration of the latter correlated excellently with that of total morphine [linear regression: $Y = 0.063X + 0.121$, where Y and X are the concentrations of free and total morphine (μg/l), respectively; correlation coefficient, 0.964; residual standard deviation, 1.730]. Apparently, 6-acetylmorphine is eliminated from the body more rapidly than morphine, a conclusion that is supported by pharmacokinetic studies carried out in dogs[4]. From this the conclusion can be drawn that increasing the sensitivity beyond what has been achieved in the method presented above does not contribute to a better chance of detecting heroin use.

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SIMULTANEOUS DETERMINATION OF MULTIPLE ADDITIVES IN COSMETICS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An high-performance liquid chromatographic method for the simultaneous determination of multiple additives in cosmetics was studied by using a methanol gradient with a multi-wavelength detector capable of scanning a range of wavelengths. For accurate determinations, it was important to use the same solvent composition both for the sample and standard, and to minimize the injection volume as well as to employ peak-area integration in the construction of the calibration curve. The simultaneous determination of twelve additives in cosmetics was carried out and satisfactory results were obtained both for the recovery and the coefficient of variation. The method was also applied to the determination of these additives in commercial cosmetics and the results showed a good agreement with the conventional method. This method would be suitable for routine, quality control analysis of cosmetic products.

INTRODUCTION

The use of an elution gradient and a multi-wavelength detector in high-performance liquid chromatography (HPLC) seems most effective in the simultaneous determination of multiple additives in cosmetics. Gradient elution has the following advantages in overcoming general elution problems in HPLC[1]: (1) the total analysis time can be significantly reduced; (2) the effective sensitivity is very high because of a negligible variation in the peak shape; (3) elution and separation of multiple components having a wide range in polarity can be accomplished simultaneously.

There are two types of multi-wavelength detector, the variable wavelength scanning detector and the photodiode array detector. The use of this type of detector overcomes the most difficult problems arising from extreme differences in the composition or the responses of multiple additives to be determined simultaneously.

This paper presents the simultaneous determination of multiple additives in cosmetics by a methanol gradient in conjunction with a multi-wavelength detector. The optimum wavelength for each component may be selected by this detector during an experiment. This method has been successfully utilized for routine analysis and quality control analysis of micro amounts of additives in cosmetics.

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EXPERIMENTAL

Reagents

Table I shows the twelve additives which are frequently present in the cosmetics used in this work. The samples were all cosmetic grade, chromatographically pure and, therefore, were used without further purification. Methanol for HPLC and phosphoric acid were obtained from Wako Pure Chemical (Osaka, Japan). Deionized water was used for the mobile phase, which was degassed by sparging it with helium gas[2].

TABLE I

ADDITIVES FREQUENTLY PRESENT IN COSMETICS

A	Pantothenyl ethyl ether
B	Methyl *p*-hydroxybenzoate
C	Salicylic acid
D	Benzyl nicotinate
E	4-Isopropyl-3-methylphenol
F	Butyl *p*-hydroxybenzoate
G	Monoammonium glycyrrhizinate
H	Trichlorocarbanilide
I	2,6-Di-*tert*.-butyl-4-methylphenol
J	Pyridoxine dioctanoate
K	Tocopheryl acetate
L	Stearyl glycyrrhetinate

Apparatus

The HPLC equipment used was an LC4A chromatograph (Shimadzu, Kyoto, Japan), a Shimadzu SPD2AS variable wavelength scanning detector and a Shimadzu SPDM1A photodiode array detector. The column (150 mm × 4 mm I.D.) was slurry-packed by using methanol with Nucleosil $5C_{18}$ (5 μm) from Macherey-Nagel (Düren, F.R.G).

Procedures

For the gradient from 35 to 99.9% methanol, a mixture of water and methanol (65:35), adjusted to pH 2.5 with phosphoric acid, was prepared as the initial eluent. From $t = 0$ to $t = 26$ min the eluent composition was linearly changed to methanol–phosphoric acid (1000:1). This composition was maintained for an additional 12 min. Throughout the flow-rate was set at 1.0 ml/min and the column temperature was maintained at 40°C. After the gradient had been started, it took 5 min to detect the eluent passed through the column and after completion of an experiment the initial eluent composition was restored in 17 min. Other HPLC conditions were based on our previous work[3]. All the samples were dissolved in methanol at concentrations from 10 to 80 μg/ml, and 20 μl of the sample solution were injected.

RESULTS AND DISCUSSION

Choice of detector for quantitative analysis

In general, small amounts of various additives with differing solubilities are present in cosmetics. Some materials are soluble in water and others are soluble in oil. Fig. 1 shows the gradient elution profile of the twelve additives selected for this study. Two detectors, SPD2AS and SPDM1A, having different detection characteristics, but giving identical chromatograms were used. The wavelength for the detection was selected so that the peak response of each component is nearly equal. The coefficients of variation of the retention time of each component were below 0.3% in five repeated analyses. Table II shows the reproducibility of the peak heights for the quantitative analysis.

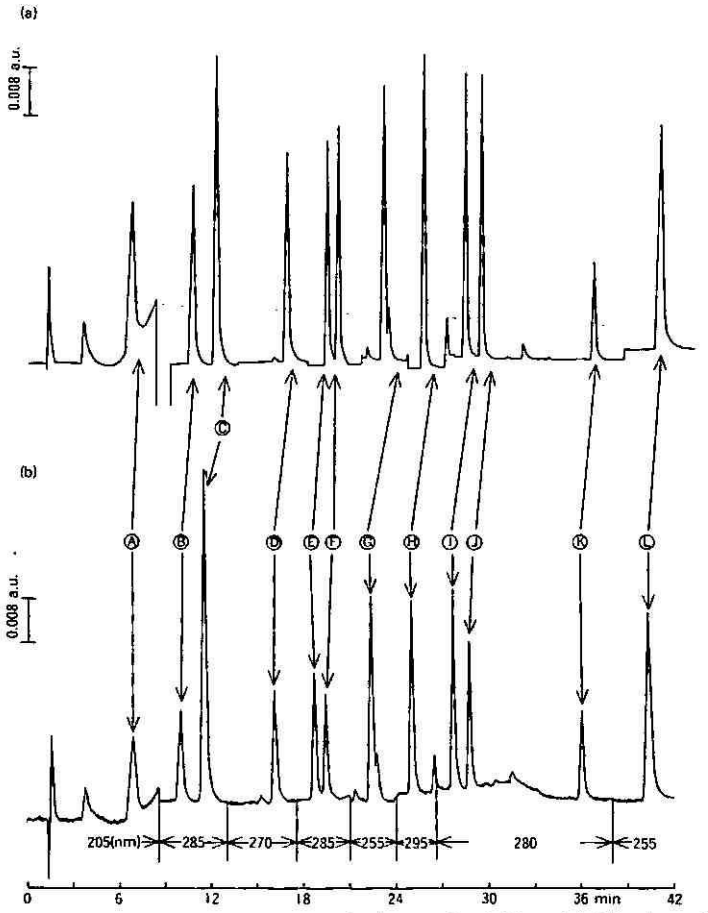

Fig. 1. Gradient elution profile of typical cosmetic additives. Mobile phase: 26-min linear gradient starting with water–methanol (65:35), adjusted to pH 2.5 by phosphoric acid, and ending with methanol–phosphoric acid (1000:1) adjusted to pH 2.5 with phosphoric acid followed by 12 min of the final eluent. Sample size: 20 μl, 50 μg/ml of each of component. Detectors: (a) SPD2AS (scanning); (b) SPDM1A (diode array).

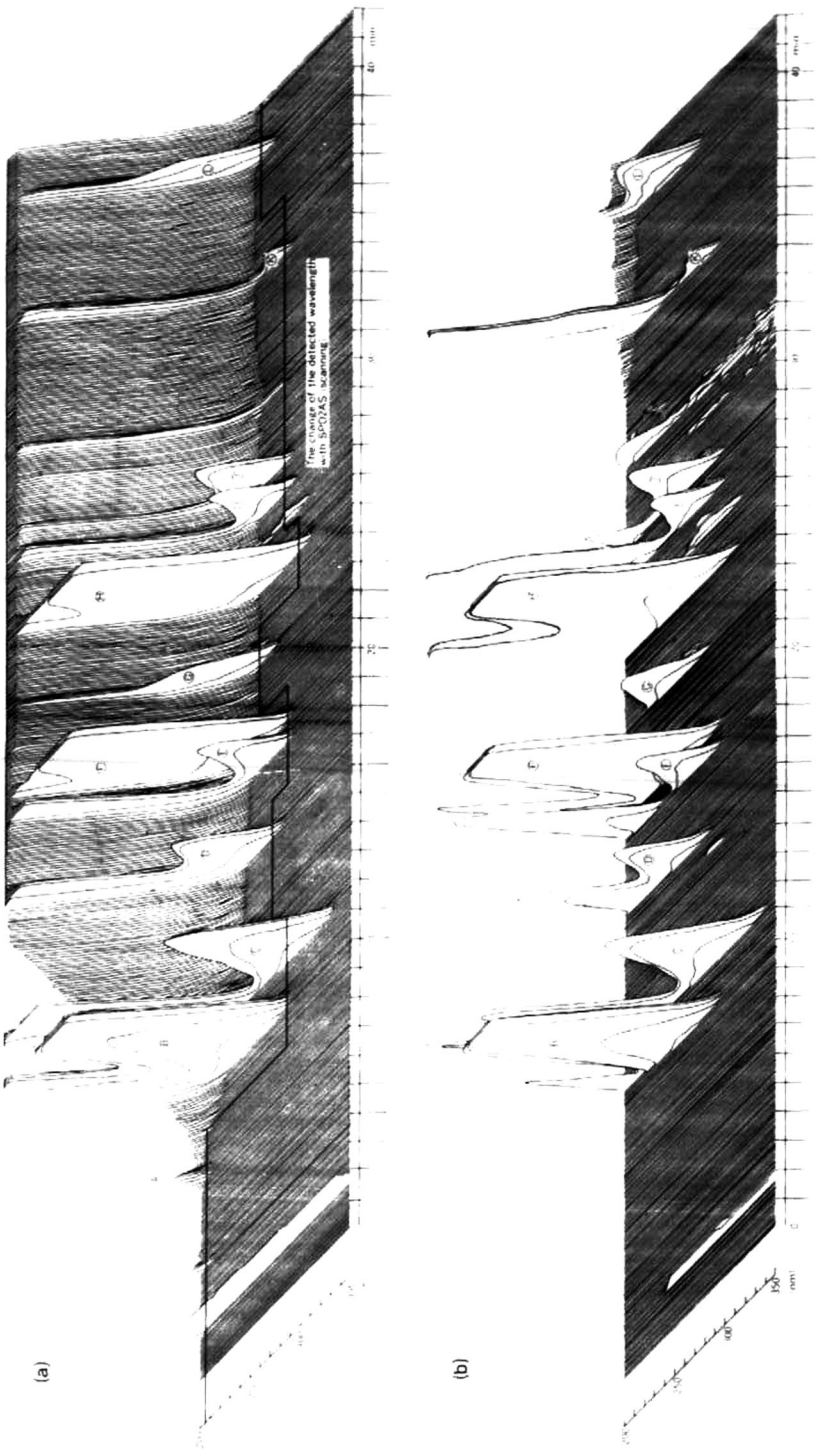

Fig. 2. Three-dimensional projections for typical additives obtained by gradient elution. Analytical conditions as in Fig. 1. (b) Projection after subtracting the methanol spectrum from (a).

TABLE II

COEFFICIENTS OF VARIATION (%) FOR THE PEAK HEIGHTS OF ADDITIVES OBTAINED BY TWO MULTI-WAVELENGTH DETECTORS

Analytical conditions as in Fig. 1. The reproducibility test was effected on five replicates.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
SPD2AS	1.2	0.8	0.8	0.9	1.5	1.3	1.4	1.5	1.9	2.1	1.7	1.8
SPDM1A	2.7	4.9	0.7	3.1	4.1	4.8	1.9	2.6	1.7	2.1	1.0	2.0

Since the coefficients of variation with the SPDM1A were higher than those obtained with the SPD2AS, probably due to the long observation time required for the measurement and the low sensitivity of the SPDM1A, the SPD2AS detector was chosen for simultaneous determination. However, this conclusion is not always applicable, as it seems that the properties of diode array detectors differ according to the equipment suppliers[4,5]. Nevertheless, there is no doubt about the advantages of this type of detector which gives much information and has wide application.

Fig. 2 shows the three-dimensional projections of the UV spectra of the twelve additives upon gradient elution. The changes in the detection wavelength with time with the SPD2AS are shown in Fig. 2a. The UV absorption of methanol in the mobile phase is significant at lower wavelengths and interferes with the UV absorption of the compounds. The spectrum of the ghost peak which often appears at methanol concentrations near 100% during a gradient elution could also be obtained by this projection[6]. Fig. 2b shows the projection after subtracting the spectrum of methanol from Fig. 2a. The spectrum of pantothenyl ethyl ether which is similar to that of methanol cannot be distinguished in Fig. 2b, but those of the other components become much clearer than in Fig. 2a.

Effect of the water content in the sample solution on the determination

Table III shows the calibration curves and the correlation coefficients for the additives obtained through six measurements from 10 to 80 μg/ml. Each plot was linear and the correlation coefficients were greater than 0.9995. The recoveries of the additives were also studied by adding each compound to a known lotion at a concentration of 500 μg/g. Table IV shows the results. The coefficients of variation were relatively low, but the recoveries of pantothenyl ethyl ether, methyl *p*-hydroxybenzoate and salicylic acid were found to be over 100%. In this experiment the sample preparation was carried out by 10-fold dilution in methanol, however, the water content of the lotion was about 85%. Therefore, the effect of the water content in the sample solution on the peak height was investigated further under isocratic condition for nine representative additives. The results are shown in Fig. 3. The peak height of each component was greatly affected by the water content in the sample solution, and this phenomenon was more marked when the injection volume was increased. In case of 40-μl injections, a change in the peak height of pantothenyl ethyl ether became apparent and an asymmetric peak similar to those obtained in overloading was obtained when the sample was diluted in methanol: however, the retention time and the peak area were not affected by the water content of the sample

TABLE III

CALIBRATION CURVES FOR THE ADDITIVES

Y = Peak height (mm); X = concentration (μg/ml).

Component	*Calibration curve*	*Correlation coefficient*
A	$Y = 1.02X - 0.6$	1.0000
B	$Y = 1.13X + 1.3$	0.9999
C	$Y = 2.34X - 3.1$	1.0000
D	$Y = 1.28X + 0.1$	0.9999
E	$Y = 1.36X + 1.2$	0.9997
F	$Y = 1.31X + 2.0$	0.9995
G	$Y = 1.82X - 2.5$	1.0000
H	$Y = 1.93X + 0.2$	0.9998
I	$Y = 1.89X + 0.2$	1.0000
J	$Y = 1.71X + 0.9$	0.9999
K	$Y = 0.67X - 0.4$	0.9998
L	$Y = 1.49X + 0.1$	0.9997

solution. This band-broadening phenomenon is probably due to the increasing diffusion of the solutes caused by introduction of a solution with lower polarity than that of the mobile phase, while the sample had a reasonable retention time[7,8]. Therefore, the recoveries over 100% obtained for some of the components are due to the fact that the sample solution contained 8.5% water, while the standard solution only contained methanol. Table V shows the results of a recovery test which was performed after adjusting the difference in water contents of the sample and standard solution. Satisfactory results were obtained for both the recoveries and the coefficients of variation.

Fig. 4 shows the effect of the column diameter on the peak height for two components. It was found that the effect of the solvent composition of the sample solution could be reduced by using a wider column. This effect was independent of the flow-rate. From these results, several conclusions can be drawn for the accurate determination by HPLC, when the sample was diluted in solvent: (1) the sample solution and the standard solution should be in the same solvent; (2) the injection volume should be as low as possible, less than 10 μl for a 4 mm I.D. column, and below 20 μl for a 6 mm I.D. column; (3) the peak-area method should be chosen for the calibration.

TABLE IV

RECOVERIES OF ADDITIVES ADDED TO A KNOWN LOTION AT 500 μg/g

C.V. = Coefficient of variation. The recoveries test was effected on five replicates.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
Recovery (%)	111.4	107.2	110.4	97.4	99.6	97.1	100.6	96.7	98.6	96.8	100.8	101.0
C.V. (%)	1.1	2.9	1.1	1.5	0.9	2.2	0.6	0.9	0.9	1.0	1.1	0.3

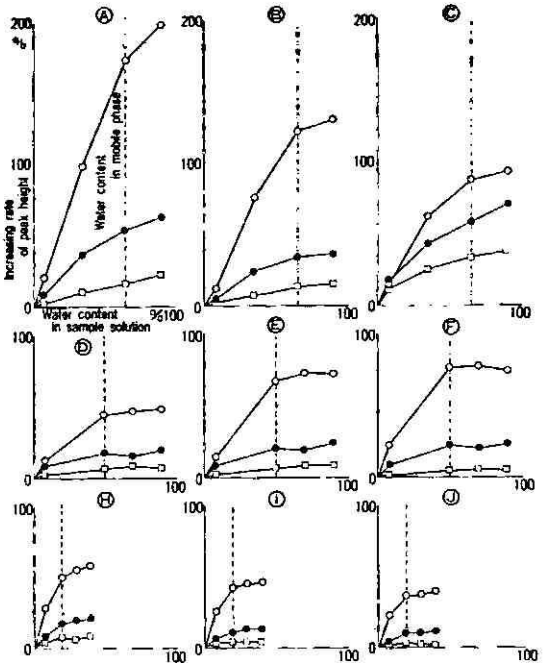

Fig. 3. Effect of the water content in the sample solution on the peak height. Injection volumes: ○, 40 μl; ●, 20 μl; □, 10 μl.

Application for routine or quality control analyses

The results of the investigation described above were applied to the simultaneous determination of micro amounts of additives in commercial cosmetics. Table VI shows the results of these simultaneous determinations and those obtained by conventional isocratic HPLC methods established independently for each component. The two sets of results agreed with each other and the fluctuation of the measurements obtained by each method are small enough. Therefore, it is concluded that this method is applicable to routine or quality control analyses. A problem remaining unresolved was the employment of the same pretreatment procedure for all samples, which is an important factor for the simultaneous determination of multiple components. However, this still depends upon the sample matrix. This method has the

TABLE V

RECOVERY TEST PERFORMED AFTER ADJUSTING THE DIFFERENCE IN WATER CONTENTS OF THE SAMPLE AND THE STANDARD SOLUTION

Conditions as in Table IV.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
Recovery (%)	100.0	100.5	100.3	99.4	99.4	99.3	100.7	99.4	98.9	99.7	101.3	100.7
C.V. (%)	2.2	3.1	0.9	1.6	1.5	2.9	0.5	1.6	0.3	1.5	0.7	0.5

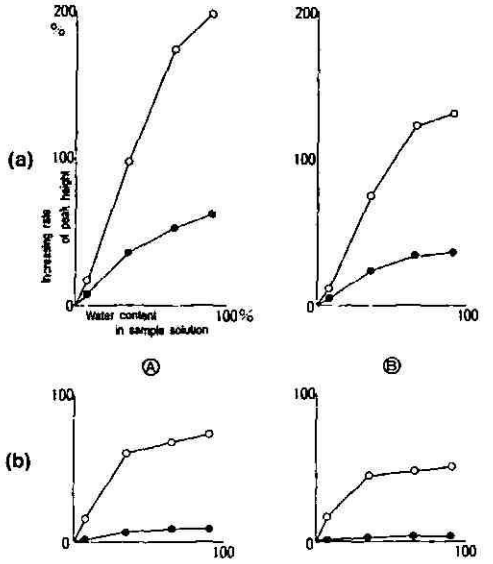

Fig. 4. Effect of the column diameter on the peak height. Column diameters: (a) 4 mm; (b) 6 mm. Other symbols as in Fig. 3.

following advantages for the quality control of cosmetic products, even though an universally effective pretreatment procedure cannot be proposed: (1) it may be possible to design an analyzer to meet the requirement, and avoid the need to change the HPLC conditions; (2) the effectiveness of the autosampler for HPLC can be improved. It is concluded that this method is suitable for quality control by the cosmetic industries which manufacture many kind of products in small quantities.

TABLE VI

DETERMINATION OF ADDITIVES IN THE COMMERCIAL COSMETICS

$\bar{X}$ = Average of two replicate analyses; R = the difference between the two measurements.

	Component	*Simultaneous determination (%, w/w)*		*Conventional method*[9–11] *(%, w/w)*	
		$\bar{X}$	R	$\bar{X}$	R
Lotion 1	A	0.0502	0.0006	0.0502	0.0005
	G	0.0488	0.0006	0.0487	0.0003
	K	0.0497	0.0004	0.0490	0.0005
Lotion 2	A	0.0502	0.0006	0.0535	0.0008
	G	0.0516	0.0006	0.0527	0.0008
	K	0.0494	0.0004	0.0492	0.0001
Shampoo	H	0.291	0.003	0.298	0.001
Hair tonic	E	0.1007	0.0007	0.1040	0.0020
	F	0.0514	0.0012	0.0521	0.0015

CONCLUSION

A method for the simultaneous determination of multiple additives in cosmetics by using gradient HPLC with a multi-wavelength detector was described. It was found that this method could be utilized for the routine, quality control analyses of multiple additives in cosmetics. In Japan, it is required under Good Manufacturing Practice (GMP) regulation that all manufactured batches of medicated cosmetics (Quasi-Drugs) be guaranteed for the content of active ingredients approved by the Ministry of Health and Welfare. The accuracy of the proposed method was adequate enough for this purpose and may be considered as a useful means for the routine analysis of active ingredients and additives.

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Note

Effect of end-capping of reversed-phase high-performance liquid chromatographic matrices on the analysis of vitamin A and its metabolites

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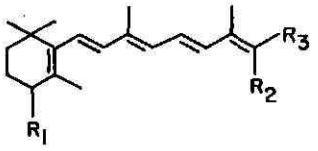
All-*trans*-retinoic acid (see Fig. 1) is a metabolite of retinol (vitamin A) capable of supporting the functions of retinol in the maintenance of normal growth and epithelial cell differentiation[1]. Retinoic acid and some of its analogues (retinoids) have generated much interest as agents useful for the treatment of skin disorders[2] and as potential cancer chemopreventive or chemotherapeutic compounds[3,4]. At present, the mechanism of action of these materials remains obscure although a number of possibilities have been proposed[5].

Recently, we have been engaged in the synthesis and biological evaluation of analogues of all-*trans*-retinoic acid and its natural isomer 13-*cis*-retinoic acid[6,7]. Our current efforts in the area involve the synthesis of electrophilic affinity label analogues derived from the metabolite 4-hydroxyretinoic acid in order to probe the cellular



1 R_1 = H, R_2 = H, R_3 = CH_2OH
2 R_1 = H, R_2 = H, R_3 = CHO
3 R_1 = H, R_2 = H, R_3 = COOH
4 R_1 = H, R_2 = COOH, R_3 = H
5 R_1 = OH, R_2 = H, R_3 = COOH

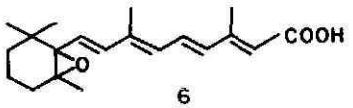


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Fig. 1. Structures of retinol and metabolites. 1 = Retinol; 2 = retinal; 3 = all-*trans*-retinoic acid; 4 = 13-*cis*-retinoic acid; 5 = 4-hydroxyretinoic acid; 6 = 5,6-epoxyretinoic acid.

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retinoic acid-binding protein potentially mediating the actions of all-*trans*-retinoic acid[5,7,8]. Successful isolation of these analogues will require the separation of retinoid isomers produced by our synthetic procedures[9].

Reversed-phase high-performance liquid chromatography (HPLC) has been extensively used to resolve retinol and its metabolites. Many of the most useful methods employ aqueous methanol-based mobile phases[10–13]. The nucleophilicity of methanol[14], however, makes its use as a mobile phase component incompatible with the planned electrophilic analogues of 13-*cis*-retinoic acid. Alternative approaches utilizing aqueous acetonitrile-based mobile phases seemed more likely to suit our needs[15,16].

In the present communication, we report a comparison of the effect of end-capping of the reversed-phase stationary phase on the chromatographic resolution of a number of retinoids of varying polarity. In addition, the ability of the two previously outlined mobile phases to resolve the representative group of retinol metabolites already mentioned, as well as retinal and 5,6-epoxyretinoic acid, has been evaluated.

EXPERIMENTAL

Materials and methods

The retinol, retinal, and all-*trans*-retinoic acid employed in this study were purchased from Aldrich (Milwaukee, WI, U.S.A.). 4-Hydroxyretinoic acid, 13-*cis*-retinoic acid, and 5,6-epoxyretinoic acid were generously provided by Dr. P. F. Sorter, Hoffmann La-Roche (Nutley, NJ, U.S.A.). Retinyl palmitate was obtained from Sigma (St. Louis, MO, U.S.A.). Methyl retinoate was prepared by treatment of retinoic acid with diazomethane according to standard procedures. HPLC grade water, methanol, acetonitrile, tetrahydrofuran, and ammonium acetate were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Retinoid solutions at a concentration of 1 mg/ml were prepared in HPLC grade acetonitrile and stored under an argon atmosphere at −35°C until use. All manipulations were performed under yellow light (Sylvania F40 gold fluorescent lamps).

Reversed-phase HPLC

HPLC was carried out on a Beckman Model 332 gradient liquid chromatograph system equipped with a Beckman Model 164 ultraviolet detector (Beckman Instruments, San Ramon, CA, U.S.A.) monitoring eluent at 340 nm and a Kipp and Zonen (Delft, The Netherlands) Model BD41 dual-pen recorder.

Retinoids were analyzed by isocratic reversed-phase chromatography on two columns: a 10-μm, irregular particle, non-end-capped octadecylsilane column (Ultrasil-ODS, 250 mm × 4.6 mm I.D.; Beckman Instruments) and a 5-μm, spherical particle, fully end-capped octadecylsilane column (Ultrasphere-ODS, 250 mm × 4.6 mm I.D.; Beckman Instruments). Mobile phases employed in specific separations are indicated in the legend of the appropriate figure. All columns were eluted at ambient temperature and a flow-rate of 1.5 ml/min.

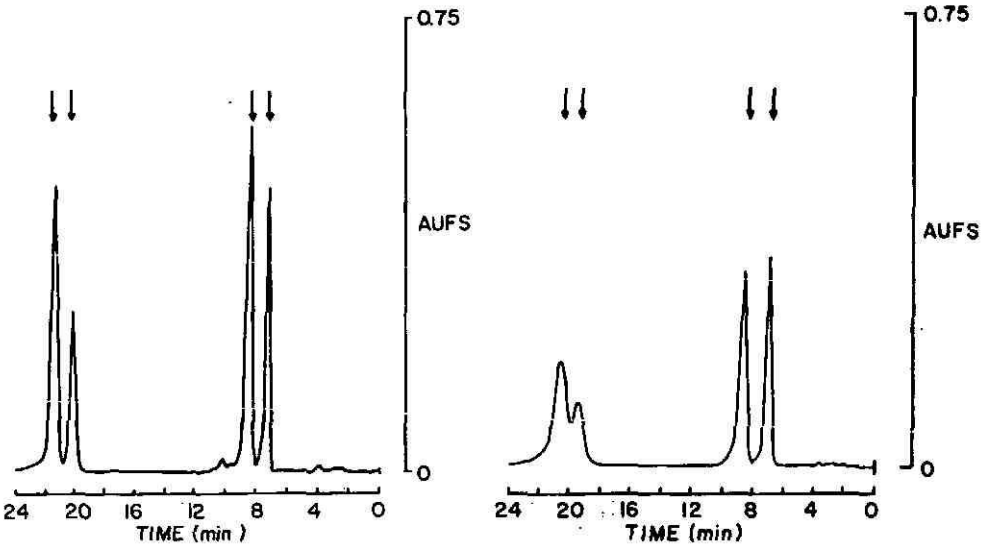

Fig. 2. Reversed-phase HPLC of retinoids on Ultrasphere-ODS with 0.01 *M* ammonium acetate in methanol–0.01 *M* ammonium acetate in water, pH 7 (88:12) as the mobile phase. Arrows indicate elution positions of (from right to left), 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

Fig. 3. Reversed-phase HPLC of retinoids on Ultrasil-ODS with 0.01 *M* ammonium acetate in methanol–0.01 *M* ammonium acetate in water, pH 7 (83:17) as the mobile phase. Arrows indicate elution positions of (from right to left), 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

RESULTS

Separations with aqueous methanol-based mobile phases

The separation of retinol and its prominent metabolites retinal, all-*trans*- and 13-*cis*-retinoic acid by isocratic reversed-phase HPLC with aqueous methanol-based mobile phases is shown in Figs. 2 and 3. Fig. 2 demonstrates their resolution on the Ultrasphere-ODS column. Fig. 3 shows the chromatography of the same four retinoids on the Ultrasil-ODS column with an aqueous methanol mobile phase designed to give retention times similar to those in Fig. 2.

Separations with aqueous acetonitrile-based mobile phases

Fig. 4 represents the most favorable separation of the four retinoids obtained with the Ultrasphere-ODS column and an aqueous acetonitrile-based mobile phase. The chromatogram shown in Fig. 5 displays the results obtained for the resolution of the same four retinoids on the Ultrasil-ODS column with an aqueous acetonitrile mobile phase designed to give retention times similar to those in Figs. 2 and 3. The effects of addition of a small amount of relatively non-polar organic modifier (in this case 5% tetrahydrofuran) on the resolution observed in Fig. 5 is shown in Fig. 6. This figure also demonstrates that the chromatographic system will resolve some of the more polar metabolites of retinoic acid, *i.e.* 4-hydroxyretinoic acid and 5,6-epoxyretinoic acid.

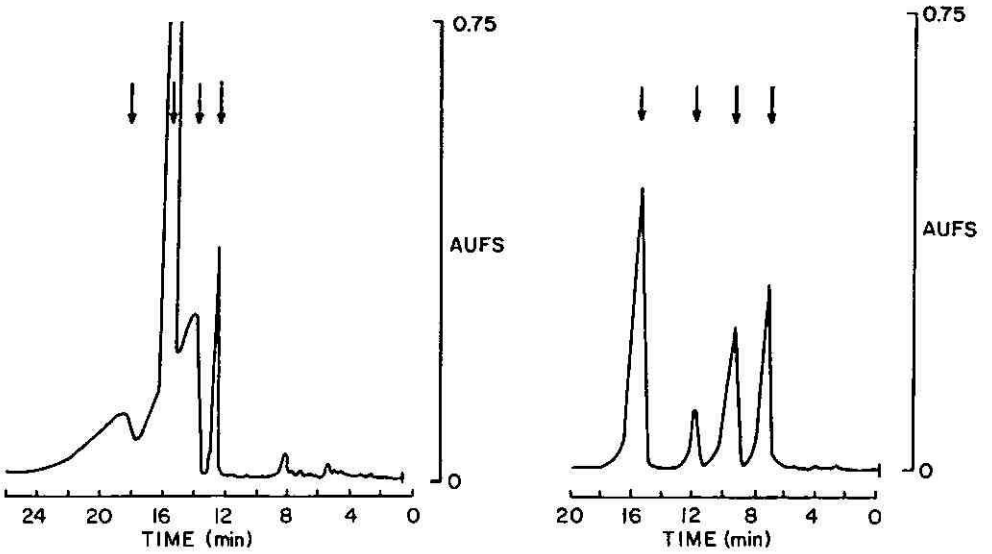

Fig. 4. Reversed-phase HPLC of retinoids on Ultrasphere-ODS with acetonitrile–0.05 *M* ammonium acetate in water, pH 7 (90:10) as the mobile phase. Arrows indicate elution positions of (from right to left), retinol, 13-*cis*-retinoic acid, retinal, and retinoic acid.

Fig. 5. Reversed-phase HPLC of retinoids on Ultrasil-ODS with acetonitrile–0.05 *M* ammonium acetate in water, pH 7 (80:20) as the mobile phase. Arrows indicate elution positions of (from right to left), 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

DISCUSSION

For separations of retinol and its oxidation metabolites in this aqueous methanol-based mobile phase the fully end-capped, 5-μm octadecylsilane column is preferable to the non-end-capped, 10-μm octadecylsilane stationary phase (*cf.* Figs. 2 and 3). Although adequate for resolving all-*trans*-retinoic acid and 13-*cis*-retinoic acid, the 10 μm non-end-capped column does not resolve the later eluting retinol and retinal as well as the 5-μm end-capped matrix.

There is a reversal of apparent matrix superiority upon changing to aqueous acetonitrile-based mobile phases. In this case, even at relatively high concentrations

TABLE I

STATIONARY PHASE PROPERTIES

Property	*Ultrasphere-ODS*	*Ultrasil-ODS*
Average particle size (μm)	5	10
Particle shape	Spherical	Irregular
Particle surface area (m^2/g)	200	350
Carbon loading (%)	12	20
End-capping	Exhaustive	None

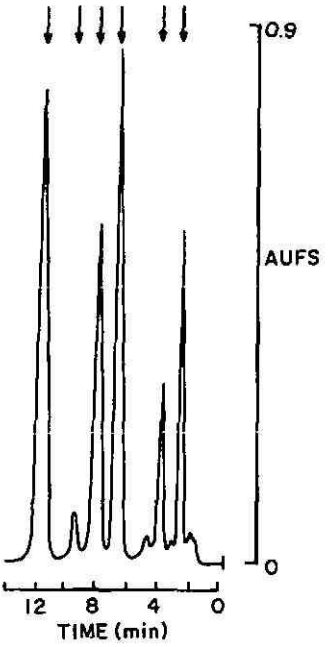

Fig. 6. Reversed-phase HPLC of retinoids on Ultrasil-ODS with acetonitrile–0.05 *M* ammonium acetate in water, pH 7–tetrahydrofuran (76:19:5) as the mobile phase. Arrows indicate the elution positions of (from right to left), 4-hydroxyretinoic acid, 5,6-epoxyretinoic acid, 13-*cis*-retinoic acid, retinoic acid, retinol, and retinal.

of acetonitrile, there is an undesired increase in the retention times of the retinoids on the Ultrasphere-ODS column. In addition, an unanticipated change in elution order of the four retinoids occurs as well as an excessive amount of peak tailing for the ionizable retinoic acids. These undesirable chromatographic effects were not moderated by an increase in ionic strength (0.1 *M* ammonium acetate), a change to the more hydrophobic salt triethylamine acetate, or addition of tetrahydrofuran (data not shown).

Alternatively, chromatography on Ultrasil-ODS is very acceptable in the aqueous acetonitrile mobile phase. Although there is a minimal amount of peak tailing in this system, this isocratic method should prove ideal in situations where methanol must be avoided as a component in the mobile phase. In addition, the resolution in this system can be improved along with a reduction in retention time by addition of a small proportion of tetrahydrofuran. Even with this added less polar organic modifier, relatively polar metabolites of retinol can still be determined (Fig. 6).

Addition of 5% tetrahydrofuran to the mobile phase employed with the Ultrasil-ODS column will also permit analysis of relatively non-polar esters of retinol and all-*trans*-retinoic acid. For example, the methyl ester of all-*trans*-retinoic acid is eluted with a retention time of 20 min with the mobile phase employed in Fig. 6 (data not shown). With a change in the acetonitrile–water–tetrahydrofuran proportion to 85:10:5, this stationary–mobile phase combination will even permit ready analysis of

retinyl palmitate, a very non-polar metabolite of retinol (t_R = 34 min; data not shown).

The apparent superiority of Ultrasil-ODS for chromatography of retinoids in aqueous acetonitrile is most likely related to the chemistry of the stationary phases. The general properties of the two modified silicas are listed in Table I. Although the Ultrasil particles are larger than those of the Ultrasphere matrix, the former material has a greater surface area due to a higher particle porosity. This property, along with the absence of end-capping of the matrix, may account for the favorable utility of the Ultrasil matrix in the present application.

In summary, we have developed an efficient, isocratic, reversed-phase chromatographic procedure for the resolution of a wide range of retinol metabolites. This procedure may prove to be generally useful as well as being particularly suited to use in separation problems which require the avoidance of nucleophilic solvents such as methanol.

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Note

Paper electrophoresis of the dansyl derivatives of amino acids and amines

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Paper electrophoresis has been widely used for separating free amines[1–3] and free amino acids[4–6], but electrophoresis of their derivatives has been limited to the study of dinitrophenyl[7] and dansyl amino acids[8,9]. Gray and Hartley[8,9] separated the dansyl derivatives of all the protein amino acids except for those of serine, proline and alanine by high-voltage electrophoresis at pH 4.4 (Whatman 3 mm paper, 80 V cm^{-1} 3 h) and completely resolved the remainder at pH 12.7 (30 V cm^{-1} 2 h). Klimek *et al.*[10] determined 18 dansyl protein amino acids at pH 12.7 using the spot area approach. There are no reports of the electrophoresis of dansylated amines (defined as amino and imino compounds lacking COOH groups) or of dansylated non-protein amino acids.

The present work had its origin in the isolation and characterisation of minute quantities of unusual tissue amines as their dansyl derivatives[11]. These derivatives are much easier to purify than the original amines because they chromatograph more cleanly, can be separated from salts by solvent extraction and can be detected at much lower levels. It was hoped that electrophoresis might provide an alternative method for purifying these derivatives and would help to identify the functional groups of the isolates obtained. In particular, by using buffers having a wide range of pH values it was hoped to distinguish between the derivatives of amines and those of basic amino acids since both classes of parent compound appear in the same basic nitrogen fraction[12].

EXPERIMENTAL

Electrophoresis equipment

Low-voltage electrophoresis: a Shandon Southern Kohn Model U77 electrophoresis tank was used with a Shandon 500 V/50 mA power pack.

High-voltage electrophoresis: a Shandon high-voltage electrophoresis chamber Model Q11 was operated with a Shandon 5 kV/200 mA power supply.

Preparation of buffers

pH 2.0: 90% (v/v) formic acid, 16 ml; glacial acetic acid, 74 ml; water, 910 ml. pH 4.0: 90 ml 0.2 *M* sodium acetate + 410 ml 0.2 *M* acetic acid. pH 5.7: 250 ml 0.1 *M* sodium dihydrogen phosphate + 18.0 ml 0.1 *M* sodium hydroxide + 232 ml

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water. pH 9.4: 50 ml 0.1 *M* sodium carbonate + 450 ml 0.1 *M* sodium bicarbonate. pH 10.2: 450 ml 0.1 *M* sodium carbonate + 50 ml 0.1 *M* sodium bicarbonate.

Preparation of dansyl derivatives

This was a modification of the method developed by Seiler and Wiechmann[13]. To 200 μl aqueous amine or amino acid (2 mg ml^{-1}) was added 400 μl 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride, 5 mg ml^{-1} in acetone). After leaving for about 15 h in darkness at 20 ± 5°C the amino acid reaction products were electrophorised without further treatment. However, mixtures containing dansylated amines were further reacted with 400 μl aqueous proline (15%) for 30 min at 20°C, followed by extraction with 2 × 2.5 ml ethyl acetate. These organic fractions were combined and evaporated to dryness in a stream of air at 50°C, the residue being redissolved in 200 μl ethyl acetate for spotting.

Paper electrophoresis

Dansylated samples (2 μl = 4 μg free amino/imino compound) were spotted on Whatman No. 1 paper together with aqueous samples of glucose (20 μg) to monitor mass buffer flow, and potassium chloride (20 μg) to act as a standard so relative mobilities at different pH values could be calculated. K^+ was selected as it remained soluble and completely ionised in all buffers.

The dansyl amino acids were routinely electrophorised for 30 min at 300 V and the dansyl amines for 2 h at 300 V (paper size 10–20 cm wide × 18 cm between wicks). In the latter case dansyl methylamine was used as a secondary standard and its mobility was related to that of K^+ in a separate experiment, because conditions giving optimum separation of dansyl amines ran K^+ off the paper.

After electrophoresis the papers were dried and the dansyl derivatives detected by their fluorescence under 366 nm UV light.

Glucose[14] was detected by spraying the relevant parts of the electrophoretograms with 1% aqueous potassium permanganate containing 2% sodium carbonate. Glucose appeared as a brown spot on a purple background, changing to grey on a brown background.

Potassium[15] was detected by spraying the relevant parts of the electrophoretogram with a reagent prepared in the following way: 5.7 g hydrated cobalt acetate, 8.1 g hydrated lead(II) acetate, 10 g sodium nitrite and 2 ml glacial acetic acid were dissolved in 75 ml water and mixed with 20 ml methanol. K^+ appeared as a yellow spot on a brown background. Electrophoretograms run in the pH 9.4 and 10.2 buffers were dipped in glacial acetic acid and dried before treating with this cobalt hexanitrite reagent.

RESULTS AND DISCUSSION

The results are given in Tables I–III and illustrated by Fig. 1. The standardized data shows actual mobilities, adjusted to average conditions as judged by the movement of the standards. This first set of figures has been corrected by subtracting mass buffer flow and then calculating the distance each dansyl derivative would have moved as K^+ electrophoresed 100 mm.

Dansyl derivatives are normally handled in organic solvents like benzene, ethyl acetate and acetone so it is perhaps surprising that all except seven proved sufficiently

TABLE I

ELECTROPHORETIC MOBILITIES OF DANSYL AMINO ACIDS

Negative values indicate movement towards anode.

Dansyl amino acid	*Standardized data (distances from origin in mm)*					*Corrected mobilities (mm)*				
	pH 2	*pH 4*	*pH 5.7*	*pH 9.4*	*pH 10.2*	*pH 2*	*pH 4*	*pH 5.7*	*pH 9.4*	*pH 10.2*
β-Alanine	17	2	−10	−7	0	23	−1.5	−18	−28	−24
Arginine	19	4	3	8	11	26.5	1.5	1.5	0	0
α-Aminobutyric acid	15	−5	−9	−6	0	20	−11.5	−17	−24.5	−24
γ-Aminobutyric acid	17	3	−9	−6	2	23	0	−17	−24.5	−20
α-Aminoisobutyric acid	16	−3	−10	−6	0	21.5	−8.5	−18	−24.5	−25
β-Aminoisobutyric acid	16	5	−9	−6	0	21.5	3	−17	−24.5	−24
Asparagine	15	−4	−8	−6	0	20	−10	−15	−24.5	−24
Aspartic acid	13	−10	−28	−21	−10	17	−20	−45	−53	−47
Azetidine-2-carboxylic acid	14	5	−11	−6	0	19	3	−20	−24.5	−24
Carnosine	17	7	3	2	3	23.5	6	1.5	−10.5	−18
Cysteic acid	2	−14	−24	−19	−13	0	−24	−38	−49	−56
Glutamic acid	14	−7	−8	−9	−9	19	−16	−15.5	−31.5	−44
Glutamine	14	1	−8	−5	0	19	−3	−15.5	−23	−22
Glycine	9	−5	−14	−5	0	11.5	−12	−24	−23	−24
Histidine	25	7	1	−1	0	35	6	−1.5	−16	−24
Homocysteine	21	3	−6	−11	−10	28	0	−12	−35	−46
Homoserine	16	0	−10	−4	0	22	−4.5	−18.5	−21	−22

4-Hydroxyproline	15	−3	−5	0	2	20	−9	−10	−14	−18
Isoleucine	16	9	−1	−1	0	21	9	−4	−16	−24
Leucine	19	10	−2	−3	2	26	10.5	−6	−19	−18
Lysine	24	8	6	2	0	33	7.5	7	−12	−24
Methionine	15	−6	−8	−2	1	19	13.5	−15	−18	−20
3-Methylhistidine	23	5	−3	−5	0	32	3	−7	−23	−24
Ornithine	24	8	6	−1	1	33	7.5	7	−16	−20
Phenylalanine	17	−2	−4	−1	0	24	−8	−8.5	−16	−24
Pipecolic acid	15	−6	−9	−4	0	19	−13.5	−17	−21.5	−22
Proline	13	−4	−11	−6	−3	17	−11	−20	−24.5	−31
Serine	14	−5	−10	−5	0	17.5	−12	−18	−23.5	−22
Taurine	2	−14	−11	−12	−6	0	−26	−20	−37	−38
Threonine	10	−4	−9	−3	1	12	−10.5	−17	−20	−20
Tryptophan	16	4	−8	−1	−1	21	1.5	−15	−16	−27
Tyrosine	17	2	−1	−1	0	23	−1.5	−4	−16	−24
Valine	15	6	−8	−5	0	20	4.5	−15	−23.5	−24
Standards										
Glucose	2	3	2	8	10.5					
K^+	67	70	69	63	54					

TABLE II

ELECTROPHORETIC MOBILITIES OF DANSYL AMINES AT pH 2 AND 4

Dansyl amine	*Standardized data (distances from origin in mm)*		*Corrected mobilities (mm)*	
	pH 2	*pH 4*	*pH 2*	*pH 4*
N-Acetyl-5-hydroxytryptamine	60	32	18	4
Agmatine	112	88	35	12
6-Aminohexanol	74	54	22	7
2-Aminoimidazole	110	51	34	7
5-Aminopentanol	79	54	24	7
Isoamylamine	80	37	24	5
Benzylamine	69	33	21	4
Isobutylamine	78	45	24	6
n-Butylamine	81	43	25	5
sec.-Butylamine	79	45	24	6
tert.-Butylamine	82	55	25	7
Cadaverine	74	0	22	–
Cystamine	86	54	26	7
1,6-Diaminohexane	88	54	27	7
1,2-Diaminopropane	96	59	29	8
1,3-Diaminopropane	88	54	27	7
Dimethylamine	82	55	25	7
Dopamine, didansyl derivative	89	44	27	6
Dopamine, tridansyl derivative	111	60	34	8
Ephedrine	70	32	21	4
Norephedrine	72	50	22	6
Epinephrine	0	0	–	–
Norepinephrine	0	0	–	–
Ethanolamine	76	54	23	7
Ethylamine	72	48	22	6
Gramine	70	41	21	5
n-Hexylamine	61	0	18	–
Histamine	93	0	28	–
Hordenine	110	76	34	10
5-Hydroxydopamine	0	0	–	–
β-Hydroxyphenylethylamine	78	51	24	6
2-Hydroxyphenylethylamine	64	0	19	–
5-Hydroxytryptamine	71	0	21	–
Metanephrine	73	0	22	–
Normetanephrine	78	0	24	–
3-Methoxy-4-hydroxy-phenylethylamine	69	0	21	–
3-Methoxy-4-hydroxy-phenylmethylamine	65	0	19	–
6-Methoxytryptamine	63	49	18	6
Methylamine	83	54	25	7
N-Methyl-3,4-dimethoxy-phenylethylamine	71	49	21	6
N-Methyldopamine	0	0	–	–
N-Methyl-4-methoxy-phenylethylamine	63	0	19	–
N-Methyl-β-phenyl-ethylamine	68	0	20	–
N-Methyltryptamine	61	0	18	–

TABLE II (*continued*)

Dansyl amine	*Standardized data (distances from origin in mm)*		*Corrected mobilities (mm)*	
	pH 2	*pH 4*	*pH 2*	*pH 4*
p-Octopamine	74	54	22	7
n-Octylamine	48	0	14	–
β-Phenylethylamine	69	0	20	–
Phenylpropanolamine	71	46	21	6
Isopropylamine	74	44	22	6
n-Propylamine	75	44	22	6
Putrescine	55	68	16	9
Spermidine	0	0	–	–
Spermine	0	0	–	–
Synephrine	0	0	–	–
Tryptamine	56	0	17	–
p-Tyramine, monodansyl derivative	76	15	23	1
p-Tyramine, didansyl derivative	106	18	32	2
Standards				
Glucose	5.5	5.5		
K^+	310*	700*		

* Calculated from position of dansylmethylamine.

TABLE III

ELECTROPHORETIC MOBILITIES OF DANSYL AMINES AT HIGHER pH VALUES

Most of the amines listed in Table II are not shown here because they were immobile at pH 5.7 and all higher pH values. Figures in brackets are the apparent R_F values, relative to glucose.

Dansyl amine	*Standardized data (distances from origin in mm)*		
	pH 5.7	*pH 9.4*	*pH 10.2*
Agmatine	19	24	15
6-Aminohexanol	7 (0.9)	8 (0.7)	11 (0.9)
2-Aminoimidazole	8 (1.0)	0	0
5-Aminopentanol	8 (1.0)	11 (0.9)	12 (1.0)
1,2-Diaminopropane	4 (0.5)	0	0
Dimethylamine	4 (0.5)	9 (0.7)	10 (0.8)
Dopamine, didansyl derivative	8 (1.0)	0	0
Ethanolamine	8 (1.0)	7 (0.6)	8 (0.7)
Ethylamine	5 (0.6)	8 (0.7)	10 (0.8)
Gramine	7 (0.9)	0	0
Hordenine	5 (0.6)	0	0
Methylamine	8 (1.0)	11 (0.9)	12 (1.0)
Phenylpropanolamine	8 (1.0)	3 (0.2)	0
Isopropylamine	8 (1.0)	4 (0.3)	0
n-Propylamine	7 (0.9)	4 (0.3)	0
p-Tyramine, monodansyl derivative	7 (0.9)	0	0
p-Tyramine, didansyl derivative	3 (0.4)	0	0

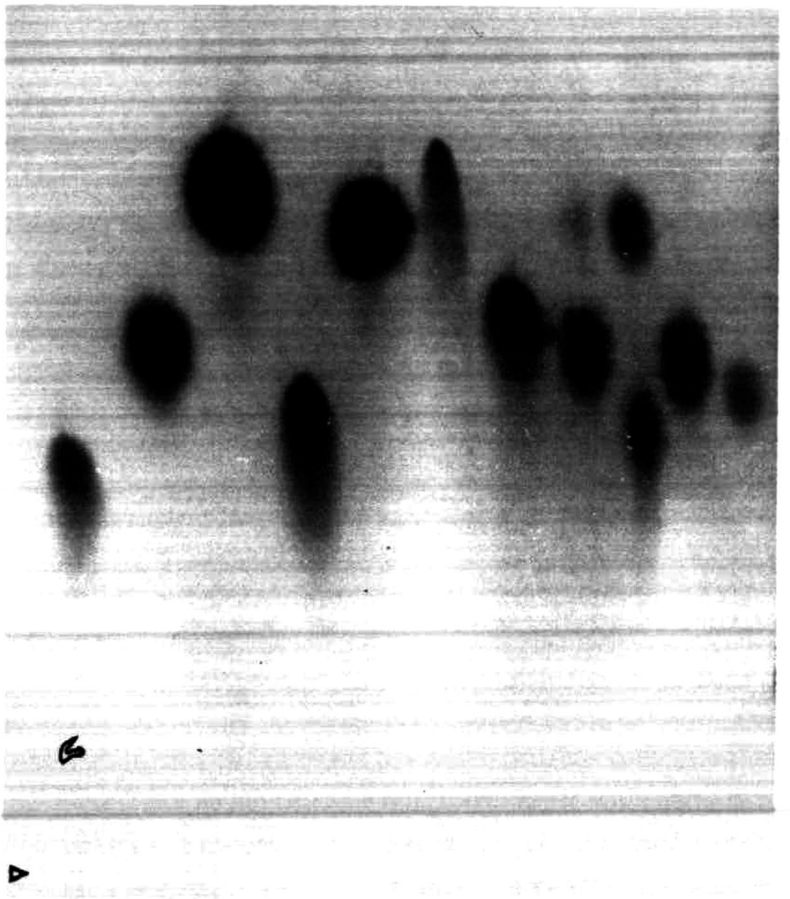

Fig. 1. Negative print of electrophoretogram run at 18 V cm^{-1} for 5.5 h. Samples, in order, from left to right, were the dansyl derivatives of tryptamine, norephedrine, methylamine, *n*-hexylamine, ethanolamine, 1,3-diaminopropane, benzylamine, valine, tyrosine (slower spot, monodansyl derivative), serine and glutamine.

water soluble to electrophorese at pH 2. The seven exceptions probably all bear more than one dansyl group. However, all dansyl amines become insoluble as the derivatising group loses its positive charge unless the molecular weight is comparatively low (*e.g.* methylamine, ethylamine) or there are other polar groups present, as in 6-amino-hexanol and agmatine. Corrected results are not given for dansyl amines which have precipitated at the origin or are moving slower than glucose since these would give the misleading impression that the sample was carrying a negative charge. The precipitation effect itself allows separation of some dansyl amines at alkaline pHs because a few are carried by the mass buffer flow and are subject to a form of adsorption chromatography.

Initial experiments at pH 2, not reported in detail here, showed that high voltage electrophoresis at 40 V cm^{-1} gave quite satisfactory results for dansyl amino acids, but caused unacceptable streaking of many dansyl amines, notably the phenylethylamines, tryptamines and polyamines. Streaking became progressively worse for all compounds as the voltage was increased to 200 V cm^{-1}. However, all derivatives shown as being mobile gave satisfactory, compact, spots under the conditions finally adopted.

Overall the results show that low-voltage electrophoresis is a useful supplementary technique for the separation of amines–amino acids, especially when the amounts available are very small. For example, electrophoresis is more efficient than thin-layer chromatography for resolving didansylputrescine/monodansyl-*p*-tyramine as well as the dansyl derivatives of ephedrine/N-methyl-4-methoxyphenylethylamine and benzylamine/phenylethylamine.

The reaction of low-molecular-weight amino and imino compounds with a higher-molecular-weight label makes the method very useful for functional group detection, since the net number of positive or negative charges carried by a monodansyl derivative at a given pH can be estimated fairly accurately. Thus a corrected mobility figure of about 20 mm corresponds to a single charge. Additional dansyl groups destroy this simple relationship: for example, they only increase mobility marginally at pH 2 because the extra charge introduced is largely balanced by an increase in molecular size.

Electrophoresis is a reliable though not quite infallible method of distinguishing between dansylated amino acids and amines: amino acids almost always show a negative charge at pH 10.2, while amines do not. Even the exceptions, amino acids containing guanidino groups, would probably arouse suspicion because they would still be soluble and mobile at pH 10.2. Moreover, electrophoresis can give indications of the presence of SH groups, unreacted NH and aromatic OH groups. This is useful because the dansylation of natural basic nitrogen fractions often gives products less completely derivatised than theoretically possible and dansyl amines bearing free phenolic hydroxy groups are sometimes isolated[11].

The dansyl chloride reaction can generate fluorescent breakdown products, monodansylated aldehydes, from lysine and ornithine[16]. However, here this has not happened as these basic amino acids have both yielded the expected didansyl derivatives, according to their corrected mobilities.

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Note

Determination of heteroatoms in organic compounds by ion chromatography after Schöniger flask decomposition*

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The Schöniger flask combustion technique is a very useful method for decomposing organic matter[1]. In our laboratory it has been used, in conjunction with titrimetry, for determination of the halogen, phosphorus and sulphur contents of organic materials. During the combustion and the trapping process, the halogens are converted to the halides, phosphorus and sulphur are oxidized to phosphate and sulphate, and are subsequently determined as such.

Ion chromatography (IC) is a powerful technique for the sequential determination of anions. Initial work using the suppressor mode with carbonate/bicarbonate eluent has indicated its usefulness when employed with the Schöniger flask decomposition[2,3]. This report describes our experience, with emphasis on comparison with titrimetry, in using the non-suppressed, single column mode with the Schöniger decomposition.

EXPERIMENTAL

The apparatus for ion chromatography consisted of a dual-piston reciprocating pump (Waters, Model 6000A), a loop injector (Rheodyne, Model 7125), a small particle diameter, silica based column for anion separation (Vydac 302 IC), a precolumn of similar material, an ultraviolet detector (Waters, Model 450) and a conductivity detector (LDC, Model 701) connected in series.

The effluent was 0.002 *M* phthalic acid adjusted to pH values ranging from 3.5 to 5.5 with sodium tetraborate. The usual flow-rate was 1 ml/min and most injections were made with a 20-μl sample loop.

For Schöniger flask decomposition, an accurately weighed sample (*ca.* 30–50 mg) was wrapped in a piece of carrier paper, mounted on the platinum holder and combusted in the usual manner. Two absorber solutions: (1) 6% hydrogen peroxide in 0.18 *M* sodium hydroxide, and (2) 2% hydrazine sulphate in distilled deionized water, were used routinely. The absorber solution was diluted to 50 ml with water after combustion.

* NRCC 26415.

TABLE I

TYPICAL ACCURACIES

X = Heteroatoms. A = *p*-dichlorobenzene, B = α,α′-dibromo-*p*-xylene, C = sulphosalicylic acid, D = combination sample of the three compounds.

Standards	*ppm X expected*	*ppm X found*	*Rel. error (%)*
A	34.6	35.2	1.7
B	31.8	312.3	1.6
C	7.99	7.47	6.5
D-Cl^-	11.3	10.3	8.8
D-Br^-	13.1	13.0	0.8
D-SO_4^{2-}	9.01	8.62	4.3

RESULTS AND DISCUSSION

There is much discussion in the literature as to the best absorber solution for Schöniger flask decomposition[4,5]. We find both the hydrogen peroxide and the hydrazine sulphate solutions adequate. The latter is often preferred when titrimetry is to be used after decomposition[4]. For IC determination, the hydrazine sulphate solution is less desirable as it contains a high concentration of sulphate, which not only makes sulphate determination impossible but also overloads the column. The hydrogen peroxide solution is preferred since it does not contain any interfering ions or cause column overloading.

The accuracy and speed of the IC determination are demonstrated in the analysis of some standards: *p*-dichlorobenzene, α,α′-dibromo-*p*-xylene and sulphosalicylic acid. The results are shown in Table I. The accuracy obtained (1–9%) is typical. The total analysis time for the three anions determined in a single run is *ca.* 15 min, which is considerably shorter than that required for three determinations in titrimetry (*ca.* 70 min). In addition, IC requires virtually no operator interaction once the sample has been injected and frees the analyst to perform other tasks between injections. Another point of practical importance is that each IC run requires only a minute portion of the decomposed sample (normally about 20 μl) whereas titrimetry often consumes the whole sample. Thus IC allows replicate analyses of one burn while titrimetry does not. Table II shows the results of several chloride analyses performed both by IC and titrimetry. Agreement between the two is good.

TABLE II

CHLORIDE ANALYSIS

Sample	*ppm* Cl^-	
	IC	*Titrimetry*
E	35.3	38.3
F	11.7	11.7
G	15.1	15.8

TABLE III

DETECTION LIMITS

Detection limits are expressed as amounts in the original sample.

Heteroatom	*IC (ng)**	*Titrimetry (ng)*
Cl	0.1	0.7**
Br	0.1	1.6**
S	0.1	0.5***

* UV detection.
** Silver nitrate titration.
*** Turbidimetry.

The detection limits for both IC and titrimetry are estimated in Table III. IC is certainly a more sensitive technique than titrimetry. Further, with on-column preconcentration, the sensitivity of IC may readily be enhanced ten-fold or more. In general, the precision of titrimetry (*ca.* 1%) is considerably better than that of IC (*ca.* 5%). However, the superior precision of titrimetry is not necessarily realized when used in conjunction with the Schöniger flask decomposition as error larger than 1% can easily be introduced in the combustion process.

In summary, ion chromatography is an accurate, rapid and sensitive method for heteroatom analysis after the Schöniger flask decomposition.

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We thank Vincent P. Clancy for helpful discussion.

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Note

Characterization of organolead polymers in trace amounts by element-specific size-exclusion chromatography

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The synthesis of interesting and significant new organometallic polymers incorporating vinylic monomers has been reported[1,2]. Conventional techniques of polymer characterization, including mass sensitive size-exclusion chromatography (SEC) can be used to estimate molecular parameters such as number- and weight-average molecular weights (M_n and M_w), molecular distribution (MWD = M_w/M_n), and conversion of monomer to polymer, provided the material is soluble. Such methods, however, are not designed to directly show the distribution of the metal-containing moiety in fractions of these new copolymers, a property of vital importance to their physicochemical and biological properties and utility in many applications. Parks and co-workers[3,4] have described an element-specific method for the SEC of a precise controlled-release copolymer of biocidal tributyltin methacrylate and methyl methacrylate. Using a dual ultraviolet absorption–differential refractive index (UV–ΔRI) detector coupled on-line with tin-specific graphite furnace atomic absorption spectrometry (GFAA), they obtained self-consistent tin-sensitive and mass-sensitive chromatograms. They found it essential to precondition SEC columns in order to prevent dissociation of the labile tin-bearing moeity, which affords tailored bioproperties, from the polymer chain during the chromatography[4]. Since tin-bearing cations were used in the pretreatment, this constraint necessitated injecting relatively large (5 mg) samples in order to keep the analyte absorption relatively large compared to the intensity of the background signal.

Weiss *et al.*[5] demonstrated multielement speciation of natural shale oils by means of coupled SEC–UV–GFAA. Using a 1:1 flow splitter and two GFAA instruments, they showed that at least one oil contained two iron-bearing species of different mol. weight, one of which incorporated arsenic and was chromophoric.

This paper describes new element-specific SEC studies of a lead-containing copolymer which is not subject to dissociation by the chromatographic packing surface. The result is a highly informative chromatogram requiring the injection of less than 1.0 μg of polymer, a quantity quite sufficient for UV and GFAA chromatograms but insufficient to be detected by differential refractive index, presaging potential applications for trace molecular speciation of many synthetic or natural metal(loid)-containing compounds.

EXPERIMENTAL

The analyte was prepared by copolymerization of (4-vinylphenyl)triphenyllead and octadecylmethacrylate in 5:1 molar proportions. The copolymer (12.7 mg) was dissolved in 1.0 ml of tetrahydrofuran (THF) and further diluted with THF (10 μl dissolved in 10 ml) to give a final concentration of $1.27 \cdot 10^{-5}$ g ml^{-1}. A 50-μg portion of this solution (0.635 μg) was injected into the SEC mobile phase (THF, 1.0 ml min^{-1}). Polymer fractions, separated by SEC (μStyragel* columns, pore size 10^3 A, 250 × 4.6 mm I.D.; three columns in tandem), were detected by lead specific GFAA (hollow cathode lamp operating at 217 nm) and a UV detector (254 nm, 0.0125 a.u.f.s.). Aliquots of the eluent were transferred automatically at 0.82 min intervals to the GFAA furnace to obtain the chromatogram shown in Fig. 1.

The SEC–UV–GFAA system has been described in detail[6]. The SEC μStyragel column was calibrated with polystyrene standard samples[6] to correlate the elution volume of analyte fractions with the estimated molecular weight.

RESULTS AND DISCUSSION

Fig. 1 displays the SEC–UV–GFAA chromatogram of the polymer, and MW assignments based on polystyrene standard samples. The calculation of M_n, M_w, and MWD is based on the elution volumes corresponding to the individual GFAA spikes and the measured height[6]. Based on the number average- and the weight average-molecular weights (4000 and 9000 daltons, respectively) the MWD is 2.25. The conversion of lead-containing monomer to polymer is 93.9%.

A striking feature of the coupled UV–GFAA chromatograms is the absence of UV absorptivity in the high polymer fraction, whereas the low-MW fraction (190 daltons), apparently unreacted monomer, absorbs very strongly. Given such loss of optical properties on polymerization, there remains only the lead specific GFAA signals for quantitation of polymer parameters. Mass-sensitive differential refractive index chromatography would provide supplementary information. The latter, however, would require a quantity of this analyte higher by three orders of magnitude than that actually used.

Very importantly, sample recovery (as lead) after injection was complete, indicating no retention or dissociation on the packing. For two consecutive injections, sample recoveries of 109.8 and 95.6% were calculated based on integration of all observed peaks in the GFAA chromatogram, and compared to the measured absorptivity of stock solution (taking the average of seven consecutive spikes, having a relative standard deviation of 3.2%).

The SEC–UV–GFAA method thus is capable of characterizing trace quantities of an organometallic polymer, such as may be obtained during preliminary stages of synthetic research. The requirements for successful characterization include polymer solubility in a solvent which is compatible with the column packing; absence or pre-

* Certain commercial materials and equipment are identified in this paper in order to specify the experimental procedures. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

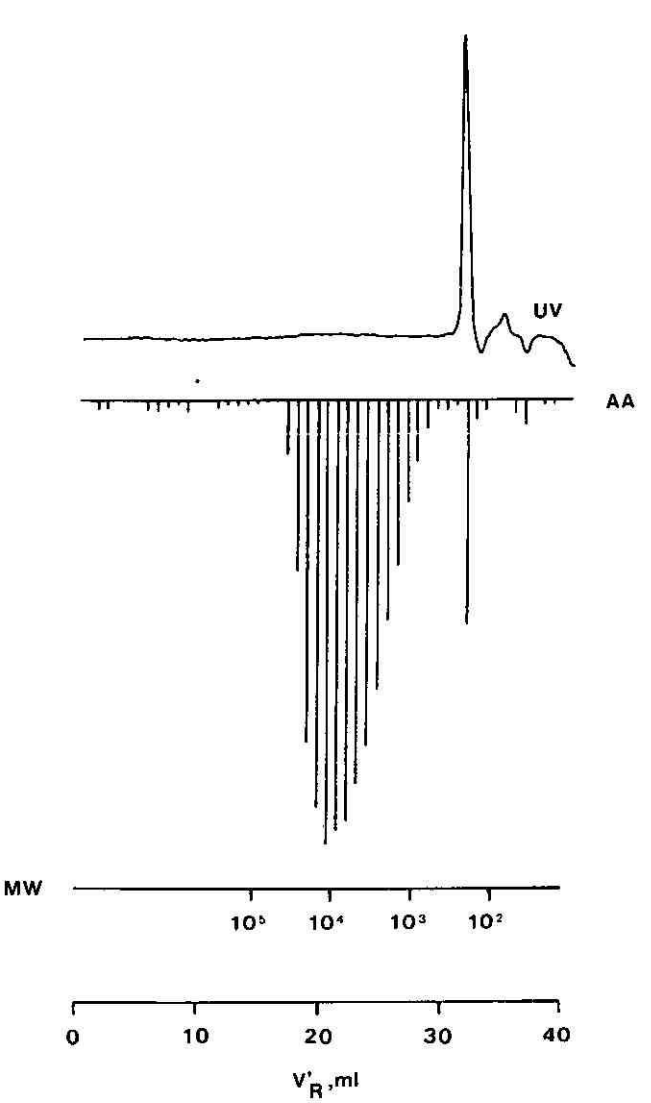

Fig. 1. SEC–UV–GFAA chromatogram of a 5:1 copolymer of 4-(vinylphenyl)triphenyllead and octadecylmethacrylate. Eluent, THF; flow-rate, 1.0 ml min^{-1}. Injected volume, 50 μl. Injected analyte, 0.635 μg.

vention of polymer decomposition on column packing materials, and an element-specific detector for the incorporated metal. Finally, the method demonstrated here for macromolecular lead is readily extended to multimetallic macromolecules as shown by Weiss *et al.*[5].

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Note

Assay of phenylmercuric acetate and nitrate in pharmaceutical products by high-performance liquid chromatography with indirect photometric detection

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Phenylmercury salts are widely used in pharmaceutical products as bactericides at concentrations of 0.001–0.002% (w/v)[1]. Their assay in these products may be performed by colorimetric methods[2–4], atomic absorption spectrometry[5–7], polarography[8,9], potentiometric titration[10], neutron activation analysis[11] or high-performance liquid chromatography (HPLC)[12,13]. The HPLC methods, to obtain the requisite sensitivity, involve either an extraction and concentration step prior to injection[12] or monitoring at 210 nm[13] which presumably must limit the applicability of the method.

Recently, widespread use has been made of indirect photometric methods for the detection and quantitation of poorly-absorbing or non-UV-absorbing compounds eluting from HPLC columns[14–20]. Methylmercury and phenylmercury form complexes with a variety of organic and inorganic ligands, those formed with thiol containing compounds having high association constants[21,22]. The adduct formed between phenylmercury (PM) and 6-mercaptopurine (6-MP) has been used for extraction of the latter from biological samples[23,24].

This paper reports the development of an HPLC method which utilises the phenylmercury–6-mercaptopurine complex (PM–6-MP) to provide a sensitive and specific method for the analysis of PM salts in pharmaceutical products.

EXPERIMENTAL

Reagents and chemicals

Phenylmercuric acetate and nitrate were obtained from BDH (Poole, U.K.) and the 6-mercaptopurine from Aldrich (Milwaukee, WI, U.S.A.). The methanol and acetonitrile were HPLC grade (Mallinckrodt, Melbourne, Australia).

Chromatographic equipment

The liquid chromatograph consisted of a pump (6000A, Waters Assoc., Milford, MA, U.S.A.), 20-μl loop injector (Rheodyne 7125, Cotati, CA, U.S.A.), variable-wavelength detector (LC-3, Pye-Unicam, Cambridge, U.K.), integrating recorder (Hewlett-Packard 3380A, Palo Alto, CA, U.S.A.) and a μBondapak C_{18} column (30 cm × 6.4 mm I.D., 10 μm particle size) (Waters Assoc., Sydney, Australia).

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Chromatographic conditions

The mobile phase consisted of methanol–acetonitrile–0.005 *M* KH_2PO_4 in water (1:4:5) containing $5 \cdot 10^{-4}$% (w/v) 6-mercaptopurine monohydrate.

RESULTS AND DISCUSSION

The PM–6-MP adduct has spectral characteristics such that it can be quantitated by monitoring at 293 nm (Fig. 1). At this wavelength the $5 \cdot 10^{-4}$% 6-MP in the eluting solvent results in a background absorbance of approximately 0.1 which results in a satisfactory baseline. Injection of an aqueous solution of PM acetate resulted in perturbation of the 6-MP concentration resulting in a negative peak at the retention time of 6-MP (immediately after the void volume) and the formation of a peak arising from the PM–6-MP adduct at 6.4 min (Fig. 2). The adduct is unstable under the chromatographic conditions employed and the injection of a solution of the PM–6-MP adduct using chromatographic solvent without 6-MP as mobile phase results in a peak due to 6-MP at 2.5 min and a small variable peak at 6.4 min. The presence of 6-MP in the mobile phase ensures that the PM–6-MP complex does not dissociate during chromatography.

The assay afforded a linear response over the range $0–5 \cdot 10^{-3}$% of PM acetate: area response = $618.9 \cdot 10^6$ [conc. % (w/v)] + $28.6 \cdot 10^3$ ($n = 5$, $r = 0.9998$). The coefficient of variation based on six replicate determinations of an 0.002% (w/v) solution of PM acetate was found to be 0.7%. Indentical results are obtained with PM nitrate and the calibration graph can be used for either bactericide provided correction is made for their relative molecular weights. The limit of detection (defined as peak height/noise = 3) of the assay is $1 \cdot 10^{-5}$%. Mercuric ion (Hg^{II}) can also be

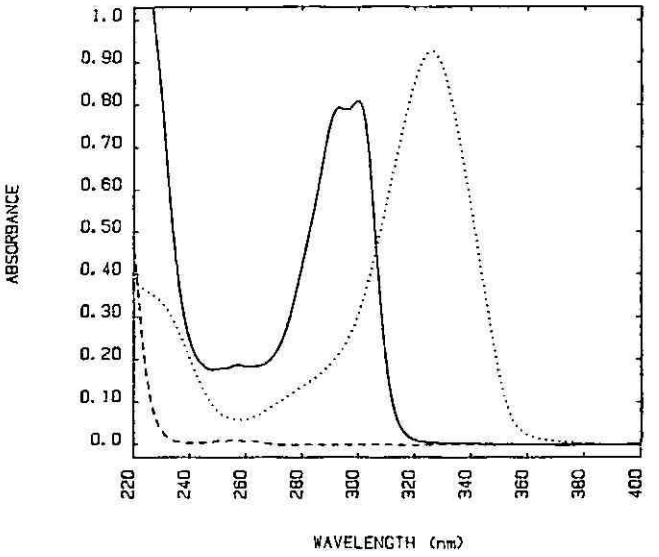

Fig. 1. Spectra of phenylmercuric acetate ($6.0 \cdot 10^{-5}$ *M*) (---------), 6-mercaptopurine ($5.9 \cdot 10^{-5}$ *M*) (.....) and the phenylmercury–6-mercaptopurine adduct made by mixing phenylmercuric acetate ($6.0 \cdot 10^{-5}$ *M*) with 6-mercaptopurine ($5.9 \cdot 10^{-5}$ *M*) (———). Solvent, 40% acetonitrile in 0.005 *M* KH_2PO_4.

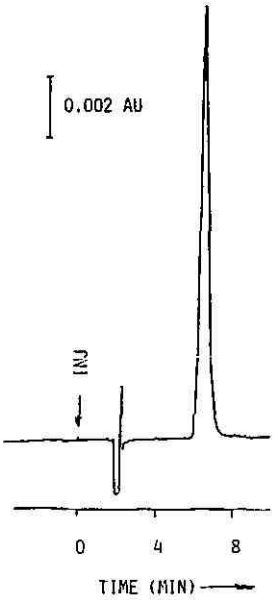

Fig. 2. Chromatogram of 0.002% (w/v) phenylmercuric acetate (20 μl). Solvent, methanol–acetonitrile–0.005 *M* KH_2PO_4 (1:4:5) containing $5 \cdot 10^{-4}$% (w/v) 6-mercaptopurine; flow-rate, 1.5 ml/min; monitoring wavelength, 293 nm. The peak at 6.4 min is due to the phenylmercury–6-mercaptopurine adduct.

detected by this system eluting (presumably as the dimercaptide adduct) at approximately 3 min. The assay can therefore quantitate both PM acetate and nitrate in the presence of free mercuric ions.

The stability of the adduct under column conditions has been evaluated by the use of stop-flow methods. Retention on the column for a period of 4 hr showed no loss of peak area demonstrating that the PM-adduct was unreactive to the metal column walls and was chemically stable.

To assess the scope of the method it was used to quantitate PM salts in pharmaceutical eye drops the formulae of which were derived from the *Australian Pharmaceutical Formulary and Handbook*[25] (Table I). In these cases all other UV absorb-

TABLE I

ANALYTICAL RESULTS OBTAINED FROM THE ANALYSIS OF EYE DROPS OF THE *AUSTRALIAN PHARMACEUTICAL FORMULARY*

Product	*Nominal content PM nitrate (%)*	*Found (%)*
Neomycin eye drops	0.002	0.00192
Fluorescein eye drops	0.004	0.00403
Chloramphenicol eye drops*	0.002	0.00195

* A commercially available brand of these drops showed minor peaks which co-eluted with the PM–6-MP adduct. Freshly prepared drops could be assayed by this method.

ing components in the formulations eluted prior to the PM–6-MP and did not interfere with the assay. The method would appear to have broad general utility and be suitable for the assay of phenylmercury salts in a range of pharmaceutical systems.

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Note

Separation of certain triglyceride isomers by argentation thin-layer chromatography with flame ionisation detection by the Iatroscan TH 10

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Several chromatographic techniques have been used for triglyceride separation. Non-aqueous reversed-phase high-performance liquid chromatography (HPLC) on C_{18} columns has now been developed to such a degree that separation of triglycerides can be achieved not just according to equivalent carbon number (ECN) but peaks of each ECN can be resolved into their constituent carbon number[1–3]. However, resolution of glyceride isomers differing only in the positions of the acyl group [*e.g. rac* 1,3-distearoyl, 2-oleoylglycerol (SOS) and 1,2-distearoyl, 3-oleoylglycerol (SSO)], cannot be achieved by HPLC on C_{18} columns, and is only possible on silver nitrate impregnated columns at subambient temperatures[4]. This is a laborious and expensive procedure and column life is very short.

Dallas and Padley[5] successfully separated symmetrical (*e.g.* SOS) and unsymmetrical (*e.g.* SSO) mono-unsaturated triglycerides on a silica gel thin-layer chromatography (Si-TLC) plate impregnated with silver nitrate using chloroform–toluene as a developing solvent. However, as with all TLC techniques, quantification of the components cannot readily be achieved.

The separation of lipid classes using the Iatroscan has been extensively reported in the literature[6–12]. However, separation of lipids, particularly triglycerides, according to their degree of unsaturation has been less extensively reported. Sebedio *et al.*[13] used silver nitrate impregnated Chromarods to separate fatty acid methyl esters according to double bond configuration. The same authors[14] also separated mono acid triglycerides and studied response factors and the effect of column re-use on these factors. Other authors[15,16] have applied the system to intact triglycerides. This paper describes our attempts to achieve separations of mono-unsaturated triglyceride positional isomers using the Iatroscan system, which had previously only carried out by argentation Si-TLC.

EXPERIMENTAL

Equipment

The Iatroscan TH10 and all ancillary equipment (Chromarods, frame, tanks, spotting guide) were obtained from Iatron's U.K. agent, Trivector Scientific, Sandy, U.K. Silica gel SII (high-performance) Chromarods were employed throughout.

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Materials

All lipid standards were obtained from Sigma Chemicals, Poole, U.K., except for mixed acid triglycerides which were synthesised in-house. Solvents, all analytical grade, were obtained from BDH, Poole, U.K., who also supplied the silver nitrate (analytical grade).

Method

Chromarods were activated by passing through a flame ionisation detector, then impregnated with silver ions by immersion for 15 min in a 2.5% (w/v) solution of silver nitrate in acetonitrile. They were then removed from the solution, drained, dried and re-activated in an oven at 120°C for 3 h. Samples were applied and rods developed in the solvent under investigation over their whole length. Following use, the rods were cleaned by soaking overnight in concentrated nitric acid, which was then removed by immersion sequentially in two portions of distilled water followed by rinsing with acetone. The rod preparation procedure is broadly the method described by Sebedio *et al.*[13].

For triglyceride separations, the following solvent systems were studied: (1) chloroform*–toluene (50:50) (The solvent system employed by Dallas and Padley[5] for TLC separations of this type); (2) chloroform*; (3) chloroform; (4) dichloromethane; (5) benzene (single development); (6) benzene (triple development); (7) chloroform*–benzene–diethyl ether (70:30:1.5).

Where the chloroform in the above list is marked* it was purified to remove ethanol (added as stabiliser to most commercial chloroform) by triple extraction with 0.2 volumes of water, followed by drying (with calcium sulphate) and distilling. Otherwise chloroform was as received, *i.e.* containing 1–2% (v/v) ethanol.

For all analyses, 2-μl samples were applied as a 1% (m/v) solution in dichloromethane (selected because of its volatility), using a 10-μl Hamilton GC syringe. Rods were rotated during application to ensure even distribution of the sample.

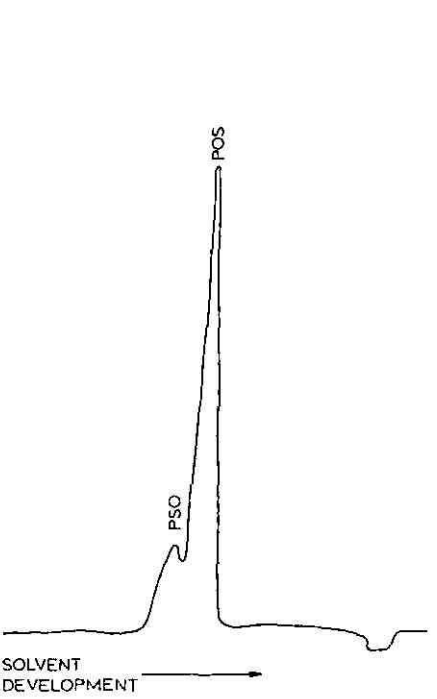
RESULTS AND DISCUSSION

Impregnation of Chromarods with copper sulphate had previously been shown to shorten the life of the rods[15]. For this application, however, rods could be repeatedly re-used for argentation separations, generally without significant loss of resolution, by washing off the silver nitrate in concentrated nitric acid, drying and re-impregnating with silver nitrate as before. However, once silver-impregnated, rods could not be completely washed free of silver salts, even with overnight soaking in aqua regia, so they could not therefore be used subsequently for conventional adsorption type separations, which would require the rods to be free of silver salts.

Of the seven solvent systems studied, six (*i.e.* all except dichloromethane) successfully resolved components based on their degree of unsaturation. Of these six, only chloroform–toluene gave no separation of symmetrical and unsymmetrical mono-(*cis*-)unsaturated triglycerides. Ethanol-free chloroform initially gave partial resolution of these isomers, but separation steadily deteriorated with subsequent runs and was not restored either by using new Chromarods or by further purification and stabilisation of the chloroform using 13X molecular sieve (which removes ethanol, water, hydrogen chloride and carbonyl chloride, the likely impurities). The best sep-

aration of the symmetrical and unsymmetrical monounsaturated isomers was achieved using chloroform–benzene–diethyl ether (70:30:1.5) as shown in Fig. 1, which showed a separation of *rac* 1-palmitoyl, 2-oleoyl, 3-stearoyl glycerol (POS) and its isomer PSO. This solvent gave marginally superior separation to benzene (triple development), and would be preferred to benzene because only one development was required. However it did not resolve trisaturated (SSS) and *trans* monounsaturated (STS) triglycerides. Fig. 2 shows an example of a separation of trimyristoyl glycerol (MMM) and 1-palmitoyl, 2-elaidoyl, 3-stearoyl glycerol (PES) in the ratio MMM: PES = 25:75, and shows only partial resolution. Toluene, in admixture with chloroform and diethyl ether, was evaluated as a substitute for benzene. Resolution was inferior to the mixture containing benzene, and the mixture was considered unsuitable.

Quantitatively the method was inferior to TLC (used in conjunction with scanning desitometry). A calibration of 1, 2, 5, 10 and 25% PSO in POS is shown in Fig. 3 and demonstrates that at the 25% level the method reported 17.1% PSO, *i.e.* the minor component was underestimated. The detection limit was about 5%, which was again poorer than TLC–densitometry. In addition, because resolution of isomers was not as good as can be achieved by TLC, integration of lesser peaks was less reliable.



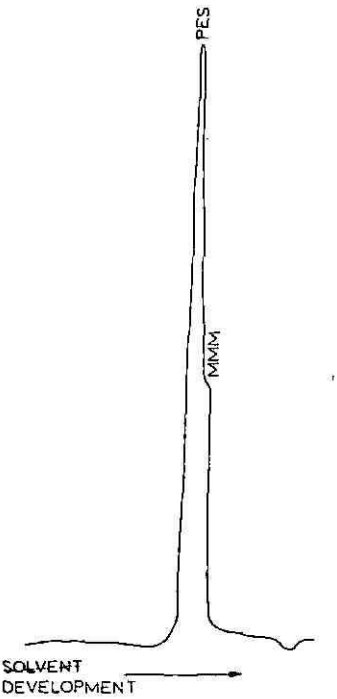


Fig. 1. Separations of POS and PSO on silver nitrate impregnated Chromarods (SII) using chloroform–benzene–diethyl ether (70:30:1.5, v/v/v).

Fig. 2. Separation of MMM and PES on silver nitrate impregnated Chromarods (SII) using chloroform–benzene–diethyl ether (70:30:1.5, v/v/v).

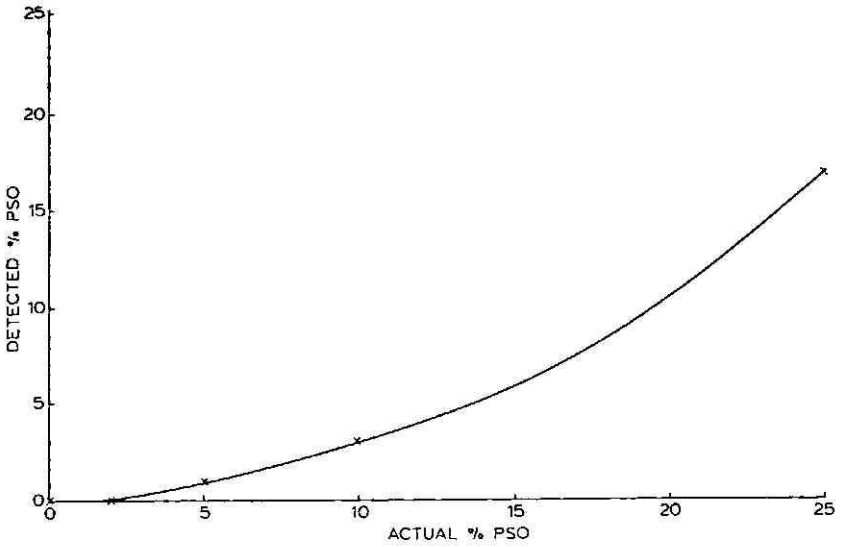


Fig. 3. Calibration of 0–25% PSO in POS on silver nitrate impregnated Chromarods (SII).

CONCLUSIONS

The method described offered a rapid method of separating and estimating the relative proportions of triglycerides according to the number and position of the double bonds, and was capable of resolving the positional isomers of the mono-unsaturated triglycerides. The method was more rapid (both in terms of operator and total analysis time) than TLC–densitometry, but was less accurate.

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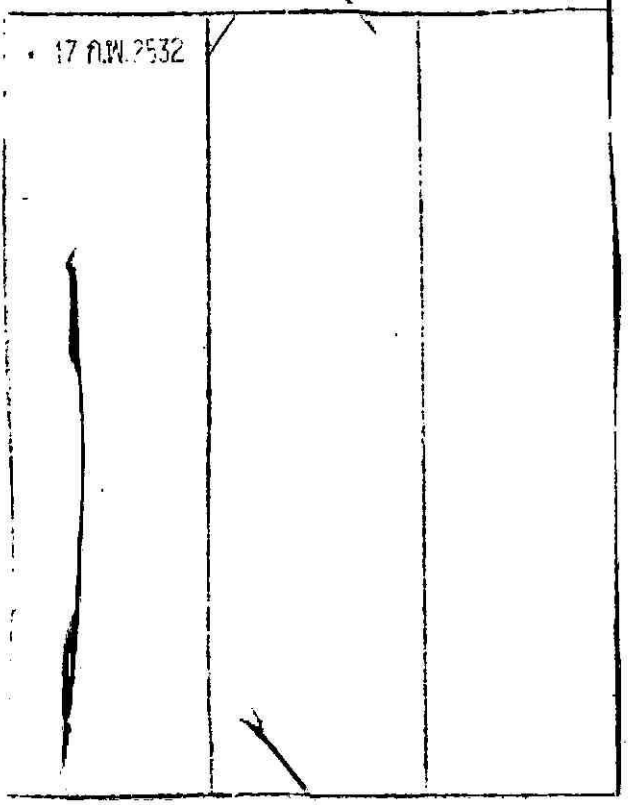

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Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

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