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edited by **J.L. Glajch** *and* **L.R. Snyder**

(Spin-off from the Journal of Chromatography Vol. 485 plus an additional chapter, index and glossary)

This book deals with the use of the computer as an aid in selecting adequate or optimum conditions for a given analytical separation. Originally published as Volume 485 of the Journal of Chromatography, it has now been reprinted in book form, since the information is so useful that many chromatographers want a copy readily available in the lab.
An extensive Introduction is added to the book edition. This surveys the field and refers to the pages where particular items are discussed in the book. The addition of a Glossary of Terms, an Author Index and a Subject Index make this book an invaluable source of easily consulted information for the practising chromatographer.
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Influence of uncharged mobile phase additives on retention and enantioselectivity of chiral drugs using an α_1-acid glycoprotein column

MÄRIT ENQUIST* and JÖRGEN HERMANSSON[a]
Apoteksbolaget AB, Central Laboratory, Department of Biomedicine, 105 14 Stockholm (Sweden)
(First received November 1st, 1989; revised manuscript received May 22nd, 1990)

ABSTRACT

The influence of uncharged mobile phase additives on retention and enantioselectivity on a chiral α_1 acid glycoprotein (AGP) column was investigated. It was observed that it is possible to induce chiral selectivity for several drugs by adding to the mobile phase uncharged modifiers with different hydrophobicities and different hydrogen bonding properties. Modifiers with different hydrogen bonding properties affect the enantioselectivity in different ways. The solute enantiomers seem to compete with the modifier molecules for binding to the chiral stationary phase. The adsorption of 1-propanol and acetonitrile on the AGP column was measured. A monolayer was obtained at mobile phase concentrations of 1.3 *M* (10%) and 2.8 *M* (15%) for 1-propanol and acetonitrile, respectively. These concentrations are in the ranges usually used for chromatographic studies. The effect of 2-propanol on the protein conformation was studied using circular dichroism spectroscopy. It was not possible to detect any change in the conformation of AGP, even in the presence of 40% 2-propanol.

INTRODUCTION

It is common that the biological activity of racemic drugs resides predominantly in one of the enantiomers. Differences between enantiomers are not limited to pharmacological and toxicological effects, but may also occur in absorption, distribution, metabolism and excretion [1–3]. As a consequence, interest in chiral separations by chromatographic methods has grown considerably in recent years. The AGP column is a chiral column based on immobilization of the human plasma protein α_1-acid glycoprotein (AGP) [4,5]. The protein consists of a single peptide chain containing 181 amino acids and five carbohydrate units [6]. There are numerous binding groups on the protein which can be involved in the binding of solutes. The column has been used for the separation of enantiomers of many kinds of chiral drugs such as amines, acids and non-protolytic compounds, which has been reviewed recently [7]. The column has also been used for the separation and determination of the enantiomers of disopyramide, atenolol, chloroquine and metoprolol in biological materials [8–13].

[a] Present address: ChromTech AB, Box 512, 145 63 Norsborg, Sweden.

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Capacity factors and separation factors obtained on the AGP column can easily be regulated by adjusting the mobile phase composition. Dramatic effects on the separation factors have been observed after the addition of charged modifiers such as N,N-dimethyloctyl amine [14] and octanoic acid [15]. This paper describes the influence of uncharged modifiers, with different hydrophobicities and different hydrogen-bonding properties, on the retention and the enantioselectivity for hydantoins, barbituric acid derivatives, N,N-diethylaminosuccinimides and 1′-alkyl 2′,6′-pipecoloxylidides. In order to obtain a deeper understanding of the uncharged organic modifier-induced changes on the enantioselectivity, the adsorption isotherm of two of the studied modifiers, acetonitrile and 1-propanol, were determined.

Circular dichroism (CD) studies of AGP were also performed in order to elucidate the effect of the modifiers on the protein conformation.

EXPERIMENTAL

Apparatus

Liquid chromatography was performed with a Waters M 6000 A pump, a Waters U6K injector and a Shimadzu SPD-2A variable-wavelength UV detector. UV detection was carried out at 215 nm. For the gas chromatographic (GC) determinations, a Hewlett-Packard HP 5890 chromatograph with a flame ionization detector was used. The pH was measured with an Orion Research Model 701 digital pH meter equipped with a Ross 8104SC pH electrode. CD spectra were registered using a JASCO (Tokyo, Japan) J600 spectropolarimeter.

Chemicals

Racemic mephenytoin, secobarbital, proxibarbal, thiopental, methylphenobarbital and (−)-methylphenobarbital were kindly supplied by Dr. Jacek Bojarski (Nicolaus Copernicus Academy of Medicine, Krakow, Poland). The 1-alkyl 2′,6′-pipecoloxylidides and N-aminoalkylsuccinimides model compounds were gifts from Dr. R. Sandberg (Astra Alab, Södertälje, Sweden). Other test compounds were obtained from their manufacturers. Analytical-reagent grade 1- and 2-propanol were obtained from E. Merck (Darmstadt, F.R.G.). Acetonitrile and methanol (UV grade) were obtained from FSA Laboratory Supplies (Loughborough, U.K.). Racemic 2-butanol, (*S*)-2-butanol and propionitrile were purchased from Fluka (Buchs, Switzerland), 1-butanol (Aristar) from BDH (Poole, U.K.) and ethanol (95.5%) from Kemethyl (Stockholm, Sweden).

Liquid chromatographic conditions

Two different AGP columns were used, one prepared in our laboratory [16] and a commercially available CHIRAL-AGP column (ChromTech, Norsborg, Sweden). The mobile phases were prepared by adding appropriate concentrations of uncharged modifiers in a sodium hydrogenphosphate buffer. The phosphate concentration was 0.01 *M*. The mobile phases were degassed in an ultrasonic bath before being used. The hold-up volume of the column (V_m) was determined by injection of water or a mixture deviating slightly in composition from the mobile phase. The flow-rate was 0.9 ml/min and the chromatographic experiments were performed at room temperature.

Determination of adsorbed 1-propanol and acetonitrile

The AGP column was equilibrated with mobile phases of phosphate buffers (pH 7.2) containing 0.665–5.32 *M* 1-propanol or 0.951–7.61 *M* acetonitrile. The adsorbed modifier was eluted from the column with 50 ml of 20% ethanol (95.5%) in water. Fractions of 10 ml were collected and analysed. The assays of 1-propanol and acetonitrile were performed by GC using a glass column (4 m × 2 mm I.D.) containing 20% Carbowax 1500 on Chromosorb (80–100 mesh) at 120°C with helium as the carrier gas (20 ml/min). A calibration graph was constructed from peak areas of known concentration of 1-propanol or acetonitrile. The amount of modifiers adsorbed on the AGP column was calculated from the concentration in the eluate after compensation for the content of the solvent in the void volume. The precision of the determination of the modifiers was <2% at all concentrations, expressed as relative standard deviation.

RESULTS AND DISCUSSION

The addition of uncharged modifiers to the mobile phase is known to decrease the retention and to affect the enantioselectivity on an AGP column [5,7]. In order to obtain a deeper understanding of the mechanism behind these observations, the adsorption of two modifiers to the column, with different hydrophobicities and different hydrogen bonding properties, was studied.

Adsorption of 1-propanol and acetonitrile on the AGP column

The amount of 1-propanol and acetonitrile adsorbed on the AGP column was measured by elution of the column with 20% ethanol in water. The concentration of 1-propanol and acetonitrile was then determined by GC as described under Experimental. The concentration range 0.13–5.3 *M* 1-propanol (corresponding to 1–40%, v/v) is much wider than that used in the chromatographic studies (0.13–0.77 *M* or 1–6%, v/v).

Table I summarizes the results for the adsorption of 1-propanol and acetonitrile. The amount of adsorbed modifier increases with increasing concentration of the studied modifier in the mobile phase. At a concentration of 1.3 *M* (10%, v/v) 1-propanol and 2.8 *M* (15%, v/v) acetonitrile the increase levels off, but the amount of adsorbed modifier continues to increase at higher modifier concentrations. This indicates that the modifiers produce multilayers. If the surface area (S) per gram of the solid phase is known, the number of layers (n) can be calculated by the equation [17]

$$n = \frac{mAN_A}{S \cdot 10^{20}} \tag{1}$$

where A is the area of one solvent molecule, the area of an acetonitrile molecule being *ca.* 21 Å^2 [18], N_A is Avogadro's number and m is the amount of adsorbed modifier in moles per gram of solid phase. According to the adsorption studies a monolayer ($1.3 \cdot 10^{-3}$ mol/g solid phase) of acetonitrile is obtained at a mobile phase concentration of 2.8 *M*. If eqn. 1 is used to calculate the solid phase area that is occupied by this amount of acetonitrile, a value of 167 m^2/g is obtained, which is in good agreement with the value given for the underivatized silica (100 m^2/g). AGP has a molecular weight of

TABLE I

ADSORPTION OF ACETONITRILE AND 1-PROPANOL ON AN AGP COLUMN

Column, AGP (100 × 4.0 mm I.D.); mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing acetonitrile or 1-propanol.

Modifier	Modifier concentration in mobile phase		Modifier adsorbed (mmol/g solid phase)
	M	% (v/v)	
Acetonitrile	0.96	5	0.23
	1.9	10	0.58
	2.8	15	1.3
	3.8	20	1.4
	5.7	30	1.7
	7.6	40	2.4
1-Propanol	0.13	1	0.24
	0.67	5	0.34
	1.3	10	0.96
	2.0	15	0.99
	2.7	20	1.2
	4.0	30	2.3
	5.3	40	3.5

40 000 [6] and the tertiary structure makes the protein very porous and accessible to small molecules, which obviously increases the surface area available for solvent molecules. For 1-propanol a mobile phase concentration of 1.3 *M* is sufficient to produce a monolayer if the area of the 1-propanol molecule is assumed to be equal to that of 2-propanol, which has been determined to be *ca.* 28 Å^2 [18].

Several different classes of chiral compounds have been resolved on the AGP column. The concentrations of uncharged modifiers used in these chromatographic studies are usually lower than those giving a monolayer. Normally 1-propanol concentrations below 2 *M* are used on this column as higher concentrations give too low retentions of most compounds. However, it is interesting that chiral recognition can still be achieved at modifier concentrations that give multilayers. Separation factors of 1.12 and 1.25 have been observed for trimipramine and alprenolol, respectively, in the presence of 7.61 *M* (40%, v/v) acetonitrile.

Influence of uncharged modifiers on retention and chiral selectivity

The retention and the enantioselectivity can be regulated by the addition of an uncharged modifier to the mobile phase. The effect on k' and α depends on the concentration and the properties of the modifier. 2-Propanol, with both hydrogen-donating and -accepting properties, is the most studied modifier on the AGP column (5,7,15,19). In this study the effects of 1-propanol, 2-propanol and acetonitrile on the retention and the enantioselectivity were investigated. Hydantoins and barbituric acid derivatives were used as model compounds and the structures are shown in Fig. 1.

Retention. AGP is a glycoprotein with 181 amino acids in a single peptide chain. Many different binding groups are present in the protein, *e.g.*, hydrophobic groups in the tryptophan, phenylalanine and tyrosine residues and cationic and anionic groups in the lysine and aspartic acid residues, respectively. The protein also contains many

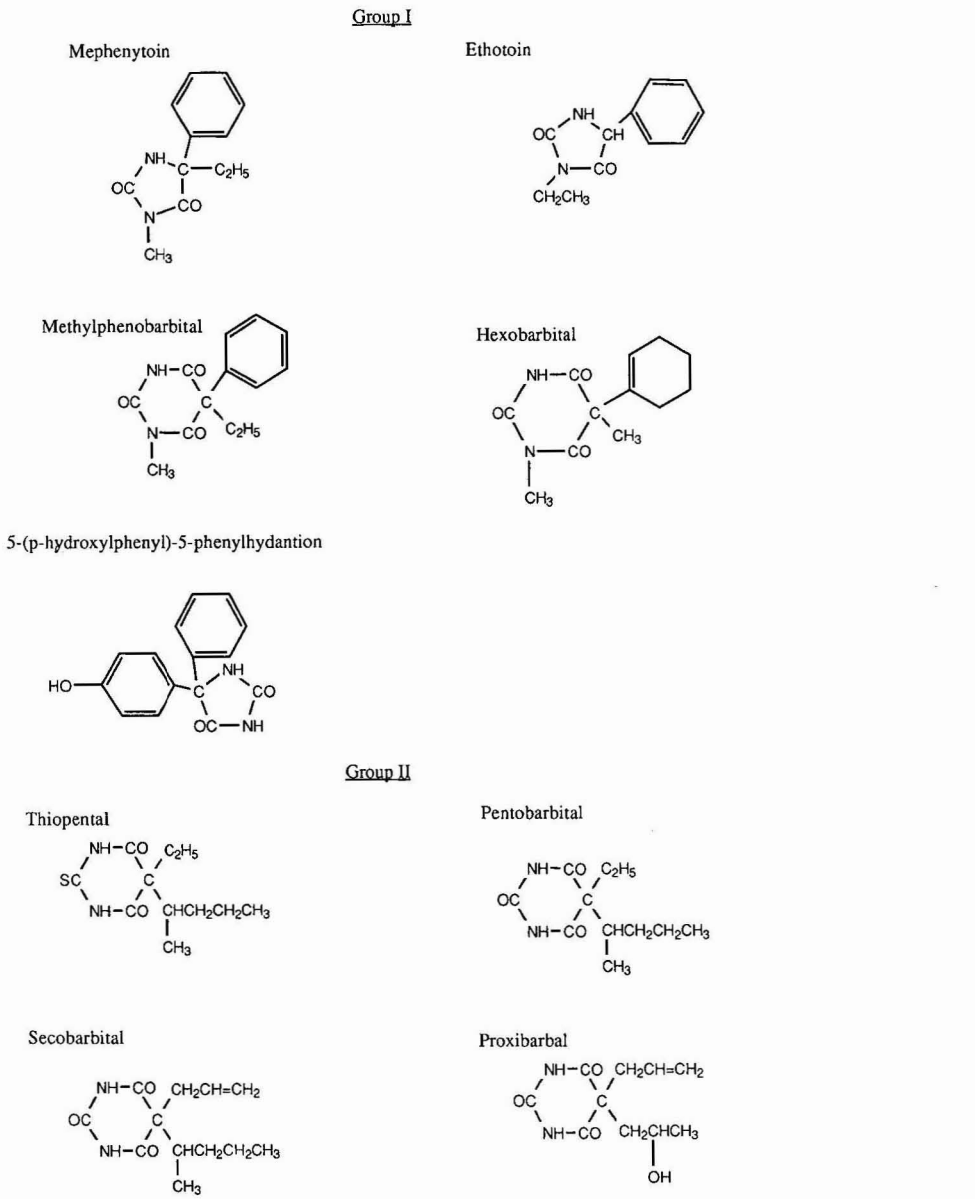


Fig. 1. Structures of the compounds studied.

hydrogen-bonding groups with different properties. All these kinds of binding abilities makes this protein useful for the separation of many compounds with different properties.

Tables II–IV demonstrate that 1- and 2-propanol decrease the capacity factors strongly for all the solutes studied. This is due to competition between the modifier and

TABLE II

INFLUENCE OF CONCENTRATION OF 2-PROPANOL ON THE RETENTION AND ENANTIOSELECTIVITY FOR HYDANTOIN AND BARBITURIC ACID DERIVATIVES

Column, AGP (100 × 4.0 mm I.D.); mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing different concentrations of 2-propanol; flow-rate, 0.9 ml/min.

Compound	Concentration of 2-propanol (*M*)									
	0		0.13		0.32		0.52		0.75	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Methylphenobarbital	49.9[a]	1.0[a]	4.28	1.12	1.84	1.22	1.10	1.27	0.708	1.24
Hexobarbital	7.76	1.95	1.83	1.67	0.89	1.50	0.56	1.42	0.43	1.33
Mephenytoin	17.6[a]	1.0[a]	1.73	1.23	0.906	1.26	0.625	1.24	0.453	1.23
Ethotoin	5.60	6.06	0.83	3.34	0.47	2.16	0.36	1.64	0.31	1.40
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	6.55	1.13	3.35	1.12	2.34	1.16	1.82	1.14	1.57	1.11
Pentobarbital	12.9	1.90	3.01	1.92	1.53	1.74	1.00	1.61	0.75	1.50
Secobarbital	19.1	1.39	4.96	1.23	2.44	1.12	1.56	1.07	1.08	1.0
Thiopental	39.4	1.96	11.7	1.82	5.81	1.64	3.18	1.63	2.16	1.47
Proxibarbal	1.32	1.0	0.309	1.0	0.185	1.0	2.30	1.0	0.076	1.0

[a] Data obtained on the CHIRAL-AGP column.

the solutes for the binding groups of the protein. More drastic effects were obtained with 1- and 2-propanol, compared with acetonitrile, which is the result of the strong adsorption of these modifiers as discussed above.

TABLE III

INFLUENCE OF CONCENTRATION OF 1-PROPANOL ON THE RETENTION AND ENANTIOSELECTIVITY FOR HYDANTOIN AND BARBITURIC ACID DERIVATIVES

Conditions as in Table II, with 1-propanol in place of 2-propanol.

Compound	Concentration of 1-propanol (*M*)									
	0		0.13		0.27		0.40		0.77	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Methylphenobarbital	49.9[a]	1.0[a]	3.14	1.14	1.50	1.17	1.05	1.16	0.642	1.0
Hexobarbital	7.76	1.95	1.46	1.49	0.708	1.37	0.363	1.32	0.350	1.0
Mephenytoin	17.6[a]	1.0[a]	1.38	1.32	0.750	1.35	0.589	1.31	0.389	1.22
Ethotoin	5.60	6.06	0.688	2.39	0.470	1.43	0.343	1.0	0.267	1.0
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	6.55	1.13	3.17	1.13	2.08	1.13	1.57	1.09	1.24	1.0
Pentobarbital	12.9	1.90	2.28	1.71	1.11	1.47	0.684	1.34	0.489	1.18
Secobarbital	19.1	1.39	3.85	1.20	2.00	1.13	1.44	1.08	0.782	1.0
Thiopental	39.4	1.96	9.32	1.65	4.98	1.45	3.58	1.32	1.84	1.13
Proxibarbal	1.32	1.0	0.234	1.0	0.108	1.0	0.124	1.0	0.077	1.0

[a] Data obtained on the CHIRAL-AGP column.

TABLE IV

INFLUENCE OF CONCENTRATION OF ACETONITRILE ON THE RETENTION AND ENANTIOSELECTIVITY FOR HYDANTOIN AND BARBITURIC ACID DERIVATIVES

Conditions as in Table II, with acetonitrile in place of 2-propanol.

Compound	Concentration of acetonitrile (*M*)									
	0		0.19		0.38		0.57		0.76	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Methylphenobarbital	49.9[a]	1.0[a]	10.0	1.0	6.32	1.0	4.50	1.0	3.49	1.0
Hexobarbital	7.76	1.95	3.71	1.67	2.62	1.57	2.00	1.48	1.55	1.41
Mephenytoin	17.6[a]	1.0[a]	3.77	1.17	2.42	1.19	1.78	1.22	1.42	1.20
Ethotoin	5.60	6.06	1.39	3.93	0.981	3.13	0.793	2.63	0.162	2.43
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	6.55	1.13	5.14	1.18	4.32	1.20	3.81	1.21	3.12	1.21
Pentobarbital	12.9	1.90	5.88	1.78	4.01	1.70	2.98	1.62	2.28	1.54
Secobarbital	19.1	1.39	10.92	1.30	7.10	1.26	5.08	1.23	3.90	1.20
Thiopental	39.4	1.96	27.4	1.90	19.3	1.83	13.5	1.76	9.38	1.74
Proxibarbal	1.32	1.0	0.649	1.0	0.386	1.0	0.284	1.0	0.221	1.0

[a] Data obtained on the CHIRAL-AGP column.

It is interesting to compare the capacity factors obtained for the enantiomers of pentobarbital and thiopental, as their structures are very similar: thiopental has a thiocarbonyl group whereas pentobarbital has a carbonyl group located in the same position in the ring structure. The thiocarbonyl group seems to play an important role in the adsorption of this solute, as this group gives the enantiomers of thiopental 3–5 times higher capacity factors than those of pentobarbital. Differences in electronegativity might influence the binding.

Chiral selectivity. Dramatic effects on the enantioselectivity have been observed with cationic and anionic mobile phase additives [14,15]. For example, the tertiary amine N,N-dimethyloctylamine (DMOA) can improve the enantioselectivity for certain cationic solutes. This was observed for propiomazine and promethazine using AGP as a chiral complexing agent in the mobile phase [20], and with immobilized protein (CHIRAL-AGP) [21]. DMOA has also been reported to improve strongly the enantioselectivity of 2-aryl propionic acids [14].

Uncharged organic modifiers can also be used in order to affect both the enantioselectivity and the retention. Usually both the retention and the enantioselectivity decrease with increasing concentration of an uncharged modifier in the mobile phase [5,19]. However, for the local anaesthetics mepivacaine and bupivacaine, it has been reported that an increase in the 2-propanol concentration from 1 to 8% did not significantly affect the separation factors, despite the fact that the retention was drastically reduced [19].

Recently, it has also been demonstrated that it is possible to induce and increase the chiral selectivity by adding uncharged modifiers to the mobile phase [7]. The effects on the enantioselectivity of three different modifiers with different hydrogen-bonding properties and hydrophobicities are presented in Tables II–IV. The test solutes can be divided into two groups, those with the chiral carbon in the ring system, group I, and

those with the chiral carbon in the attached side-chain, group II (see Fig. 1). The enantiomers of mephenytoin, methylphenobarbital and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (group I) gave separation factors between 1.0 and 1.13 using a mobile phase without modifier, whereas the separation factors obtained for ethotoin and hexobarbital were 6.06 and 1.95, respectively. Ethotoin and hexobarbital do not have as large substituents on the chiral carbon as the other solutes in group I which seems to be favourable for the chiral recognition in the absence of a modifier.

It is interesting that 1- and 2-propanol (with both hydrogen-donating and -accepting properties) improve the chiral selectivity for both methylphenobarbital and mephenytoin. However, acetonitrile, with only hydrogen-accepting properties, induces a selective increase in the separation factor, α, for only mephenytoin, as demonstrated in Figs. 2 and 3. Improvement of the enantioselectivity for 5-(*p*-hydroxyphenyl)-5-phenylhydantoin was obtained with 2-propanol and acetonitrile but not with 1-propanol. The separation factors for these solutes increased initially with increasing concentration of the modifiers in the mobile phase. The separation factors reached a maximum at about 0.3–0.5 *M* of the modifiers; at concentrations above 0.5 *M* the enantioselectivity decreased slightly. For ethotoin and hexobarbital, closely related to mephenytoin and methylphenobarbital, respectively, the enantioselectivity decreased with increasing concentration of all three modifiers tested.

For the solutes in group II (pentobarbital, thiopental and secobarbital), separation factors $\geqslant 1.39$ were obtained in phosphate buffer (pH 7.2). The enantioselectivity for these solutes decreased with increasing concentration of organic modifier in the mobile phase. The least hydrophobic modifier, acetonitrile, decreased the enantioselectivity less than 1- and 2-propanol. The resolution of the enantiomers of secobarbital and mephenytoin is demonstrated in Fig. 4a and b.

For proxibarbal no enantioselectivity could be observed using mobile phases containing 1-propanol, 2-propanol or acetonitrile and the enantiomers of proxibarbal did not separate in pure phosphate buffer.

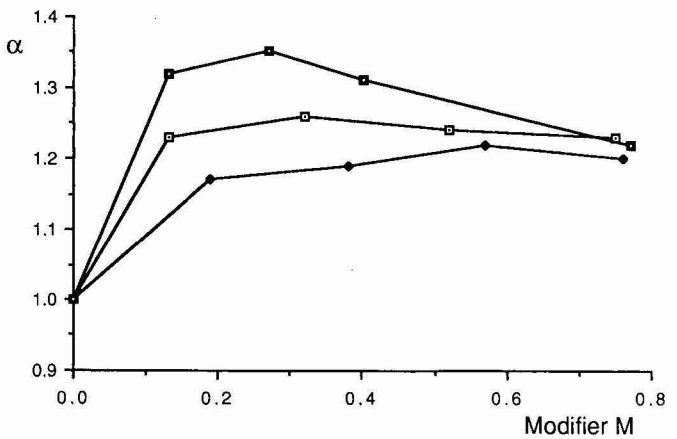


Fig. 2. Influence of mobile phase additives on the separation factor of mephenytoin. Column, AGP (100 × 4 mm I.D.); mobile phase, 0.01 *M* phosphate buffer (pH 7.2) containing different amounts of uncharged modifier; flow-rate, 0.9 ml/min. ■, 1-Propanol; □, 2-propanol; ◆, acetonitrile.

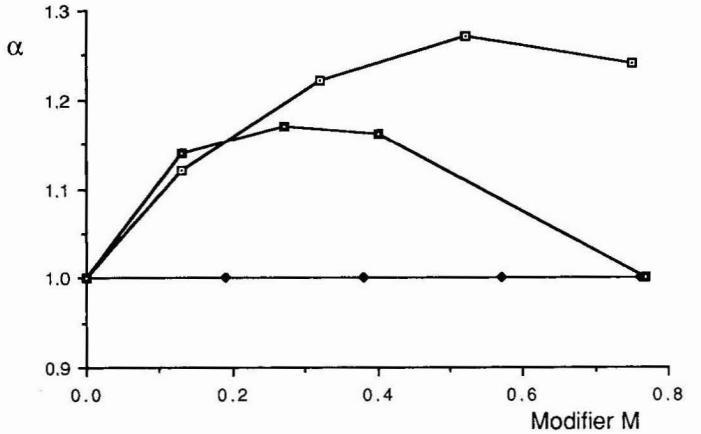


Fig. 3. Influence of mobile phase additives on the separation factor of methylphenobarbital. Conditions and symbols as in Fig. 2.

There are two reasonable explanations for the effects of uncharged modifiers on the enantioselectivity. One is that the modifier competes with the solute enantiomers for binding to groups with different hydrogen-bonding properties in the binding site(s). Therefore, modifiers with different hydrogen-bonding properties affect the enantioselectivity in a different way. The other is that the uncharged modifiers cause reversible changes in the protein conformation. The effect of uncharged organic modifiers of the protein conformation was studied using CD. CD spectra of native AGP (25 μM) were recorded in phosphate buffers (pH 7.0) with and without 2-propanol. The CD spectra were identical and are presented in Fig. 5. With this technique it was not possible to detect any change in the conformation of AGP, even in the presence of as high a concentration as 40% of 2-propanol. However, it is possible to affect the conformation of AGP by adding charged modifiers to a solution of AGP. Fig. 5 demonstrates a CD spectrum of AGP dissolved in phosphate buffer (pH 7.0) containing 0.015 M sodium dodecyl sulphate. The negative peak with a maximum at

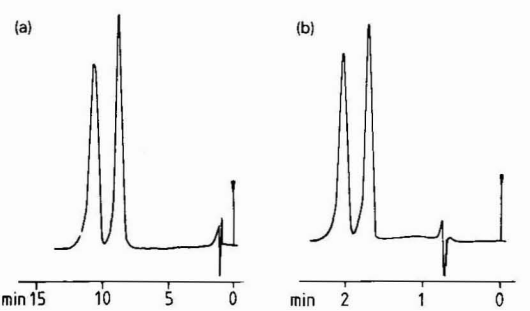


Fig. 4. (a) Resolution of the enantiomers of secobarbital. Column, AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) containing 0.36 M acetonitrile. (b) Resolution of the enantiomers of mephenytoin. Column, AGP column (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) containing 0.13 M 1-propanol.

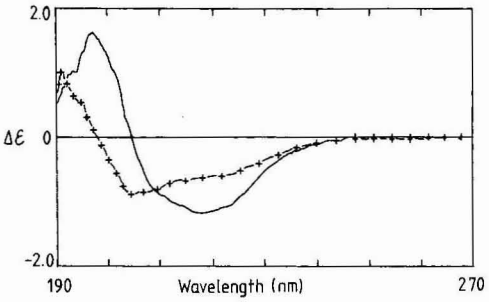


Fig. 5. (———) CD spectrum of native AGP dissolved in phosphate buffer (pH 7.0) or in phosphate buffer (pH 7.0) containing 40% (v/v) 2-propanol (spectra are identical). (+ —— +) CD spectrum of native AGP in phosphate buffer (pH 7.0) containing 0.015 *M* sodium dodecyl sulphate.

205 nm and a shoulder at about 225 nm clearly demonstrates a change in the secondary structure of AGP, probably a transformation of parts of the protein molecule with a β-conformation or an unordered structure into an α-helical form. This observation is in accordance with that obtained by Jirgensons [22]. Hence it is reasonable to assume that the uncharged organic modifier-induced changes in the enantioselectivity depend on competition between the solute enantiomers and the modifier for the binding groups present in the binding sites of the protein. However, small changes in the protein conformation resulting in the exposure of new binding groups in the binding site(s) cannot be neglected when a small molecule is bound to a protein or an enzyme [23,24].

The effects of other alcohols and nitriles than 1- and 2-propanol and acetonitrile on retention and the chiral selectivity for methylphenobarbital and mephenythoin were also studied and the results are summarized in Table V. In this study the commercially available CHIRAL-AGP column was used. All the alcohols studied, except methanol, induced chiral selectivity for both methylphenobarbital and mephenytoin. The separation factor for methylphenobarbital increased with increasing length of the alkyl chain of the alcohol and with alcohols with a branched alkyl chain. The best separation factor was obtained in presence of 2-butanol. It is interesting that the two tested nitriles induce a selective increase in α only for mephenytoin. The highest separation factor for mephenytoin was obtained by adding propionitrile to the mobile phase. It was also observed that the effect on both k' and α was equivalent with (*R,S*)-2-butanol and (*S*)-2-butanol.

The effect of different uncharged modifiers on the enantioselectivity was also studied with two other homologous series, 1-alkyl 2′,6′-pipecoloxylidides and N,N-diethylaminosuccinimides.

Table VI presents capacity factors and separation factors obtained for a series of 1-alkyl 2′,6′-pipecoloxylidides using 1-propanol, 2-propanol and acetonitrile as mobile phase additives. Mobile phases containing 1-propanol gave the best separation conditions for this series of compounds, as high enantioselectivity and low retention were obtained. A capacity factor of 15.8 and no enantioselectivity were obtained for the enantiomers of the ethyl homologue using a mobile phase containing 0.75 *M* (4.0%, v/v) acetonitrile, whereas a k'_1 value of 4.63 and a separation factor of 1.30 were obtained using 0.75 *M* (5.8%, v/v) 1-propanol.

TABLE V

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR METHYLPHENOBARBITAL AND MEPHENYTOIN

Column, CHIRAL-AGP; mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing different modifiers; flow-rate, 0.9 ml/min.

Modifier	Concentration (*M*)	Methylphenobarbital		Mephenytoin	
		k'_1	α	k'_1	α
–		49.9	1.0	17.6	1.0
Methanol	0.25	37.3	1.0	13.8	1.0
	0.44	28.6	1.0	10.9	1.0
	1.48	13.0	1.0	5.26	1.0
Ethanol	0.18	20.3	1.0	7.65	1.10
	0.88	4.52	1.15	2.20	1.13
	1.41	2.53	1.14	1.42	1.08
1-Propanol	0.13	7.42	1.17	3.24	1.24
	0.27	3.63	1.18	1.90	1.23
	0.66	1.76	1.0	1.19	1.12
1-Butanol	0.11	3.16	1.29	1.76	1.15
	0.22	1.68	1.14	1.25	1.0
	0.44	1.05	1.0	0.86	1.0
2-Propanol	0.13	10.1	1.19	4.33	1.12
	0.27	5.07	1.29	2.92	1.16
	0.66	1.95	1.29	1.30	1.09
(*R,S*)-2-Butanol	0.11	3.81	1.33	2.07	1.19
	0.22	2.09	1.33	1.37	1.17
	0.44	1.14	1.25	1.01	1.0
(*S*)-2-Butanol	0.22	2.08	1.32	1.39	1.16
Acetonitrile	0.19	20.8	1.0	8.65	1.06
	0.38	15.9	1.0	5.53	1.15
	0.95	6.22	1.0	2.72	1.16
Propionitrile	0.14	8.02	1.0	3.18	1.33

The chiral selectivity is highly affected by the length of the alkyl chain bound to the piperidine nitrogen, as discussed previously [16]. The enantioselectivity for the unsubstituted compound (PPX) was >3 times higher than for the methyl-substituted compound with 2-propanol in the mobile phase. Separation factors of 4.27 and 4.96 were obtained for the PPX enantiomers using mobile phases containing 2-propanol and 1-propanol, respectively. Acetonitrile, with only hydrogen-accepting properties, drastically reduced the enantioselectivity for the enantiomers of PPX (see Table VI), which clearly demonstrates that the hydrogen-bonding properties of the modifier strongly affect the enantioselectivity.

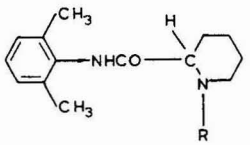
For the series of N,N-diethylaminosuccinimides, it is also more advantageous to use 1-propanol as mobile phase modifier, even though the separation factor is 1.6 times higher when using acetonitrile (see Table VII). This is due to the fact that the retention for the last eluted enantiomer is twelve times lower with 1-propanol as mobile phase additive and the separation factor is >1.4 in all instances.

In conclusion, it is important to note that it is possible to induce and increase chiral selectivity for chiral compounds by adding certain uncharged modifiers to

TABLE VI

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR 1-ALKYL-2',6'-PIPECOLOXYLIDIDES

Column, AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) containing different modifiers; flow-rate, 0.9 ml/min. S = The last-eluted enantiomer.



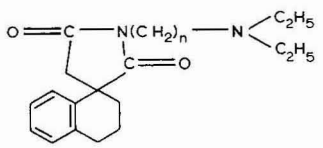
R	0.75 *M* 1-propanol		0.75 *M* 2-propanol		0.75 *M* acetonitrile	
	k'_s	α	k'_s	α	k'_s	α
H (PPX)	12.6	4.96	11.1	4.27	16.5	1.93
CH_3	3.99	1.51	3.95	1.40	12.0	1.17
C_2H_5	4.63	1.30	4.21	1.23	15.8	1.0
C_3H_7	7.83	1.67	8.56	1.40	45.6	1.37
C_4H_9	10.6	1.47	12.7	1.24	79.6	1.26
C_5H_{11}	13.8	1.19	18.9	1.07	–	–
C_6H_{13}	18.1	1.0	31.0	1.15	–	–

the mobile phase. The adsorption of methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, acetonitrile and propionitrile to the protein is reversible and the columns are very stable and can be used for long periods without being negatively affected by these modifiers. This was tested by running test compounds frequently

TABLE VII

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR DIETHYLAMINESUCCINIMIDES

Conditions as in Table VI. k'_2 = The last-eluted enantiomer.



n	0.75 *M* 1-propanol		0.75 *M* 2-propanol		0.75 *M* acetonitrile	
	k'_2	α	k'_2	α	k'_2	α
2	12.1	1.48	23.2	1.60	147	2.40
3	6.84	1.75	10.5	2.12	85.7	3.26
4	5.95	1.41	9.37	1.70	71.9	2.51
5	9.73	1.54	18.2	1.83	124	2.21

during this study for the determination of the capacity factors and the enantioselectivity for the test compounds.

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Separation of the enantiomers of β-receptor blocking agents and other cationic drugs using a CHIRAL-AGP® column

Binding properties and characterization of immobilized α_1-acid glycoprotein

MÄRIT ENQUIST* and JÖRGEN HERMANSSON[a]
Apoteksbolaget AB, Central Laboratory, Department of Biomedicine, S 105 14 Stockholm (Sweden)
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ABSTRACT

The effect of the immobilization procedure on the conformation of α_1-acid glycoprotein (AGP) was investigated by recording the fluorescence spectra of native and immobilized AGP. A 20-nm red shift was obtained for the immobilized form of AGP compared with the emission maximum of 338 nm obtained for native AGP. This demonstrates that the tryptophan residues are exposed on the protein surface after immobilization, indicating that the immobilized form of AGP has a more unfolded structure than the native AGP. The effect of N,N-dimethyloctylamine on the enantioselectivity for some fentiazine derivatives, observed with immobilized AGP, was equal to that obtained with AGP as a chiral complexing agent in the mobile phase. This demonstrates that even though the immobilization procedure affects the conformation of the protein there still exist large similarities between native and immobilized AGP concerning chiral recognition.

The adsorption isotherm of (−)-terodiline was studied by use of the breakthrough technique. The adsorption isotherm indicates that (−)-terodiline is adsorbed to one site with high affinity and at least one more site with lower affinity. It was also observed that the enantiomers of amines, acids and non-protolytic compounds compete with the cationic compound, (−)-terodiline, for binding to the same sites.

The β-receptor blocking agents atenolol, metoprolol, pindolol, alprenolol, oxprenolol and propranolol were resolved on a CHIRAL-AGP® column. The retention and enantioselectivity are highly influenced by the structure of the solute and the nature of the uncharged mobile phase additives. Separation factors of 1.2–1.8 were obtained for the β-blockers under the studied conditions.

INTRODUCTION

An α_1-acid glycoprotein (AGP) column has been used for the resolution of a large number of chiral drugs [1]. The broad applicability of the chiral phases can be ascribed to the fact that the binding sites contain many different binding groups, giving the possibility of stereoselective interactions with a broad range of solutes, such as amines, acids and non-protolytes. Further, it has been demonstrated that it is possible to affect the chiral stationary phase reversibly with organic modifiers in such a way

[a] Present address: ChromTech AB, Box 512, 145 63 Norsborg, Sweden.

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that enantioselectivity can be induced. The effects of mobile phase additives of different character (hydrogen bonding properties, hydrophobicity and charge) and concentration and of the mobile phase pH and temperature on the retention and enantioselectivity have been discussed in several papers [2–6] and have also been reviewed recently [1]. This paper discusses the effects of uncharged modifiers, with different bonding properties, on the retention and enantioselectivity for a series of β-receptor blocking agents, with the purpose of establishing optimum separation conditions for these solutes.

It is well known that the conformations of proteins can be affected when they are bound to solid phases with different character [7]. Such conformational changes can be characterized using fluorescence spectroscopy. This technique was used in this study to determine differences in conformation between immobilized and non-immobilized AGP.

It has been suggested that amines, acids and non-protolytic compounds are bound to a single binding site on the AGP molecule [8,9]. Others [10,11], in contrast, have reported two different binding sites on AGP. In order to characterize the binding site(s) of immobilized AGP, adsorption studies were performed using (−)-terodiline as the model compound. Chromatographic experiments using (−)-terodiline as a mobile phase additive were also performed in order to obtain a deeper insight into the nature of the binding site(s).

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Waters Assoc. M 6000 A pump, a Waters Assoc. U6K injector and a Shimadzu SPD-2A variable-wavelength UV detector operated at 215 nm. The chiral column was a commercially available CHIRAL-AGP® column from ChromTech (Norsborg, Sweden). Fluorescence spectr a of AGP were obtained with a Shimadzu RF-500 spectrofluorimeter.

Chemicals

(+)- and (−)-terodiline were kindly supplied by Kabi (Stockholm, Sweden). The other test compounds were obtained from their manufacturers. Analytical-reagent grade 1- and 2-propanol were obtained from E. Merck (Darmstadt, F.R.G.) and acetonitrile and methanol (UV grade) from FSA Laboratory Supplies (Loughborough, U.K.). Racemic 2-butanol and propionitrile were purchased from Fluka (Buchs, Switzerland), 1-butanol (Aristar) from BDH (Poole, U.K.), ethanol (95%) from Kemethyl (Stockholm, Sweden) and N,N-dimethyloctylamine (DMOA) from ICN Pharmaceuticals (Plainview, NY, U.S.A.).

Chromatographic conditions

Chromatography was performed at room temperature and the flow-rate was 0.9 ml/min. The mobile phases used were phosphate buffers containing different concentrations of uncharged modifiers, DMOA or (−)-terodiline. The concentration of phosphate was 0.01 *M* in the mobile phases containing uncharged modifiers and 0.02 *M* in those phases containing charged modifiers. The pH was adjusted to 7.0 with sodium hydroxide. The mobile phases were degassed in an ultrasonic bath before being

used. The hold-up volume of the column (V_m) was determined by injection of water or mobile phase with a slight difference in composition.

Fluorescence spectra of AGP

AGP in solution. AGP was dissolved in 0.01 *M* phosphate buffer (pH 7.0) at a concentration of 100 μg/ml ($2.5 \cdot 10^{-6}$ *M*). An excitation wavelength of 295 nm was used. Emission spectra were recorded between 300 and 400 nm.

Immobilized AGP. Packing material from the CHIRAL-AGP column was packed into a 1-mm flow cell and the material was equilibrated with 0.01 *M* phosphate buffer (pH 7.0) [7]. The fluorescence was measured as above.

Adsorption of (−)-terodiline on the CHIRAL-AGP column

The amounts of (−)-terodiline adsorbed by the chiral stationary phase from the mobile phase were determined by the breakthrough technique [12]. The column was first equilibrated with a mobile phase of phosphate buffer (pH 7.0) (0.02 *M* phosphate). A second mobile phase (with the same phosphate concentration and pH) containing (−)-terodiline was then pumped onto the column. The loading of the amine was followed by UV detection at 215 nm. The amount of (−)-terodiline, $[T]_s$, in moles adsorbed on the column was calculated with the equation

$$[T]_s = (V_R - V_m)C_m \quad (1)$$

where the breakthrough volume (V_R) is the net retention volume corresponding to the front. V_R is measured as the volume that has been pumped through the column from the moment the mobile phase containing (−)-terodiline is introduced onto the column to the inflection point of the breakthrough curve. C_m is the concentration of (−)-terodiline in the mobile phase. After each loading occasion the column was washed with 50 ml of 20% (v/v) 1-propanol in water and reconditioned with 50 ml of phosphate buffer (pH 7.0).

RESULTS AND DISCUSSION

Characterization of AGP

AGP is a human plasma protein which consists of a single peptide chain containing 181 amino acids. Five carbohydrate units are linked to the peptide chain via the asparagine residues. AGP contains many binding groups of different character, acidic and basic groups and hydrogen-bonding groups. The protein has a moderate hydrophobic character as it contains many hydrophobic amino acid residues such as tryptophan, lysine and phenylalanine [13].

In the CHIRAL-AGP column, AGP is immobilized onto silica particles by a covalent linkage and cross-linking of adjacent protein molecules [1]. Some of the binding groups which are free for interaction in native AGP are utilized in the immobilization and cross-linking procedures. The conformation of both native and immobilized AGP was studied using fluorescence spectroscopy as described under Experimental.

The fluorescence spectra of native AGP in solution and of AGP in the immobilized form are given in Fig. 1a and b. The idea behind this experiment was to

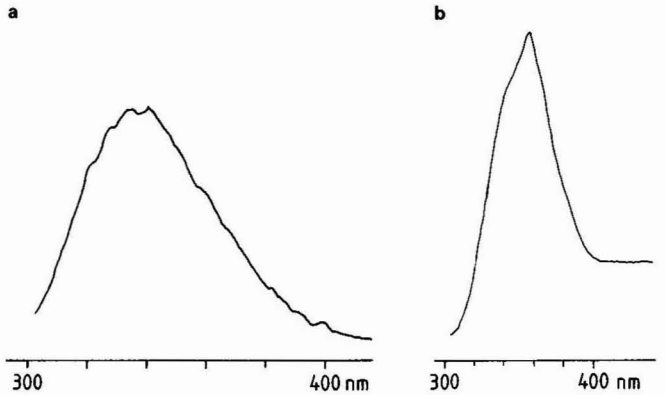


Fig. 1. (a) Emission spectrum of AGP dissolved in a 0.01 *M* phosphate buffer (pH 7.0). AGP concentration, 100 μg/ml; excitation, 295 nm; band width, 1.5 nm; scan speed, low. (b) Emission spectrum of AGP immobilized on silica in 0.01 *M* phosphate buffer (pH 7.0). Conditions as in (a).

study the emission of the three tryptophan residues in the AGP molecule, as differences in the emission maximum of the tryptophans reflect differences in the protein conformation. A buried tryptophan residue has another emission maximum compared with the one that is exposed on the surface of the protein molecule [7]. An emission maximum of 338 nm was obtained for AGP in solution, indicating that the tryptophans in native AGP are buried within the hydrophobic microenvironment of the molecule. However, a 20-nm red shift was obtained for the immobilized form of AGP. Obviously, the immobilization of AGP onto the silica surface influences the protein conformation in such a way that one, two or all three tryptophans are exposed on the surface of the protein molecule. In order to simulate a surface-exposed tryptophan residue, a fluorescence spectrum was also recorded for N-acetyltryptophanamide, which gave an emission maximum of 360 nm, which is in good agreement with the value obtained for tryptophan of immobilized AGP.

Despite the fact that the conformation of AGP is affected by the immobilization procedure, there are still large similarities between native and immobilized AGP with respect to chiral recognition. This was demonstrated by comparing the effect of DMOA on the enantioselectivity for some fentiazine derivatives (Fig. 2) on a column with immobilized AGP (CHIRAL-AGP) (Fig. 3) and with AGP as a chiral complexing agent in the mobile phase (Fig. 4, data from ref. 14). The enantioselectivity increased for propiomazine and promethazine whereas it decreased for trimipramine and alimemazine on increasing the DMOA concentration in the mobile phase. The fentiazines were affected in the same way by DMOA both when using the protein as an immobilized chiral selector and when using AGP as a chiral complexing agent in the mobile phase.

Adsorption of (−)-terodiline on the CHIRAL-AGP column

In order to study the binding of enantiomers to the chiral stationary phase, the adsorption isotherm of the cationic compound (−)-terodiline was determined using the breakthrough technique. In Fig. 5 the amount of (−)-terodiline adsorbed to a CHIRAL-AGP column is plotted against the concentration of (−)-terodiline in the

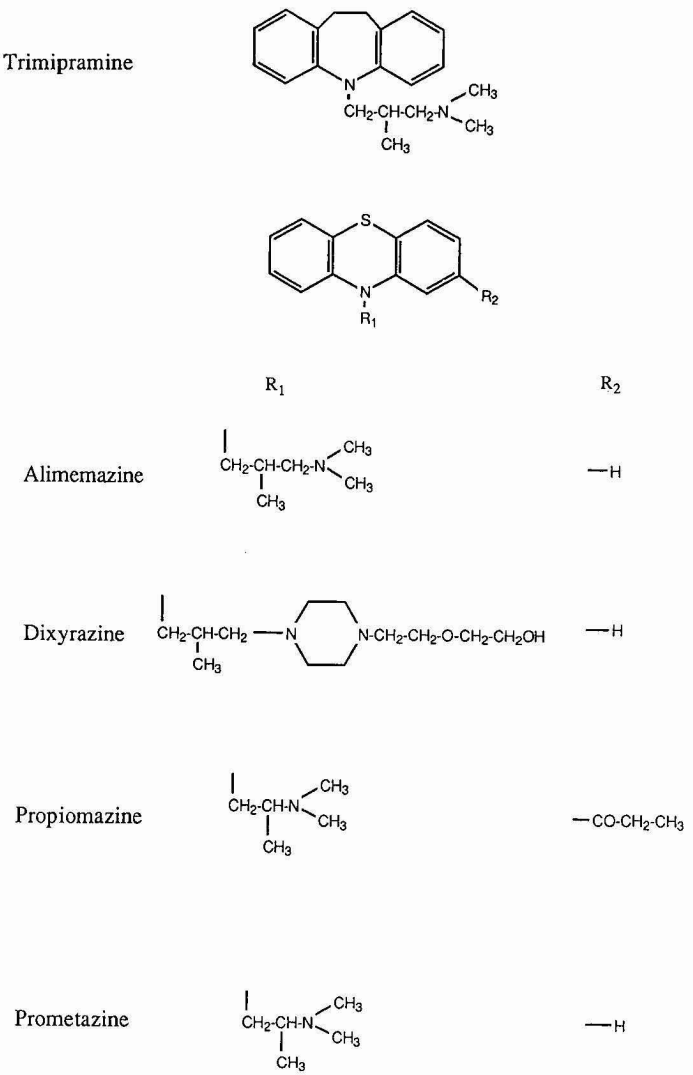


Fig. 2. Structures of fentiazine derivatives and trimipramine.

mobile phase (0–79.5 μM). At low concentrations of (−)-terodiline in the mobile phase the amount of adsorbed (−)-terodiline increases dramatically with increasing concentration. At concentrations above 15 μM the curve levels off and the slope is changed. No saturation of the stationary phase was observed in the concentration range studied. The shape of the curve indicates that (−)-terodiline is adsorbed to more than one site, one site with high affinity and at least one more site with lower affinity. It has been demonstrated previously [6,15] that the charged compound sodium dodecyl sulphate changes the secondary structure of AGP. Thus, an alternative explanation can be that (−)-terodiline at a certain concentration affects the protein in such a way

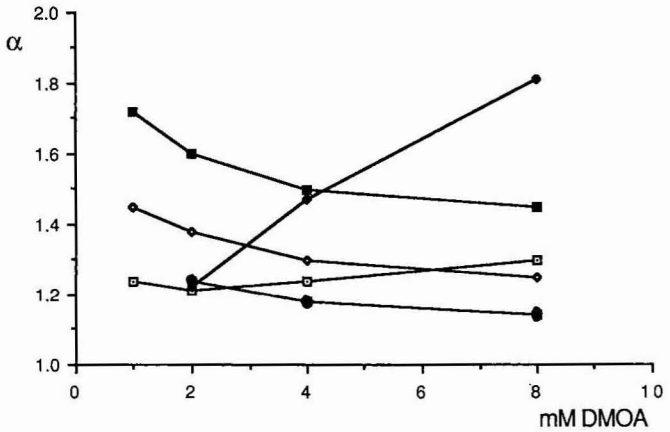


Fig. 3. Effects of the concentration of DMOA on the enantioselectivity of fentiazine derivatives and trimipramine using immobilized AGP as chiral stationary phase. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.0) (0.01 *M* phosphate) containing 2% (v/v) 2-propanol and different concentrations of DMOA; flow-rate, 0.9 ml/min. ◆, Propiomazine; ■, trimipramine; ◇, alimemazine; □, prometazine; ●, dixyrazine.

that new binding groups in the binding site(s) are exposed and become accessible for binding of solute molecules. The resolution of terodiline on the CHIRAL-AGP column using a mobile phase of phosphate buffer (pH 7.2) containing 15% (v/v) 2-propanol is demonstrated in Fig. 6.

Effects of (−)-terodiline on the retention and enantioselectivity

The effects of uncharged modifiers on the enantioselectivity and retention have been discussed recently [1,6]. It was suggested that the enantiomers compete with the

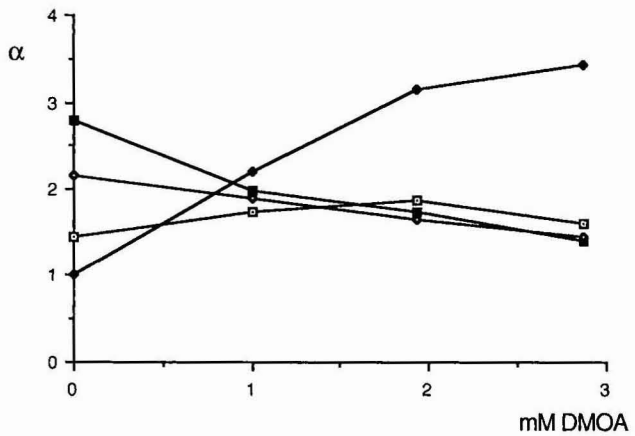


Fig. 4. Effects of the concentration of DMOA on the enantioselectivity of fentiazine derivatives and trimipramine using AGP as chiral complexing agent in the mobile phase. Column, LiChrosorb diol (150 × 3.2 mm I.D.); mobile phase, phosphate buffer (pH 7.55) (ionic strength, $\mu = 0.05$) containing 8.8 μM AGP and different concentrations of DMOA; flow-rate, 0.5 ml/min. Symbols as in Fig. 3. Data from ref. 14.

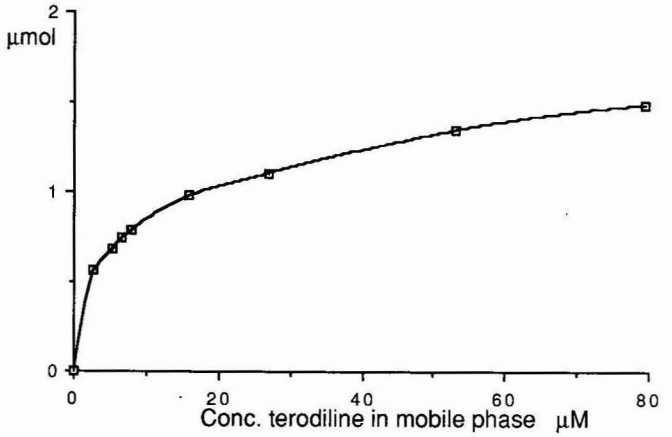


Fig. 5. Adsorption isotherm of (−)-terodiline. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.0) (0.02 *M* phosphate).

modifiers for binding to the same site(s) on the protein and that the hydrophobicity and hydrogen-bonding properties are of great importance for the effects on the enantioselectivity. The influence of charged modifiers on the retention and enantioselectivity is more complicated. The modifiers might affect the conformation of the protein as discussed above and it can also compete with the enantiomers for the binding groups of the protein. In this study the effect of the cationic compound (−)-terodiline on the retention of amines, non-protolytic compounds and strong and weak acids was examined. It was observed that the retentions of the amines metoprolol and 2-amino-5-methoxytetralin and of bendroflumethiazide and ethyl mandelate (a weak acid uncharged at pH 7 and a non-protolyte, respectively) decreased with

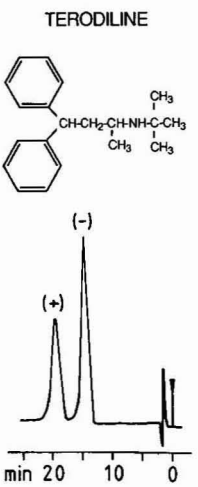
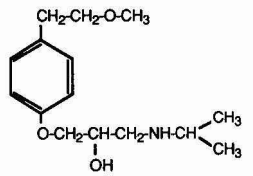
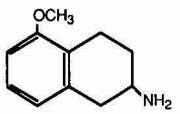


Fig. 6. Resolution of the enantiomers of terodiline. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.4) (0.01 *M* phosphate) containing 15% 2-propanol; flow-rate, 0.6 ml/min.

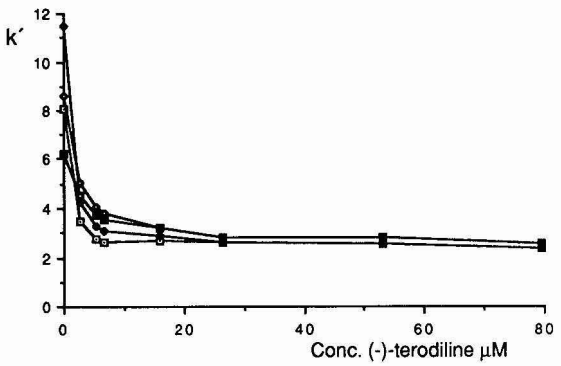
a



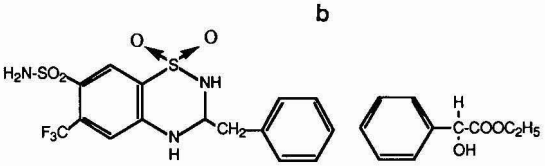
METOPROLOL



2-AMINO-5-METHOXY TETRALIN



b



BENDROFLUMETHIAZIDE

MANDELIC ACID
ETHYL ESTER

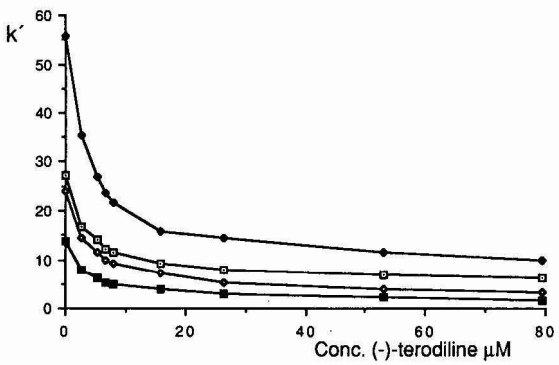


Fig. 7.

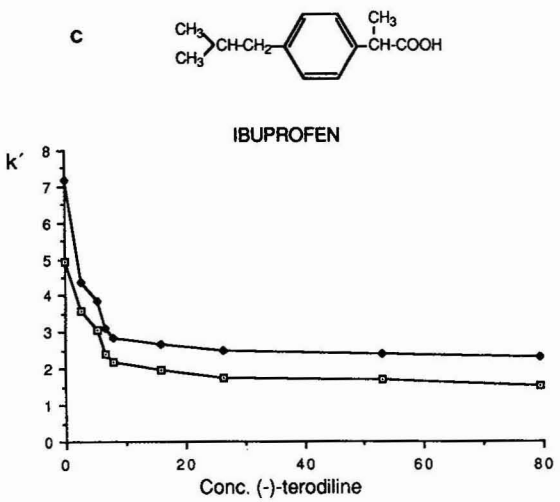


Fig. 7. Effect of (−)-terodiline content in the mobile phase on retention. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.0) (0.02 *M* phosphate) containing different concentrations of (−)-terodiline; flow-rate, 0.9 ml/min. (a) □, (*R*)-Metoprolol; ◆, (*S*)-metoprolol; ■, (1) 2-amino-5-methoxytetralin; ◇, (2) 2-amino-5-methoxytetralin. (b) □, (1) Bendroflumethiazide; ◆, (2) bendroflumethiazide; ■, (1) ethyl mandelate; ◇, (2) ethyl mandelate. (c) □, (1) Ibuprofen; ◆, (2) ibuprofen. [(1) and (2) refer to the two enantiomers.]

increasing concentration of (−)-terodiline (Fig. 7a and b). Fig. 7c shows that the retention of the enantiomers of the anionic compound ibuprofen also decreased with increasing concentration of (−)-terodiline. At (−)-terodiline concentrations below 10 μM the capacity factors for the enantiomers decreased dramatically, but at higher concentrations (10–20 μM) the decrease levelled off. The enantiomers of the test solutes are still retained with capacity factors of 1.5–9.9 at a concentration of 79.5 μM(−)-terodiline. It can be concluded that anionic, cationic and also non-protolytic compounds are bound to and compete for the same sites on the protein. The above data also suggest that the solutes, independently of their nature, are bound to one high-affinity and one low-affinity site. The high-affinity site is described by the steep part of the capacity factor curves obtained at low concentrations of (−)-terodiline demonstrated in Fig. 7a–c. The second site can be an unspecific binding area on the protein or a site created by the binding of (−)-terodiline to the protein, as discussed above.

It is interesting that the enantioselectivity of the amines metoprolol and 2-amino-5-methoxytetralin disappeared at a concentration of *ca.* 20 μM(−)-terodiline but the chiral selectivity for the uncharged solutes decreased only slightly. The separation factor for the enantiomers of ibuprofen increased with increasing concentration of (−)-terodiline, as demonstrated in Fig. 8. Similar results have been observed previously for the arylpropionic acid derivatives ibuprofen, naproxen and ketoprofen on adding another cation, DMOA, to the mobile phase [2,3].

The above effects of (−)-terodiline on the retention and the enantioselectivity may indicate that the solutes of different character interact with different binding groups in the same binding sites.

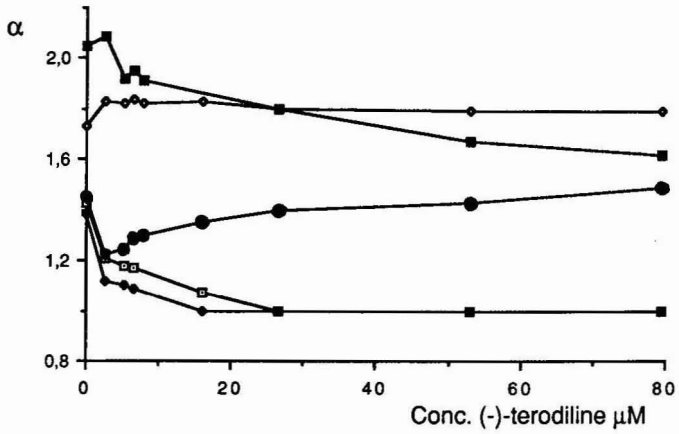
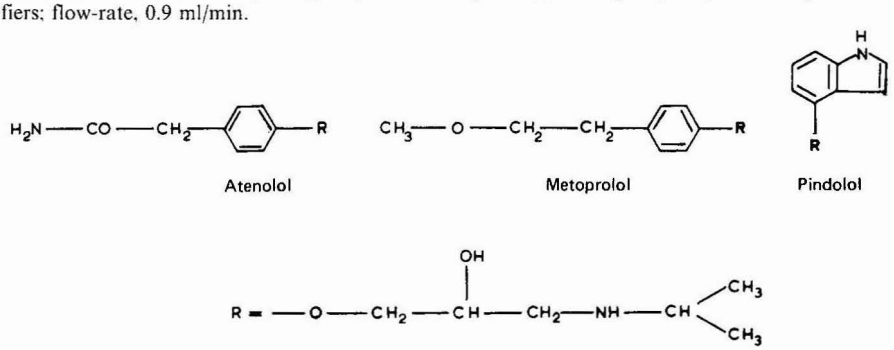


Fig. 8. Effect of (−)-terodiline on enantioselectivity. Conditions as in Fig. 7. □, Metoprolol; ◆, 2-amino-5-methoxytetralin; ■, bendroflumethiazide; ◇, ethyl mandelate; ●, ibuprofen.

TABLE I

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR β-RECEPTOR BLOCKING AGENTS

Column, CHIRAL-AGP; mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing different modifiers; flow-rate, 0.9 ml/min.

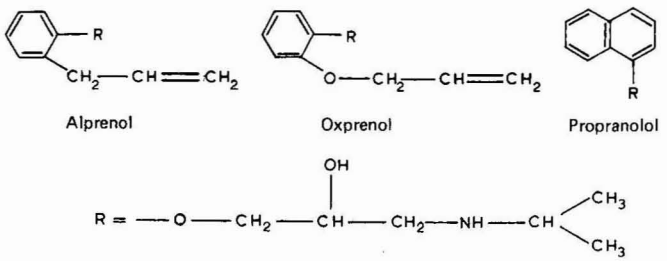


Modifier	Concentration (*M*)	Atenolol		Metoprolol		Pindolol	
		k'_1	α	k'_1	α	k'_1	α
–	–	4.58	1.31	37.1	1.36	–	–
Methanol	1.48	3.08	1.11	8.64	1.37	–	–
	2.47	2.91	1.05	6.09	1.25	32.3	1.20
Ethanol	0.88	3.21	1.0	5.55	1.15	22.5	1.21
	2.11	2.84	1.0	3.73	1.0	8.40	1.29
1-Propanol	0.66	3.09	1.0	4.14	1.0	8.98	1.0
	1.06	2.88	1.0	3.43	1.0	6.66	1.0
1-Butanol	0.22	3.32	1.0	4.68	1.0	9.01	1.0
	0.44	4.13	1.0	3.85	1.0	7.30	1.0
2-Propanol	0.13	3.19	1.05	7.93	1.22	41.1	1.08
	1.06	2.53	1.0	3.13	1.0	6.85	1.04
Acetonitrile	0.95	3.59	1.0	5.93	1.11	28.6	1.53
	2.85	–	–	–	–	6.82	1.23

TABLE II

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR β-RECEPTOR BLOCKING AGENTS

Conditions as in Table I.



Modifier	Concentration (M)	Alprenol		Oxprenol		Propranolol	
		k'_1	α	k'_1	α	k'_1	α
–	–	–	–	–	–	–	–
Ethanol	1.76	49.7	1.49	33.9	1.28	–	–
	2.11	36.3	1.47	23.1	1.25	–	–
1-Propanol	0.665	22.8	1.20	15.4	1.04	41.9	1.11
	1.06	12.9	1.0	8.15	1.0	25.41	1.0
1-Butanol	0.218	25.3	1.16	17.1	1.08	–	–
	0.436	12.21	1.0	7.77	1.0	23.4	1.0
2-Propanol	1.06	19.6	1.21	15.7	1.12	34.6	1.24
	1.60	12.8	1.07	8.7	1.05	21.5	1.03
2-Butanol	0.436	18.3	1.13	14.9	1.04	33.0	1.06
Acetonitrile	0.951	–	–	–	–	–	–
	2.85	30.6	1.83	15.6	1.11	33.9	1.11

Direct resolution of β-receptor blocking agents

Atenolol, metoprolol, pindolol, alprenolol, oxprenolol and propranolol were resolved on a CHIRAL-AGP column using a mobile phase of phosphate buffer (pH 7.2) with and without uncharged modifiers. Tables I and II summarize the capacity factors (k') and separation factors (α) for these compounds. Practolol and acebutolol are not included as no enantioselectivity was obtained under the described conditions.

Fig. 9. Separation of the enantiomers of atenolol. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) (0.01 M phosphate).

The enantiomers of atenolol have previously been reported to separate on an AGP column after preparation of an enantiomeric acetyl derivative [16]. It was observed that the retention of the first-eluted enantiomer decreased and that of the last-eluted enantiomer increased when atenolol was acetylated. The enantioselectivity thereby became dramatically improved. However, atenolol, can also be resolved in the underivatized form on the CHIRAL-AGP column using a mobile phase of phosphate buffer (pH 7.2) without modifier, as demonstrated in Fig. 9.

Atenolol and metoprolol are relatively hydrophilic compounds and the highest separation factors, 1.31 and 1.36, respectively, are obtained in pure phosphate buffer (pH 7.2). Addition of uncharged modifier decreases the retention and the enantioselectivity dramatically. However, low concentrations of methanol as mobile phase additive reduced the retention without destroying the chiral selectivity. A separation factor of 1.25 and a baseline separation were obtained within 8 min for the enantiomers of metoprolol when the concentration of methanol in the mobile phase was 10% (2.47 *M*). It is very important to study the effects on the enantioselectivity of modifiers with different bonding properties in order to obtain chromatographic systems giving high enantioselectivity and low retention. This is of special importance when determining drugs present in, for example, plasma samples at low concentrations.

Bioanalytical methods for studies of the enantiomers of metoprolol in plasma, using a CHIRAL-AGP column and mobile phases containing 2-propanol or acetonitrile, have recently been developed [17–19]. In order to improve the sensitivity in those studies the gradient technique [17,18] and the column enrichment technique [19] had to be used.

Another example of modifier effects is shown in Fig. 10, where the effects of 1-propanol and acetonitrile on the enantioselectivity of pindolol are compared. No enantioselectivity was observed for the enantiomers of pindolol, using a mobile phase containing a low concentration of 1-propanol. However, if 1-propanol, with both hydrogen-accepting and -donating properties, was replaced with 10% (2.85 *M*) acetonitrile, with only hydrogen-accepting properties, the enantiomers were baseline resolved with a separation factor of 1.36. Similar effects have been observed previously [1,5]. This demonstrates that the hydrogen-bonding properties of the uncharged modifiers can sometimes be crucial for the chiral recognition of a certain solute.

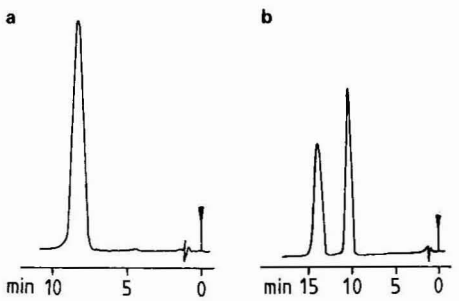


Fig. 10. Resolution of the enantiomers of pindolol. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (0.01 *M* phosphate), (a) pH 7.4 containing 5% 1-propanol and (b) pH 7.3 containing 10% acetonitrile; flow-rate, 0.9 ml/min.

The retention and the enantioselectivity obtained for alprenolol are higher than those for oxprenolol, independently of the mobile phase modifier used (see Table II). However, when the solutes were chromatographed on the AGP column as oxazolidone derivatives both the capacity factors and separation factors were higher for oxprenolol [20]. Chromatography of alprenolol and oxprenolol using a mobile phase of phosphate buffer (pH 7.2) without an uncharged organic modifier gives a very high retention for these solutes. In order to decrease the retention, uncharged modifiers must be added to the mobile phase. However, the enantioselectivity also decreases with increasing concentration and hydrophobicity of the added modifiers. Ethanol and acetonitrile can be recommended as mobile phase additives for the resolution of alprenolol and oxprenolol. Much higher concentrations of the most hydrophilic modifiers, acetonitrile and methanol, can be used with maintained enantioselectivity, but such data are not included here. For example, alprenolol was separated with a separation factor of 1.25 using a mobile phase containing 7.61 *M* (40%, v/v) acetonitrile [6].

Propranolol is a relatively hydrophobic solute and the retention on the CHIRAL-AGP column is therefore high. In Table II it is demonstrated that the best chiral selectivity for propranolol is obtained using 2-propanol as mobile phase additive.

CONCLUSIONS

Fluorescence studies have demonstrated that the immobilized form of AGP has a more unfolded structure than native AGP. However, despite this, there are large similarities between native and immobilized AGP concerning chiral recognition. The chromatographic studies using (−)-terodiline in the mobile phase suggest that chiral solutes of different character, such as amines, acids and non-protolytic compounds are bound to the same sites on the protein. Adsorption experiments with (−)-terodiline indicate that one high-affinity site and at least one more site with lower affinity are involved in the binding of the solutes.

Six β-receptor blocking agents were separated on the CHIRAL-AGP column with a separation factor between 1.2 and 1.8 using a phosphate buffer (pH 7) with or without uncharged modifiers as the mobile phase. It was observed that the hydrogen-bonding properties of the modifier to a large extent affected the enantioselectivity.

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High-performance liquid chromatography of casein hydrolysates phosphorylated and dephosphorylated

I. Peptide mapping

LISE LEMIEUX and JEAN AMIOT*

Centre de Recherche STELA, Département de Sciences et Technologie des Aliments, Université Laval, Sainte-Foy, Québec G1K 7P4 (Canada)

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ABSTRACT

A mixture of small peptides of molecular weight averaging 1000 daltons, obtained by controlled hydrolysis of casein with proteases, chymotrypsin and trypsin, was separated by size-exclusion and reversed-phase high-performance liquid chromatography. Peptides were identified and located in the known casein structures from their amino acid content and their N- and C-terminal amino acid analyses. The primary structure of peptides identified from casein hydrolysate phosphorylated and casein hydrolysate dephosphorylated is presented.

INTRODUCTION

Milk is the natural food for all newborn mammals, including humans, and it is a good source of protein and various nutrients. Caseins, accounting for about 80% of milk proteins [1], are phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-, which are present in the micelles in the proportions 38, 11, 38 and 13%, respectively [2]. One approach to obtain casein hydrolysates is by *in vitro* digestion using multiple enzymes [3].

Casein digestion in an enzymic membrane reactor has been proposed as a means of producing food ingredients. This product has been shown to have a nutritive content similar to that found in the intestine after digestion of milk proteins [4]. As *in vitro* hydrolysis of casein simulates gastric and pancreatic digestion, the hydrolysates are better absorbed than a mixture of free amino acids [5], which may be due to the size and nature of the peptides produced during the digestive process.

Phosphorylated or dephosphorylated casein hydrolysates have been used as nutritional food ingredients for people suffering from severe gastrointestinal diseases [6,7] as they have favourable proportions of small peptides and amino acids.

Some of the peptides obtained after the hydrolysis of milk proteins have been reported to have physiological and biological functions [8]. These peptidic fragments and others can be identified and located in the phosphoproteins, as the amino acid sequences of these proteins have been completely elucidated [9–12]. If the amino acid

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sequences of the peptides are known, it would be possible to synthesize peptides with dietetic and pharmaceutical properties.

Casein hydrolysates obtained by proteases, trypsin and chymotrypsin contain more than 200 peptides of different sizes. It has been proposed that these peptides could be separated by a size-exclusion high-performance liquid chromatography (HPSEC) method [13] according to their hydrodynamic volume. This study was conducted to verify if peptides obtained from casein hydrolysates phosphorylated and dephosphorylated could be efficiently separated, according to their size, by the HPSEC method. Further separations of peptides and amino acids, including C- and N-terminal amino acids, were performed by reversed-phase high-performance liquid chromatography (RP-HPLC). The identification and location of the peptides in the known casein structures were determined.

EXPERIMENTAL

Materials

Commercial casein hydrolysates phosphorylated and dephosphorylated (CHPS and CHDS) were provided by Laboratoire Sopharga (France). In the abbreviations used we have included the letter "S" to denote that it is a gift from Sopharga. Casein hydrolysates were prepared according to the procedure developed by Maubois and Brulé [14], which involves a controlled hydrolysis of casein by proteases, chymotrypsin and trypsin in a continuous-flow membrane enzymatic reactor. The molecular weight distribution profile of the hydrolysates were as follows: >5000 daltons, 3%; 1000–5000 daltons, 28%; and <1000 daltons, 69%, which includes 8% of free amino acids [4]. Isolation of phosphoserine residues from peptidic hydrolysates was possible after aggregation in the presence of added calcium and phosphate ions; phosphorylated peptides, which formed aggregates in this solution, could not pass through the ultrafiltration membrane (CHPS) whereas non-phosphorylated peptides could do so (CHDS).

Sequanal-grade triethylamine (TEA), trifluoroacetic acid (TFA), phenyl isothiocyanate (PITC) and amino acid standard mixture H were obtained from Pierce (U.S.A.). Absolute ethanol, sodium acetate trihydrate and hydrochloric acid (R.P. Normapur) were supplied by Prolabo (Paris, France). Acetonitrile "Baker Analyzed" reagent for chromatography was purchased from J. T. Baker (Deventer, The Netherlands). HPLC-grade acetic acid, ammonia (Suprapur) and 2-propanol (LiChrosolv) were obtained from Merck (Socolab, France). Doubly distilled water was purified by passing it through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Size-exclusion HPLC

Primary separation of CHPS and CHDS was performed by HPSEC on a TSK G2000 SW column (600 × 7.5 mm I.D.) with a guard column (60 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan) according to the method of Vijayalakshmi *et al.* [13] for protein hydrolysates. An LKB HPLC system equipped with a Model 2150 pump, a Model 2152 controller, a Model 2151 variable-wavelength monitor, a Rheodyne M 7010 sample injection valve with a 20-μl loop, and a Model 3390A integrator (Hewlett-Packard) was used. The mobile phase consisted of 0.1% TFA, 0.05

M phosphate buffer (pH 5.0) and 35% methanol. The system was run isocratically at a flow-rate of 0.75 ml/min at constant temperature. Polypeptides were monitored at 214 nm with an absorbance scale of 0.05. The mobile phase was filtered through a 0.45-μm filter (Millipore) and sonicated before use.

Reversed-phase HPLC

Fractions obtained by HPSEC were rechromatographed to separate the peptides on a Waters μBondapak C_{18} (10 μm) reversed-phase column (300 × 3.9 mm I.D.) according to the method of Yvon [3] for casein hydrolysates. A Waters Assoc. HPLC system equipped with two Model F-6000A pumps, a Model M 720 solvent programmer, a WISP automated sample injector, a Model M 441 fixed-wavelength detector (214 nm) and a Model M 730 two-channel chart recorder was used. The column was maintained at 40°C in a water-bath. After equilibration of the column with 0.115% TFA (solvent A) at a flow-rate of 2 ml/min, peptides were eluted by linearly increasing the concentration of solvent B [60% (v/v) acetonitrile in 0.1% TFA] as follows: 0–24 min, 0–48% B; 24–25 min, 48–100% B; 25–25.5 min, 100–0% B). The time required for one complete run, including the equilibration time, was 33 min. Prior to use, the mobile phases were degassed with helium. Peptides were monitored at 214 nm with an absorbance scale of 0.1. The fractions obtained from the RP-HPLC column were evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY, U.S.A.).

Peptides that coeluted were isolated by utilizing a second solvent system: (A) 25 m*M* ammonium acetate (pH 6.0) and (B) 60% (v/v) acetonitrile in 50 m*M* ammonium acetate (pH 6.0); all other conditions were the same as for RP-HPLC. The absorbance scale was increased to 0.2 and the flow-rate was decreased to 1 ml/min.

Peptide identification

Peptides were hydrolysed with 5.7 *M* triply distilled hydrochloric acid in evacuated, sealed tubes for 24 h at 110°C. The amino acid analyses were then performed on a Waters Pico-Tag amino acid analysis system [15] according to the manufacturer's instructions. Prior to hydrolysis, Pyrex tubes were heated at 500°C for 16 h to eliminate any contamination.

The identity of each peptide was established by comparison of its amino acid composition with that of α_{s1}-, α_{s2}-, β- and κ-caseins using Petrilli's program [16] on an Apple IIE computer, and confirmed by N- and C-terminal analyses following the methods of Tarr [17] and Ribadeau-Dumas [18], respectively.

The identification of phenylthiocarbamyl (PTC) and phenylthiohydantoin (PTH) derivatives was carried out on an LKB HPLC system using a Pico-Tag column (Waters). For PTC derivatives, 1 m*M* EDTA (Fisher Scientific) was added to the Waters Pico-Tag eluent A and the gradient was modified by increasing the concentration of the Waters Pico-Tag eluent B as follows: 0–4 min, 0–30% B; 4–12 min, 30–45% B; 12–12.5 min, 45–100% B; 14–15 min, 100–0% B. Injections were performed every 21 min. When PTC derivatization of a blank hydrochloric acid was performed, values of about 10 pmol were obtained for serine and glycine. This "background" effect was previously reported by Stone and Williams [19]. These blank values were subtracted from the amino acid analysis of the samples. The identification of PTH derivatives [20] was performed by using solvent A [35 m*M* sodium acetate (pH

5.0) (500 ml)–acetonitrile (100 ml)] and by increasing the concentration of solvent B (2-propanol, 60%) in the following manner: 0–3.5 min, 0–2% B; 3.5–6.0 min, 2–36% B; 6.0–7.0 min, 36–40% B; 10.5–10.7 min, 40–60% B; 11.0–11.5 min, 60–0% B. The time required for the analysis and equilibration was 20 min. In each instance the flow-rate was kept constant at 1.0 ml/min and norleucine was used as an internal standard.

RESULTS

According to the HPSEC elution conditions described above, 40 μg of hydrolysate could be injected per run. Fig. 1A and B represent the chromatographic patterns of casein digests CHDS and CHPS, respectively. Forty-five injections were performed for each hydrolysate of CHDS and CHPS; various fractions, as indicated in the Fig. 1A and B, were collected manually and pooled. After evaporation under nitrogen, the fractions were freeze-dried. These were solubilized in 0.115% TFA and injected onto a μBondapak C_{18} reversed-phase column. As an example, the RP-HPLC

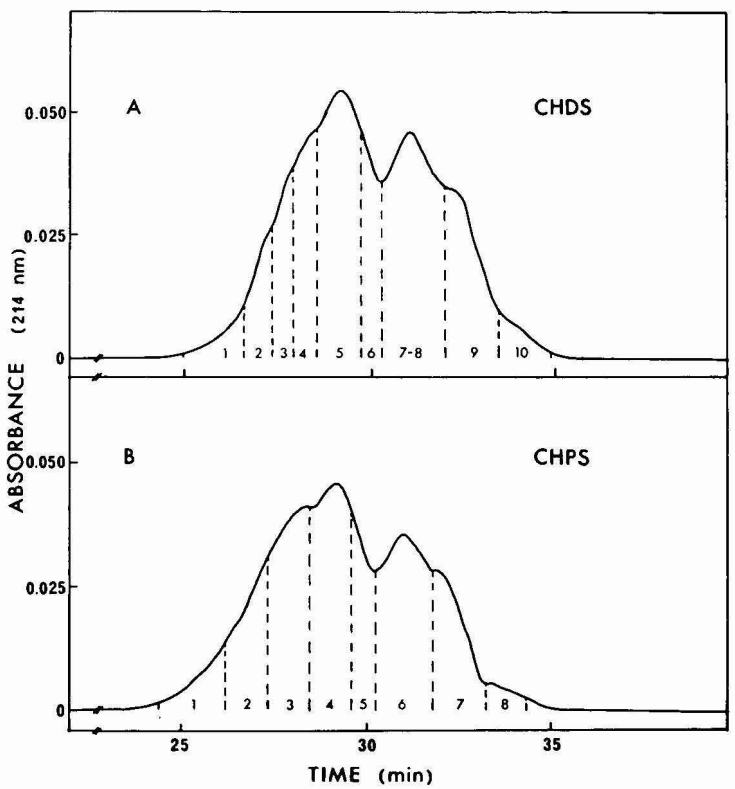


Fig. 1. Elution profiles of tryptic and chymotryptic digests of casein on a TSK G2000SW column. Each hydrolysate (2 mg) was dissolved in 1 ml of mobile phase [0.1% TFA–0.05 *M* phosphate buffer (pH 5.0)–35% methanol]. Injection volume, 20 μl. (A) CHDS (casein hydrolysate dephosphorylated); (B) CHPS (casein hydrolysate phosphorylated). 1–10, Fractions collected manually.

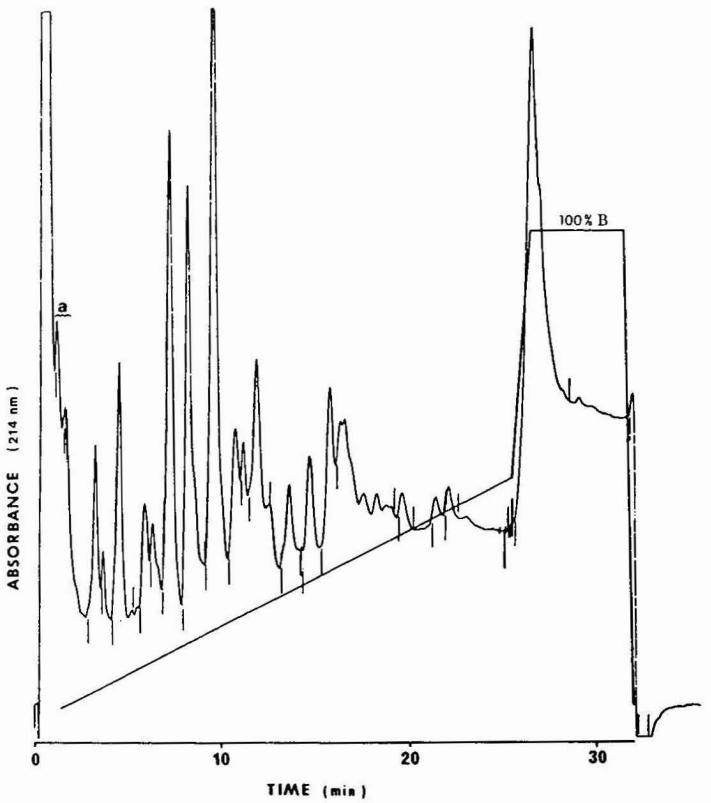


Fig. 2. Reversed-phase peptide mapping on a μBondapak C_{18} column of fraction CHPS-5. Each fraction from HPSEC was diluted with 500 μl of solvent A and filtered through an HV 0.45-μm filter (Millipore). The injection volume was 100 μl. Chromatography was performed as described under Experimental.

elution profile for fraction 5 obtained from the CHPS hydrolysate by HPSEC is shown in Fig. 2; the first peak which eluted in the isocratic mode contained salts from the phosphate buffer used in the HPSEC system, however, it was found to be devoid of peptides on further analysis. We observed that the gradient mode was effective only at 2.5 min. Four injections of the same product were performed and individual fractions were collected in separate test-tubes and evaluated for purity on the basis of symmetry and narrowness of the peak. Large peaks or those having a shoulder were further isolated by using a second RP-HPLC step as described. As the second RP-HPLC step was able, for example, to separate peak a obtained from the first RP-HPLC step (Fig. 2) into five peptides (Fig. 3), it confirms that the second solvent system was necessary for complete separation.

Seventy fractions collected from the first RP-HPLC step which were not pure were rechromatographed with a second RP-HPLC step before amino acid analysis. A picomole amino acid analysis of the peptide κ: 98–102, from fraction CHDS-4, as an example, was performed after converting its amino acids into their PTC derivatives (Fig. 4). The amino acid analysis and C- and N-terminal residue analyses were also performed for 223 pure peptides isolated from the first RP-HPLC step.

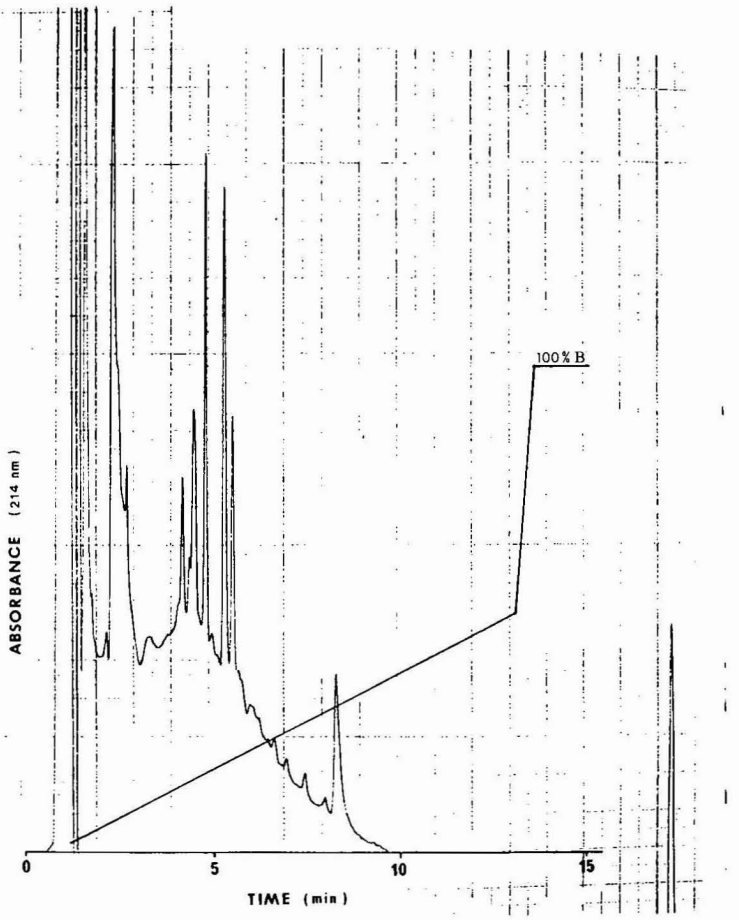


Fig. 3. Elution profile of peak a from Fig. 2 (CHPS-5), obtained as described under Experimental on a Waters μBondapak C_{18} reversed-phase column.

The identified sequences of peptides isolated from each HPSEC fraction of both casein hydrolysates are presented in Tables I and II. The sequences of α_{s1}-, α_{s2}-, β- and κ-caseins [21–23] are shown in Figs. 5 and 6. The underlined fragments indicate where the identified peptides are located in caseins and the numbers indicate in which fraction the peptides were eluted in the HPSEC for CHPS and CHDS.

Casein fragments obtained from CHDS and CHPS contained 1–24 amino acid residues with molecular weights ranging from 132 to 2600 daltons. Peptide molar composition values (Tables I and II) were determined by Pico-Tag amino acid analyses. The amounts injected onto the Pico-Tag column were determined from the peptide molar composition values and found to be 500 and 800 pmol for CHPS and CHDS, respectively (Tables I and II). As trypsin was used for hydrolysis, small peptides obtained by this method should contain only one arginine or lysine. If the peptides were devoid of arginine or lysine, these peptides would have come from the

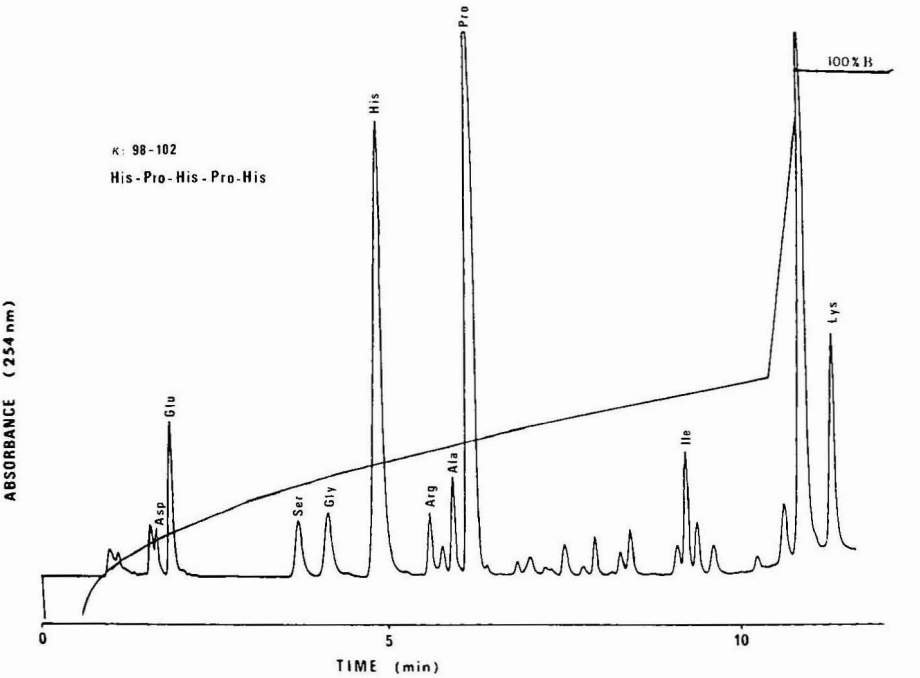


Fig. 4. Picomole chromatography of PTC-amino acids for the peptide κ: 98–102 (His–Pro–His–Pro–His). Peptide amino acids were converted into their PTC derivatives and then separated by RP-HPLC on a Pico-Tag column according to the manufacturer's instructions.

TABLE I

IDENTIFICATION OF PEPTIDES FROM A DEPHOSPHORYLATED CASEIN HYDROLYSATE (CHDS) ISOLATED BY SIZE-EXCLUSION HPLC FOLLOWED BY REVERSED-PHASE HPLC

The single-letter code for amino acids is used.

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
1			N
			G
2			N
	β: 53-68	0.20	AQTQSLVYPFPGPIPN
3			N
	κ: 98–102	0.20	HPHPH
	α_{s1}: 125–132	0.50	EGIHAQQK
	α_{s1}: 106–119	0.20	VPQLEIVPNSAEER
	α_{s1}: 174–193	0.60	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	0.30	HQGLPQEVLNENLLR
	β: 144–163	0.40	MHQPHQPLPPTVMFPPQSVL

(*Continued on p. 306*)

TABLE I (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
4			Y
			K
	α_{s1}: 35–36[c]	0.25	EK
	α_{s1}: 4–7	2.00	HPIK
	κ: 98–102	0.70	HPHPH
	α_{s1}: 125–132	1.80	EGIHAQQK
	β: 89–97	0.20	QPEVMGVSK
	α_{s1}: 106–119	0.50	VPQLEIVPNSAEER
	α_{s1}: 174–193	1.50	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	0.40	HQGLPQEVLNENLLR
	β: 49–68	0.20	IHPFAQTQSLVYPFPGPIPN
5	α_{s1}: 35–36	1.10	EK
	β: 30–32[c]	0.80	IQK
	α_{s1}: 100–102[c]	0.50	RLK
	β: 94–97[c]	2.00	GVSK
	α_{s1}: 55–58[c]	1.30	EDIK
	κ: 62–63[c]	1.60	AK
	κ: 64–65[c]	1.60	PA
	α_{s2}: 42–45[c]	0.50	EVVR
	β: 164–169[c]	1.40	SLSQSK
	β: 100–105	3.00	EAMAPK
	α_{s1}: 37–42	2.00	VNELSK
	α_{s1}: 125–132[c]	0.40	EGIHAQQK
	α_{s1}: 84–90	3.00	EDVPSER
	β: 108–113	2.00	EMPFPK
	β: 177–183[c]	3.00	AVPYPQR
	β: 170–176	2.00	VLPVPQK
	β: 33–48	0.80	FQSEEQQQTEDELQDK
	α_{s2}: 138–150	0.40	TVDMESTEVFTKK
	α_{s2}: 81–89	0.40	ALNEINQFY
	β: 194–202	1.00	QEPVLGPVR
	β: 193–202[c]	1.00	YQEPVLGPVR
	α_{s1}: 106–119	1.00	VPQLEIVPNSAEER
	β: 134–139	1.50	HLPLPL
6	α_{s2}: 110–113	0.60	DQVK
	α_{s2}: 180–181[c]	0.70	LK
	β: 30–32[c]	0.70	IEK
	α_{s2}: 171–173[c]	0.60	YQK
	κ: 62–63[c]	0.80	AK
	κ: 64–65[c]	0.50	PA
	α_{s2}: 42–45[c]	0.40	EVVR
	α_{s2}: 167–170[c]	0.50	ISQR
	β: 164–169[c]	0.50	SLSQSK
	α_{s2}: 194–197[c]	0.30	IQPK
	β: 100–105	0.35	EAMAPK
	α_{s1}: 37–42	1.00	VNELSK
	α_{s1}: 84–90	2.00	EDVPSER
	κ: 1–10	0.70	EEQNQEQPIR
	κ: 17–21[c]	0.17	FFSDK
	α_{s2}: 162–165[c]	0.30	NFLK

TABLE I (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
	β: 108–113	3.00	EMPFPK
	β: 177–183[c]	2.00	AVPYPQR
	β: 49–52	2.00	IHPF
	β: 170–176	0.30	VLPVPQK
	β: 33–48	1.00	FQSEEQQQTEDELQDK
	β: 114–123	0.25	YPVEPFTESQ
	β: 194–202[c]	1.00	QEPVLGPVR
	β: 193–202[c]	0.20	YQEPVLGPVR
	α_{s1}: 106–119	0.60	VPQLEIVPNSAEER
	β: 134–139	0.70	HLPLPL
7–8	α_{s2}: 171–173	0.30	YQK
	α_{s1}: 170–173[c]	0.40	GTQY
	α_{s2}: 182–188	0.50	TVYQHQK
	α_{s1}: 157–159	0.30	DAY
	β: 120–125[c]	1.00	TESQSL
	β: 53–58[c]	0.50	AQTQSL
	α_{s1}: 91–92[c]	1.20	YL
	κ: 1–10	0.35	EEQNQEQPIR
	κ: 39–42[c]	0.30	GLNY
	β: 193–198[c]	0.50	YQEPVL
	α_{s2}: 174–181[c]	0.20	FALPQYLK
	κ: 17–21[c]	0.20	FFSDK
	κ: 35–38	0.20	YPSY
	β: 108–113[c]	0.80	EMPFPK
	β: 177–183[c]	0.21	AVPYPQR
	β: 191–193[c]	0.55	LLY
	α_{s2}: 200–207	1.00	VIPYVRYL
	α_{s2}: 138–149	0.75	TVDMESTEVFTK
	α_{s2}: 101–109	0.40	QGPIVLNPW
	β: 184–190	4.00	DMPIQAF
	α_{s1}: 166–173[c]	1.30	YVPLGTQY
	α_{s1}: 133–142	0.20	EPMIGVNQEL
	α_{s1}: 133–144[c]	0.70	EPMIGVNQELAY
	β: 194–202[c]	0.60	QEPVLGPVR
	β: 114–119[c]	3.50	YPVEPF
	α_{s1}: 92–98[c]	0.20	LGYLEQL
	α_{s1}: 154–159[c]	0.15	YQLDAY
	α_{s1}: 166–173[c]	0.20	YVPLGTQY
	κ: 25–30[c]	0.50	YIPIQY
	α_{s1}: 133–144[c]	0.24	EPMIGVNQELAY
	β: 184–190	1.00	DMPIQAF
	α_{s2}: 92–96[c]	0.50	FPQYL
	β: 59–68	0.20	VYPFPGPIPN
	α_{s1}: 25–32	0.60	VAPFPQVF
	β: 203–209	0.60	GPFPIIV
9			Y F
	α_{s1}: 93–94[c]	0.20	GY
	α_{s2}: 206–207	0.85	YL
	α_{s1}: 92–94	0.40	LGY

(*Continued on p. 308*)

TABLE I (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
	κ: 103–105	0.30	LSF
	β: 120–127	0.50	TESQSLTL
	α_{s1}: 146–149	0.15	YPEL
	α_{s1}: 152–154[c]	3.00	QFY
	β: 191–193[c]	0.50	LLY
	α_{s1}: 23–24[c]	2.00	FF
	κ: 17–18	2.00	FF
	β: 184–191	0.40	DMPIQAFL
	α_{s2}: 100–106	0.18	YQGPIVL
	β: 114–119	0.32	YPVEPF
	β: 184–190	0.35	DMPIQAF
	α_{s2}: 92–96[c]	0.60	FPQYL
	α_{s2}: 7–20[c]	0.25	VSSSEESIISQETY
	α_{s1}: 145–150	0.12	FYPELF
10			Y
			F
	α_{s1}: 152–154	0.50	QFY
	α_{s1}: 23–24	0.50	FF

[a] Fig. 1A.
[b] Peptide molar composition determined with an injection ion volume of 10 μl.
[c] Peptides identified from the second RP system.

TABLE II

IDENTIFICATION OF PEPTIDES FROM A PHOSPHORYLATED CASEIN HYDROLYSATE (CHPS) ISOLATED BY SIZE-EXCLUSION HPLC FOLLOWED BY REVERSED-PHASE HPLC

The single-letter code for amino acids is used.

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
1	Nothing		
2	κ: 41–42	0.40	NY
	β: 146–156	0.12	QPHQPLPPTVM
	β: 144–163	0.17	MHQPHQPLPPTVMFPPQSVL
3	α_{s1}: 125–132	0.70	EGIHAQQK
	α_{s2}: 126–137	0.20	EQLSTSEENSKK
	β: 89–97	0.20	QPEVMGVSK
	κ: 61–68	0.15	YAKPAAVR
	α_{s1}: 80–90	0.20	HIQKEDVPSER
	α_{s2}: 200–205	0.25	VIPYVR
	α_{s2}: 115–125	0.15	NAVPITPTLNR
	α_{s1}: 106–119	0.25	VPQLEIVPNSAEER
	α_{s1}: 174–193	1.20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	0.42	HQGLPQEVLNENLLR
	β: 49–68	0.08	IHPFAQTQSLVYPFPGPIPN
	β: 144–163	0.65	MHQPHQPLPPTVMFPPQSVL

TABLE II (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
4	α_{s1}: 1–3	0.80	RPK
	α_{s1}: 55–58[c]	0.80	EDIK
	β: 29–32[c]	0.80	KIEK
	κ: 62–65[c]	0.50	AKPA
	α_{s2}: 42–45[c]	0.30	EVVR
	β: 164–169[c]	1.60	SLSQSK
	α_{s2}: 77–80[c]	0.40	HYQK
	α_{s1}: 80–83[c]	0.40	HIQK
	β: 100–105	3.00	EAMAPK
	κ: 80–86[c]	1.50	SNTVPAK
	α_{s1}: 125–132[c]	1.00	EGIHAQQK
	α_{s1}: 120–124[c]	0.90	LHSMK
	β: 100–105	0.50	EAMAPK
	α_{s1}: 37–42	0.50	VNELSK
	β: 89–97	0.70	QPEVMGVSK
	β: 177–183	1.40	AVPYPQR
	β: 170–176	1.00	VLPVPQK
	β: 33–48	0.50	FQSEEQQQTEDELQDK
	α_{s2}: 138–150[c]	0.50	TVDMESTEVFTKK
	β: 194–202	0.50	QEPVLGPVR
	α_{s2}: 81–89[c]	0.50	ALNEINQFY
	α_{s2}: 115–125	0.50	NAVPITPTLNR
	α_{s1}: 106–119	1.00	VPQLEIVPNSAEER
	α_{s1}: 105–119[c]	0.25	KVPQLEIVPNSAEER
	α_{s1}: 104–119[c]	0.10	YKVPQLEIVPNSAEER
	β: 192–202[c]	0.35	LYQEPVLGPVR
	α_{s1}: 174–193	0.50	TDAPSFSDIPNPIGSENSEK
	β: 33–52	0.50	FQSEEQQQTEDELQDKIHPF
	α_{s1}: 170–193[c]	0.20	GTQYTDAPSFSDIPNPIGSENSEK
	α_{s1}: 25–34[c]	0.40	VAPFPQVFGK
5	β: 98–99	0.25	VK
	α_{s2}: 204–205[c]	0.40	VR
	α_{s2}: 171–173[c]	0.50	YQK
	β: 94–97	0.30	GVSK
	α_{s2}: 42–45[c]	0.90	EVVR
	β: 164–169[c]	1.30	SLSQSK
	α_{s2}: 194–197[c]	0.50	IQPK
	β: 100–105	0.30	EAMAPK
	α_{s1}: 37–42	0.20	VNELSK
	β: 89–97	0.50	QPEVMGVSK
	β: 177–183	0.60	AVPYPQR
	β: 49–52	0.30	IHPF
	β: 108–113	1.00	EMPFPK
	α_{s1}: 146–154	0.40	YPELFRQFY
	α_{s1}: 106–119	0.30	VPELEIVPNSAEER
	α_{s2}: 100–109	0.20	YQGPIVLNPW
	β: 134–139	0.15	HLPLPL
	α_{s1}: 25–34	0.20	VAPFPEVFGK
6	α_{s2}: 171–173	0.17	YQK
	β: 126–127	0.40	TL

(*Continued on p. 310*)

TABLE II (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
	α_{s2}: 183–184[c]	0.40	VY
	α_{s1}: 91–92	0.60	YL
	κ: 17–21	0.23	FFSDK
	β: 193–198[c]	0.20	YQEPVL
	β: 177–183	0.10	AVPYPQR
	α_{s2}: 200–207	0.20	VIPYVRYL
	β: 191–193[c]	0.70	LLY
	β: 49–52[c]	0.92	IHPF
	κ: 44–50	0.13	QQKPVAL
	α_{s1}: 146–150[c]	0.15	YPELF
	α_{s2}: 142–147[c]	0.30	ESTEVF
	α_{s2}: 100–106[c]	0.30	YQGPIVL
	β: 184–190	0.40	DMPIQAF
	α_{s1}: 166–173[c]	1.50	YVPLGTQY
	β: 114–119	1.00	YPVEPF
	β: 184–190	1.00	DMPIQAF
	α_{s1}: 133–144	0.30	EPMIGVNQELAY
	α_{s1}: 152–164	0.20	QFYQLDAYPSGAW
	α_{s2}: 97–109	0.20	QYLYQGPIVLNPW
	α_{s1}: 25–32	0.25	VAPFPQVF
	β: 203–209	0.50	GPFPIIV
7			F
			Y
	α_{s2}: 206–207	0.50	YL
	α_{s2}: 99–100	0.60	LY
	α_{s1}: 152–154[c]	0.40	QFY
	β: 184–190	0.30	DMPIQAF
	β: 114–119[c]	0.80	YPVEPF
	α_{s1}: 166–173[c]	0.20	YVPLGTQY
	κ: 26–30[c]	0.16	IPIQY
	β: 184–190	0.90	DMPIQAF
	α_{s2}: 92–96[c]	0.40	FPQYL
	α_{s1}: 145–150	0.15	FYPELF
8			N
	α_{s1}: 23–24	0.15	FF
	α_{s1}: 152–154	0.30	EFY

[a] Fig. 1B.
[b] Peptide molar composition determined from an injection volume of 10 μl.
[c] Peptides identified from the second RP system.

action of chymotrypsin and the calculation was then based on the values obtained for aspartic acid, glutamic acid, proline and alanine residues.

According to the peptide molar composition values we found that some of the peptides were contaminated (data not shown). This contamination could be explained by the so-called "memory effect" [24], in which remaining hydrophobic peptides bound to the gel could elute after successive runs.

DISCUSSION

We were able to isolate and identify 213 and 187 peptides from less than 2 mg of CHDS and CHPS, respectively. Although the elution profiles of casein digests from HPSEC were almost the same, most of the identified peptides came from fraction 4 (550–840 daltons) of CHPS and fraction 7–8 (210–410 daltons) of CHDS and had comparable elution times in both hydrolysates. The identified peptides were found to originate from the four phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-caseins, in the proportions 35, 21, 34 and 9%, respectively.

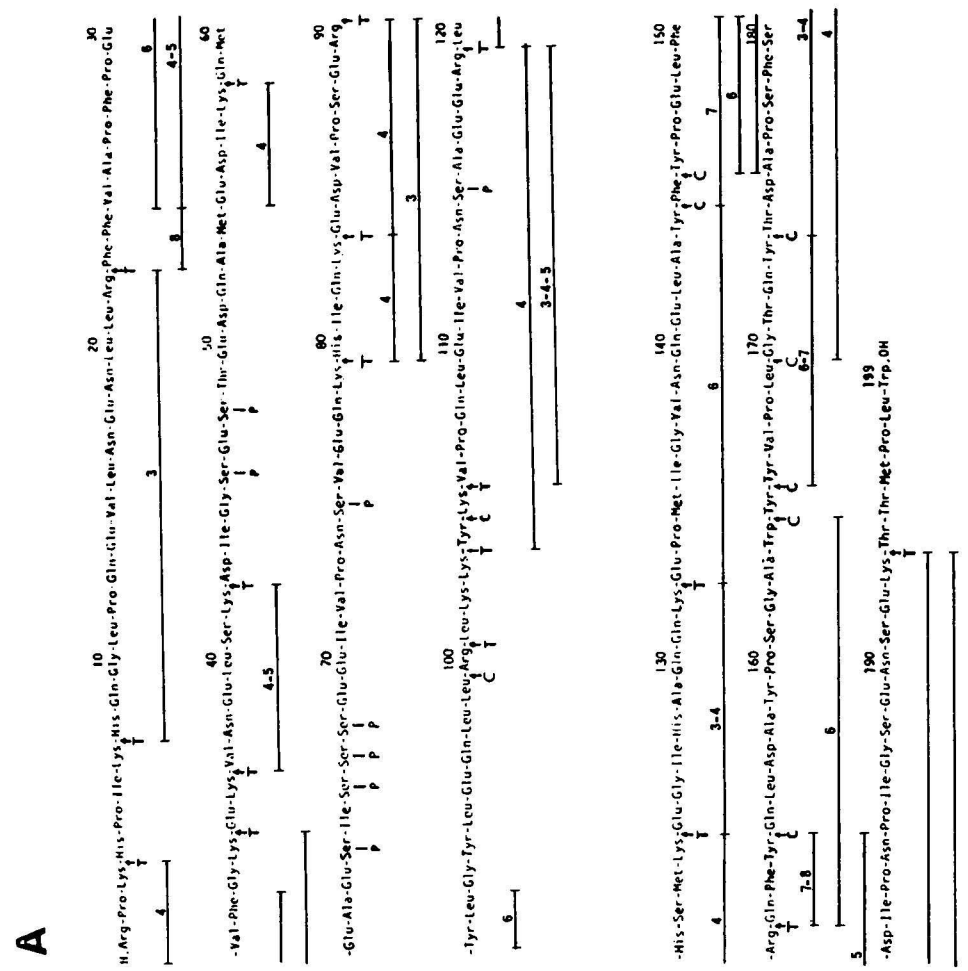
The peptides obtained from CHDS and CHPS had lysine, arginine, tyrosine, phenylalanine and valine as the C- and N-terminal amino acids as the enzymes used were endopeptidases, trypsin and chymotrypsin. Trypsin is highly specific for peptide bonds linking the carboxyl groups of two basic amino acids, lysine and arginine. However, chymotrypsin, although less specific than trypsin, has a preference for peptide bonds linking the carboxyl groups of the aromatic amino acids, phenylalanine, tryptophan and tyrosine. Keil [25] observed that chymotrypsin also cleaves proteins at the carboxylic side of other amino acids such as leucine, methionine and histidine at a slower rate. The cleavage sites of chymotrypsin and trypsin which are shown in Figs. 5 and 6 are those reported by Pelissier [26]. Although it is possible to have a number of cleavage sites for chymotrypsin [26], we observed only a few of them.

The elucidation of primary amino acid sequences of caseins was performed previously by conventional methods [9–12]. However, Stewart and co-workers [21,22] recently characterized bovine caseins at mRNA levels by cloning cDNAs. As this method provides accurate primary amino acid sequences of caseins, we used these sequences [21–23] to identify and locate the peptides isolated from CHDS and CHPS.

Peaks obtained from HPSEC using a TSK G2000SW column are not sharp and well separated (Fig. 1), as the resolution of the column is low. Hence identical peptides were eluted in two or more different fractions as given in Tables I and II. When fractions obtained from HPSEC were injected onto the reversed-phase column, these identical peptides had the same retention time (data not shown) but differed in concentration. If the HPSEC column had a better resolution, the fraction which contained the highest amount of the peptide would have had less contamination due to other peptides.

Peptide β: 184–190 was identified at two different elution times in the first RP-HPLC system; this could be explained by the oxidation of the methionine residue to a more polar sulphoxide or sulphone [27]; this was confirmed by performing PTC-amino acid analysis: methionine sulphoxide was eluted between arginine and threonine and methionine sulphone was eluted after proline.

Peptides with one (α_{s1}: 106–119; α_{s2}: 138–150; β: 33–48) or two serine residues (α_{s2}: 126–137) which were phosphorylated or dephosphorylated were found to have identical elution times in both casein digests. This finding does not agree with previous results obtained by Grego *et al.* [28], who observed longer retention times for corresponding dephosphorylated peptides. This result could be explained by the possible contamination of CHPS by dephosphorylated peptides [29]. The elution times of peptides with one or two dephosphorylated serines were lower than that of the peptide with four dephosphorylated serines (α_{s2}: 7–20) and with an intermediate hydrophobicity of 12.67 kcal/mol [30]. Thus, the elution of the peptide α_{s2}: 7–20 from


A
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-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-Ser-Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met
-Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Glu-Gln-Lys-His-Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg
-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-Ser-Ala-Glu-Glu-Arg-Leu
-His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe
-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser
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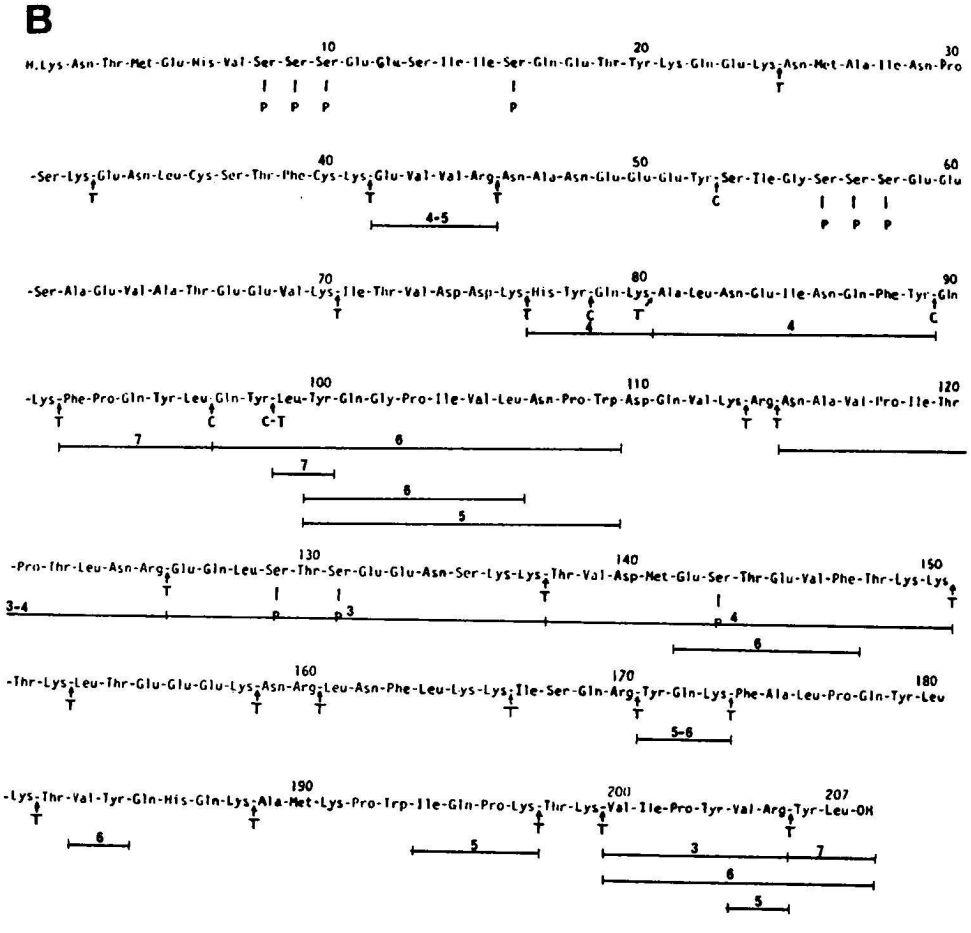
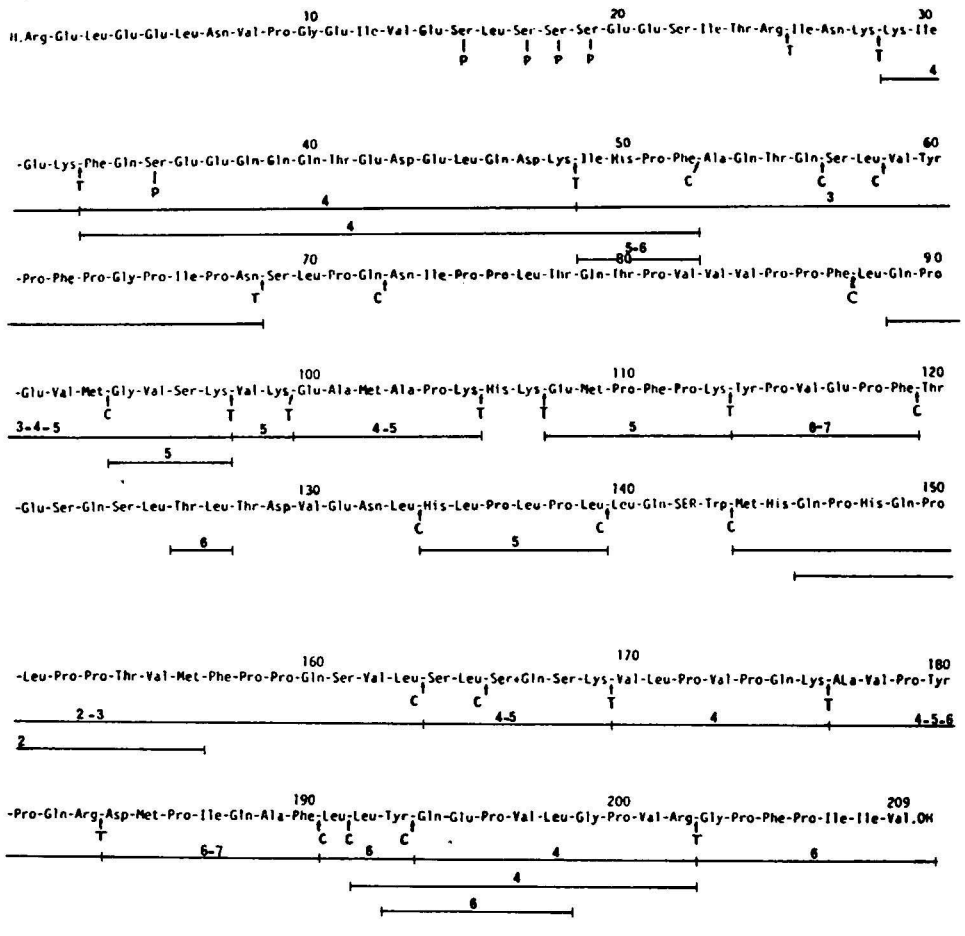


Fig. 5.

(*Continued on p. 314*)

C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70
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130
140
150
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160
170
180
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190
200
209
-Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val.OH

D

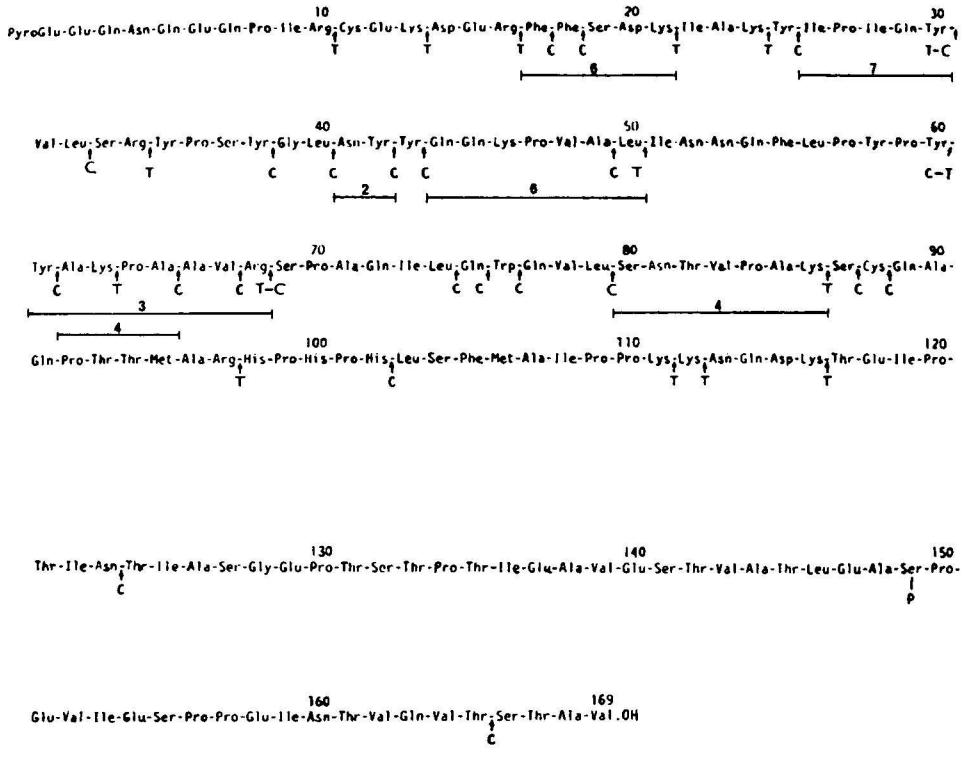
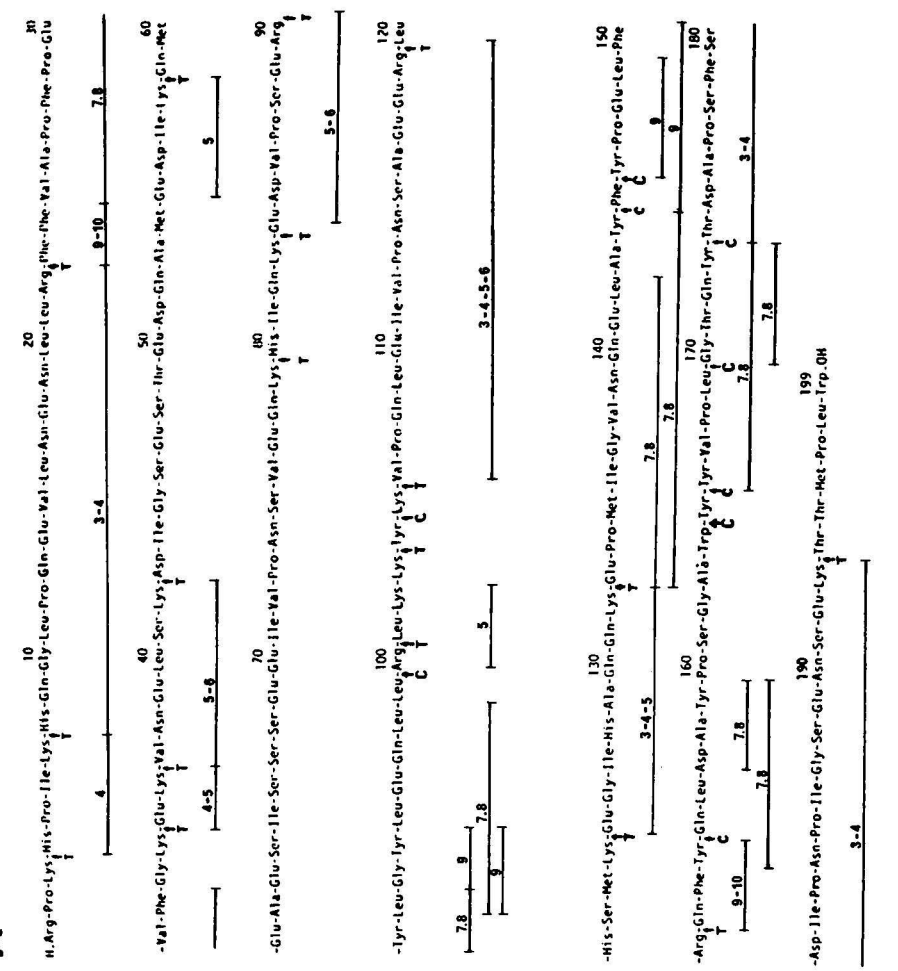


Fig. 5. (A) Underlined α_{s1}-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). (B) Underlined α_{s2}-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). (C) Underlined β-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). (D) Underlined κ-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). C and T: chymotrypsin and trypsin identified cleavage sites.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-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-Ser-Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met
-Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Glu-Gln-Lys-His-Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg
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-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser
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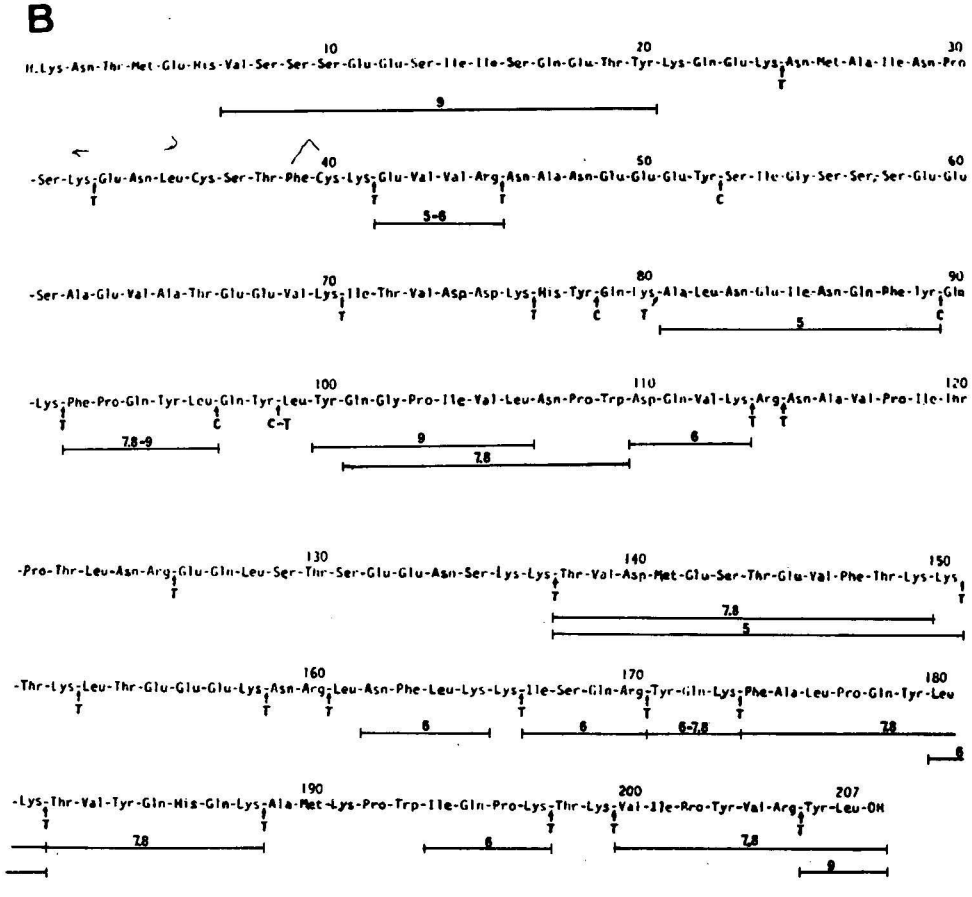

Fig. 6.

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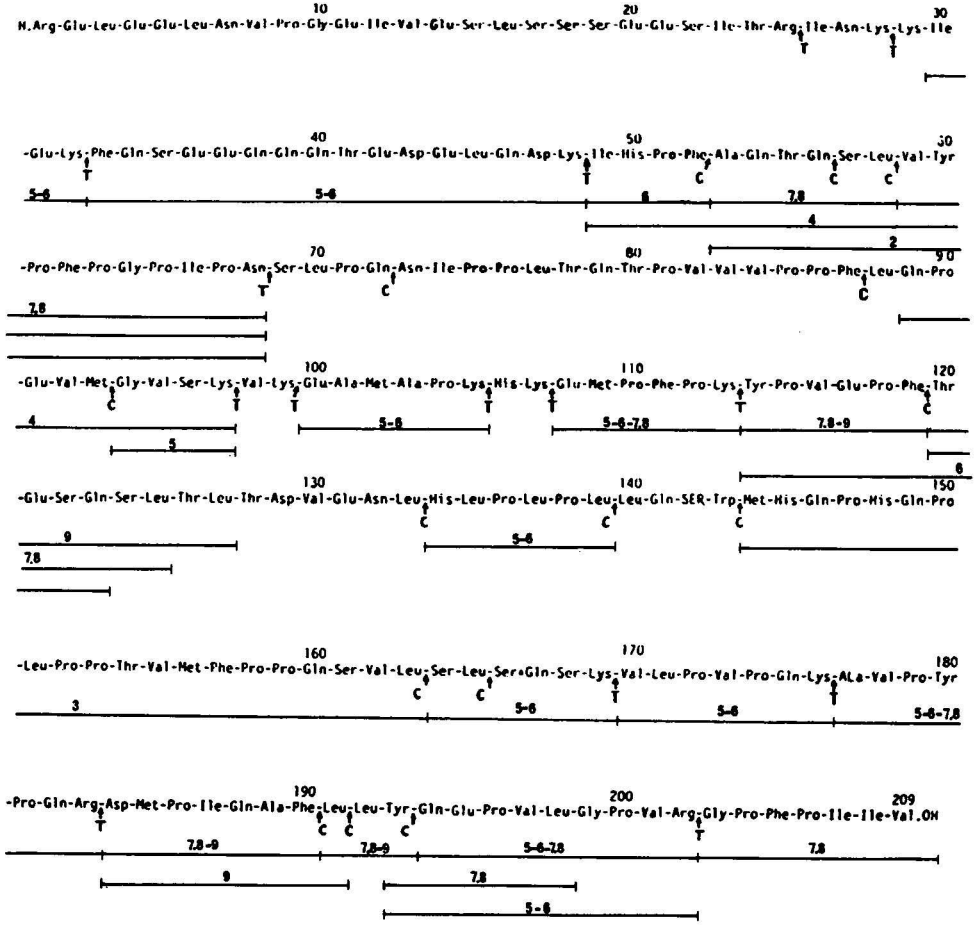
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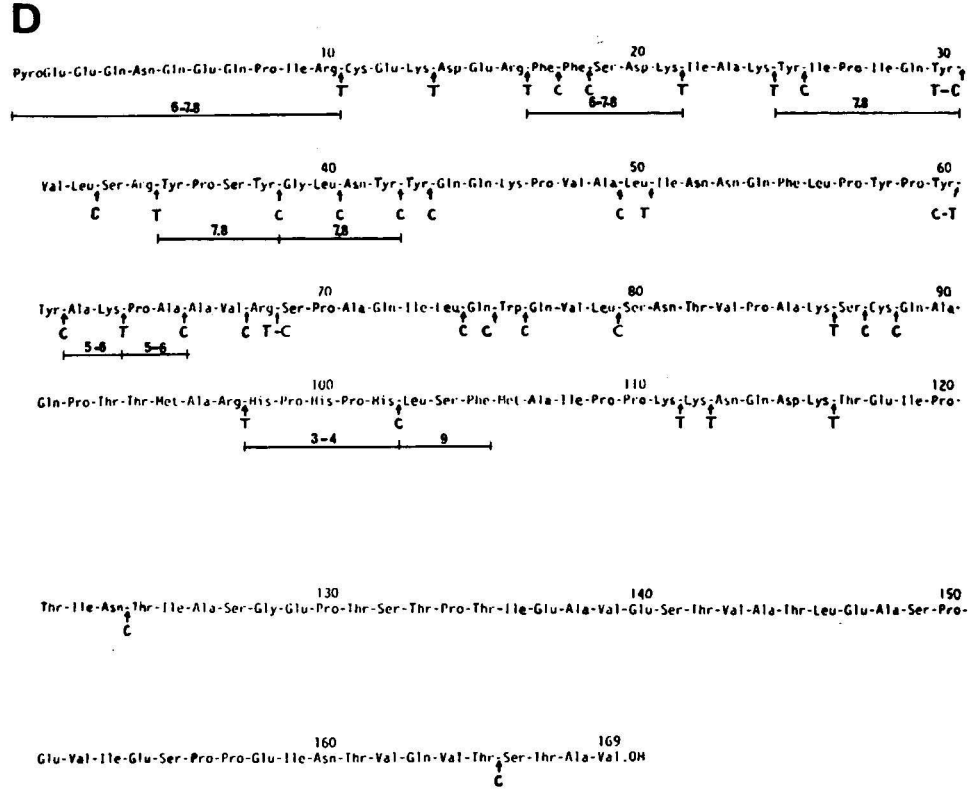

Fig. 6. (A) Underlined α_{s1}-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). (B) Underlined α_{s2}-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). (C) Underlined β-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). (D) Underlined κ-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). C and T: chymotrypsin and trypsin identified cleavage sites.

the reversed-phase column seems to be controlled not only by its hydrophobicity but also, as suggested by Juhl and Soderling [31], by its molecular weight.

The peptide, β: 203–209, isolated from CHDS and CHPS, was similar to the peptide found in bitter cheese. Pelissier *et al.* [32] have reported a number of bitter peptides originating from α_{s1}- and β-casein hydrolysates. However, we did not identify many of these bitter peptides in CHDS and CHPS, probably because of the conditions of the proteolysis.

Peptides such as β: 49–68 and β: 59–68 isolated from CHDS and CHPS may possess an immunostimulant property, since Maubois and Léonil [8] found that the peptide β: 63–68 was an immunomodulator. Other peptides have been associated with various biological functions [8] such as bioavailability of oligo-elements (β: 1–25); opioids (β: 60–66, β-casomorphin 7; α_{s1}: 90–96, α-casein exorphin), antithrombosis (κ: 106–116; κ: 106–112; κ: 113–116) and antihypertension (β: 177–183 or CEI B_7; α_{s1}: 23–34 or CEI_{12}). Among the peptides known to inhibit the angiotensin-converting enzyme, ACE (E.C. 3.14.15.1), CEI B_7 was found in both casein digests whereas CEI_{12} was identified only in CHPS. Further, peptide α_{s1}: 106–119 isolated from CHPS has previously been found to act as a mineral carrier [33] and peptide β: 191–193 from CHPS and CHDS to exert a stimulating function on the immune system [34].

Figs. 5 and 6 show that most of the peptides in the casein were identified. However, there are some missing links in the sequences. It is possible that some of the peptides produced in the enzymatic–ultrafiltration process may have been lost in the ultrafiltration step owing to some steric effects or electrostatic interactions [35]. In addition, lack of material, significant loss of polypeptides at low concentrations and adsorption on plastic surfaces or glassware may be responsible for the missing links in the sequences. Further, irreversible bonding of peptides at low concentrations can occur on the stationary phase, as reported for phosphopeptides on a C_{18} column [36].

CONCLUSION

A combination of size-exclusion and reversed-phase HPLC methodologies was efficient for the separation of protein hydrolysates and identification by Pico-Tag amino acid analysis. These methods also led to the isolation of some biologically active casein fragments. Work is in progress to evaluate the accuracy of HPSEC for the separation of peptides from protein hydrolysates according to their molecular weight.

ACKNOWLEDGEMENTS

The authors are very grateful to Dr. B. Ribadeau-Dumas (INRA–France) for helpful discussions. Part of this work was supported by grants from le Ministère de l'Enseignement Supérieur et de la Science, a Quebec–France exchange program sponsored by le Ministère des Affaires Intergouvernementales du Québec and a research contract (PPIL program) from the National Research Council of Canada.

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Rapid determination of clenbuterol residues in urine by high-performance liquid chromatography with on-line automated sample processing using immunoaffinity chromatography

W. HAASNOOT*, M. E. PLOUM, R. J. A. PAULUSSEN, R. SCHILT and F. A. HUF

Department of Biopharmaceutical Analysis, State Institute for Quality Control of Agricultural Products (RIKILT), Bornsesteeg 45, 6708 PD Wageningen (The Netherlands)

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ABSTRACT

A liquid chromatographic column-switching system for automated sample pretreatment and determination of clenbuterol in calf urine, using an immunoaffinity precolumn with Sepharose-immobilized polyclonal antibodies against clenbuterol, is described. A second precolumn packed with C_{18}-bonded silica was used for the reconcentration of desorbed clenbuterol prior to the analytical separation. Urine, after 2-fold dilution with buffer (pH 7.4), was loaded directly onto the immuno precolumn, where clenbuterol was trapped by the immobilized antibodies. This immuno precolumn has been used for more than 200 runs with standard solutions and samples. Bound analyte was desorbed with 0.01 *M* acetic acid and transferred, via the second precolumn, to the analytical column. The total runtime per sample was 35 min. Using a sample load of 27 ml of dilute urine and UV detection at 244 nm, the detection limit was 0.5 ng/ml. The mean recovery of clenbuterol added to a blank urine sample at the 5 ng/ml level was 82 ± 2% (n = 5) as determined with standard solutions loaded onto the same system. Urine samples from treated animals were analysed and the clenbuterol concentrations were comparable to those obtained by high-performance liquid chromatography using solid-phase extraction for sample clean-up.

INTRODUCTION

Clenbuterol, one of the β_2-agonistic drugs used for the treatment of obstructions of the bronchial tubes [1], has been shown to increase the carcass weight of calves and to improve the carcass quality [1,2]. In The Netherlands, the therapeutic use of clenbuterol has been forbidden for calves older than 14 weeks since November 6th 1988 [3]. For control purposes, mostly urine samples are taken.

In urine samples from male veal calves (aged 12 and 16 weeks) treated orally with the recommended therapeutic dose of clenbuterol · HCl (0.8 μg per kg body weight) twice a day over a period of 2 weeks, the highest concentration of clenbuterol found was 75 ng/ml. Eight days after the final application, the concentrations were lower than 0.5 ng/ml [4]. From this excretion study, a half-live of about 1.5 days was calculated. Therefore, to detect the misuse of clenbuterol over a longer period, highly sensitive methods are necessary.

In our institute, a method based on high-performance liquid chromatography (HPLC) in combination with both ultraviolet (UV) and electrochemical detection has been used, with a simple two-step sample pretreatment involving an Extrelut-3 column

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and a solid-phase extraction column [4]. Clenbuterol in urine can be determined above the 0.5 ng/ml level, which is better than or at least comparable to other reported HPLC methods [5–11]. Positive results obtained with the HPLC method are confirmed by gas chromatography in combination with mass spectrometry (GC–MS) in both the electron impact and chemical ionization modes [12]. In addition to these techniques, other methods have been described, involving high-performance thin-layer chromatography [10,13,14] or GC with electron-capture detection [15,16]. These procedures are not suitable for automation or lack the required sensitivity.

Yamamoto and Iwata [17] described a sensitive enzyme immunoassay (EIA) for the determination of clenbuterol in human plasma with a detection limit of 0.02 ng/ml. However, in principle, immunoassays are prone to cross-reactions, which may lead to less accurate results.

The specificity of an immunoassay can be improved by combination with HPLC for sample clean-up. Such a combination has been applied, for instance, to the determination of the steroid hormone nortestosterone in urine [18]. An alternative to such a combination is HPLC with on-line immunoaffinity chromatography, in which immobilized antibodies are used for the selective preconcentration of the analyte(s) prior to the analytical separation. This technique was successfully applied to the determination of small amounts of nortestosterone in biological samples of calves [19,20].

This paper demonstrates the potential of the above technique for the determination of clenbuterol in urine, without the need for any off-line sample pretreatment. To characterize the applied antibodies with respect to cross-reactivities, a microtitre plate enzyme immunoassay was developed and the results obtained with this procedure are also presented.

EXPERIMENTAL

Apparatus

The set-up of the HPLC system used is shown schematically in Fig. 1. The HPLC

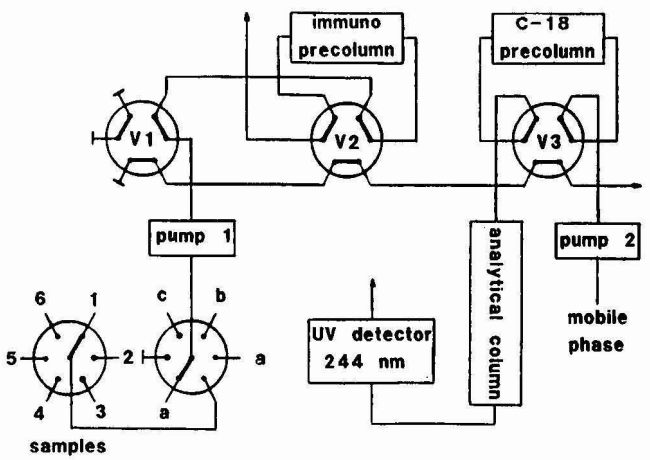

Fig. 1. Set-up of the automated HPLC system for the determination of clenbuterol in urine samples. Valves V1–V3 are all shown in position A (compare Table I).

system consisted of a Merck–Hitachi (Darmstadt, F.R.G.) Model 655-A-11 pump for the analytical column, a Kratos (Ramsey, NJ, U.S.A.) Model 400 pump for sample handling, two Kratos Model Must valve-switching units (containing two solvent-selection valves and three high-pressure six-port rotary valves), a Kratos Spectroflow 450 solvent programmer, a Merck–Hitachi L-4200 UV–VIS detector set at 244 nm and a Merck–Hitachi Model D2000 integrator. The analytical separation was achieved on a Merck 125 mm × 4 mm I.D. LiChrospher 60 RP-select B (5 μm) column with a Merck 4 mm × 4 mm I.D. LiChrospher 60 RP-select B (5 μm) guard column. The C_{18} precolumn was a 10 mm × 2 mm I.D. reversed-phase preconcentration column from Chrompack (Middelburg, The Netherlands). A 10 mm × 10 mm I.D. stainless-steel column equipped with 5-μm stainless-steel screens and PTFE rings at the column inlet and outlet, obtained from the Free University of Amsterdam, was filled with immunosorbent according to the procedure described previously [19]. The preparation of the immunosorbent is described below.

Chemicals

Tresyl-activated Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). HPLC-grade acetonitrile, HPLC-grade methanol, general-reagent grade glacial acetic acid, analytical-reagent grade sodium dodecyl sulphate, horseradish peroxidase and *Helix pomatia* digestive juice (containing a minimum of 40 U/ml β-glucuronidase and 20 U/ml arylsulphatase) were obtained from Merck (Darmstadt, F.R.G.). Sheep anti-rabbit IgG (whole molecule), *o*-phenylenediamine, Tween-20, bovine serum albumin (BSA) and fenoterol hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Clenbuterol hydrochloride was a gift from Boehringer (Ingelheim, F.R.G.) and salbutamol sulphate and terbutaline sulphate were obtained from Bufa-Chemie (Castricum, The Netherlands). Carbuterol hydrochloride was a gift from Warner-Lambert (Bornem, Belgium) and cimaterol was a gift from D.G. Mann Testing Labs. (Mississauga, Ontario, Canada). Flat-bottomed microtitre ELISA plates (96-well) were purchased from Greiner (Nurtingen, F.R.G.). Stock solutions of the β-agonistic drugs were prepared in methanol. Water was clarified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All eluents were filtered through 0.45-μm Millipore filters.

Antibodies

Clenbuterol was conjugated to BSA after diazotization, as described by Yamamoto and Iwata [17]. The conjugate (molar ratio 7.3) was used to elicit antibodies in a New Zealand White rabbit. Antibody titres were determined with an EIA procedure using BSA–clenbuterol as antigen bound to the solid phase. The IgG fraction of the collected antiserum was purified by ammonium sulphate precipitation [21] and stored at −20°C until used.

Preparation of the immunosorbent

Anti-clenbuterol IgG was bound to tresyl-activated Sepharose as recommended by the manufacturer, using 10 mg IgG/ml gel. Until use, the immunosorbent was stored at 4°C in 0.1 *M* sodium phosphate buffer (pH 7.2) containing 0.02% sodium azide as preservative.

Enzyme immunoassays

For cross-reactivity determinations, a competitive EIA was developed, applying clenbuterol–horseradish peroxidase (HRP) as the label. The enzyme conjugate (8.6 mol clenbuterol/mol HRP) was prepared as described above for BSA–clenbuterol. Microtitre plates were coated overnight with 100-μl aliquots of sheep anti-rabbit IgG [10 μg/ml in 50 m*M* sodium carbonate (pH 9.6)] at 4°C. Plates were washed four times with phosphate-buffered saline (PBS)–Tween [5.4 m*M* sodium phosphate–1.3 m*M* potassium phosphate–150 m*M* sodium chloride (pH 7.4)–0.05% Tween-20) with a Wellwash Model 4 microplate washer (Denley Instruments, Billingshurst, U.K.). Aliquots of 50 μl of serially diluted β-agonists were added to the wells, followed by 25 μl of appropriately diluted clenbuterol–HRP and finally 25 μl of antiserum (all in PBS–Tween). The plates were incubated for 2 h at 37°C. After washing (as described above), the bound peroxidase was assessed with 100 μl of a freshly prepared solution of 2.2 m*M* *o*-phenylenediamine–0.012% hydrogen peroxide in 100 m*M* citrate–200 m*M* potassium phosphate (pH 5.0). After incubation in the dark for 30 min at 20°C, the reaction was stopped by addition of 50 μl of 12.5% sulphuric acid. The product of the peroxidase reaction was determined at 490 nm with an Argus 400 microplate reader (Canberra Packard, Downers Grove, IL, U.S.A.). The limit of detection of the assay was 100 pg of clenbuterol per well (2 ng/ml). Cross-reactivities were determined at 50% displacement.

Sample materials

Blank samples of calf urine were obtained from the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands). Clenbuterol-containing urine samples were obtained from an animal experiment (at IVVO in March–April 1989), in which five calves (12–16 weeks old) were treated orally with the recommended therapeutic dose of clenbuterol · HCl (0.8 μg/kg body weight) twice a day over a period of 2 weeks. During this treatment and up to 3 weeks after, urine was collected daily in two periods, *i.e.*, from 17.00 p.m. to 6.30 a.m. and from 6.30 a.m. to 17.00 p.m. Two clenbuterol-positive samples were obtained from the Dutch General Inspection Service (Kerkrade, The Netherlands).

Sample preparation

Determination of free clenbuterol. Urine samples were filtered (Type 595 1/2 filter-paper, Schleicher & Schüll, Dassel, F.R.G.) and to 25 ml of filtered urine 25 ml of PBS–buffer (pH 7.4) were added and, if necessary, the pH was adjusted by adding a few drops of 1 *M* hydrochloric acid. Of this mixture, 27 ml were loaded onto the immuno precolumn.

Determination of total clenbuterol. To 10 ml of filtered urine a few drops of 4 *M* acetic acid were added to adjust the pH to 4.8 $\pm$ 0.2. Next, 50 μl of *Helix pomatia* juice were added, the mixture was incubated at 37°C for 2 h and the volume was adjusted to 50 ml by adding PBS–buffer (pH 7.4). Of this mixture, 27 ml were loaded onto the immuno precolumn.

HPLC procedure

The analytical procedure is summarized in Table I. The first step involves the preconditioning of the immuno precolumn with water, then the sample is introduced

TABLE I

SCHEDULE OF THE AUTOMATED ANALYSIS

For each valve, position A corresponds to the position shown in Fig. 1. For further explanation, see text.

Step	Event	Valve position		
		Valve 1	Valve 2	Valve 3
1	Flushing capillaries with water (7 ml)	B	A	A
2	Flushing immuno precolumn with water (20 ml)	A	A	A
3	Flushing capillaries with sample (7 ml)	B	A	A
4	Flushing immuno precolumn with sample (27 ml)	A	A	A
5	Flushing capillaries with water (7 ml)	B	A	A
6	Flushing immuno precolumn with water (10 ml)	A	A	A
7	Flushing C_{18} precolumn with water (10 ml)	B	A	B
8	Flushing capillaries with 0.01 *M* acetic acid (7 ml)	B	B	B
9	Flushing immuno precolumn and C_{18} precolumn in series with 0.01 *M* acetic acid (7 ml)	A	B	B
10	Desorbing C_{18} precolumn by on-line switching with the analytical column	A	B	A
11	Flushing capillaries with methanol–water (70:30) (7 ml)	B	A	A
12	Flushing immuno precolumn with methanol–water (70:30) (20 ml)	A	A	A

by pump I via the two solvent selection valves. Next, the immuno precolumn is flushed with water to displace the remaining sample. In the following step, the C_{18} precolumn is switched off-line with respect to the analytical column and preconditioned with water. Subsequently, the immuno precolumn, now containing the trapped analyte, and the C_{18} precolumn are switched in series and the transfer of the analytes is accomplished in the back-flush mode with 0.01 *M* acetic acid (pH 3.5). Subsequently, the actual separation is started by switching the C_{18} precolumn on-line with the analytical column. Simultaneously, the immuno precolumn is reconditioned by flushing with methanol–water (70:30). On the analytical column, clenbuterol is separated by using a mobile phase consisting of a mixture of acetonitrile and an ion-pair buffer (45:55) at a flow-rate of 1 ml/min. The ion-pair buffer contained 25 mmol of sodium dodecyl sulphate and 20 mmol of acetic acid and was adjusted to pH 3.5 with sodium hydroxide (1 *M*). After a total run time of 35 min, the next analysis can be started while the separation in the analytical column is still running. The system is automated for six samples by using a second solvent selection valve as sample selector.

RESULTS AND DISCUSSION

Antibodies

Antibodies were raised against a clenbuterol–BSA conjugate. The specificity of the isolated IgG fraction was determined in a competitive EIA, employing clenbuterol–HRP. Cross-reactions with related β-agonists (Fig. 2) were expressed at a 50% displacement level (Table II, Fig. 3). The observed cross-reactions of the antibodies may be useful in immunoaffinity chromatographic applications, allowing the entrapment of several β-agonists. Despite the relatively high specificity of the

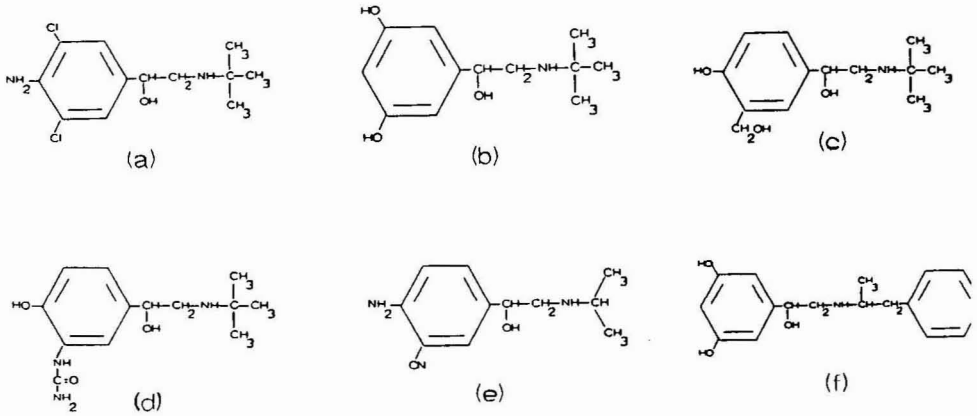

Fig. 2. Structures of (a) clenbuterol, (b) terbutaline, (c) salbutamol, (d) carbuterol, (e) cimaterol and (f) fenoterol.

antibodies for clenbuterol, these data may not be directly extrapolated towards an immunoaffinity chromatographic application. When using anti-clenbuterol IgG for the selective entrapment of analytes from a sample, there is no competition with the peroxidase conjugate, as with the EIA.

HPLC and on-line affinity chromatography

In previous experiments, several HPLC columns and mobile phases were investigated for their applicability to the separation of clenbuterol [4]. The combination of a LiChrosorb 60 RP-select B column (specially designed for basic compounds) and a mixture of acetonitrile and sodium dodecyl sulphate (SDS)–acetate buffer as the mobile phase offered the best results with respect to reproducible retention times of the analyte. The limit of detection for clenbuterol with this system, using UV detection at 244 nm, was 1 ng. The procedure also allowed the separation of clenbuterol from other β-agonistic drugs (Table II).

The column-switching system described here is based on earlier studies of the determination of 19-nortestosterone, in which selective sample pretreatment with an immunoaffinity precolumn on-line with HPLC was developed [19,20]. Agarose-based

TABLE II

RELATIVE RETENTIONS OF SOME β-AGONISTIC DRUGS, COMPARED WITH CLENBUTEROL, ON THE HPLC SYSTEM TOGETHER WITH THEIR CROSS-REACTIVITIES TOWARDS THE ANTIBODIES AGAINST CLENBUTEROL IN AN ENZYME IMMUNOASSAY

β-Agonist	Relative retention	Cross-reactivity (%)
Clenbuterol	1.000	100
Cimaterol	0.407	3.3
Fenoterol	0.386	<0.01
Terbutaline	0.342	2.8
Carbuterol	0.314	7.9
Salbutamol	0.309	3.3

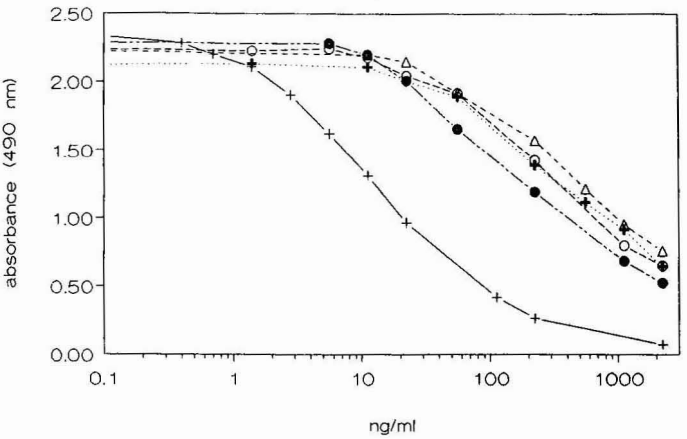

Fig. 3. Cross-reactivities of some β-agonistic drugs in the enzyme immunoassay. +, Clenbuterol; ●, carbuterol; ○, cimaterol; +, salbutamol; △, terbutaline.

precolumns were used because of their proven capability, *i.e.*, they can be loaded, flushed and desorbed with aqueous solutions with or without organic modifiers. The only disadvantage is their compressibility, which requires the application of a dual precolumn system in which the agarose-based precolumn is switched in series with a second precolumn during the desorption step. In this study we used tresyl- instead of cyanogen bromide-activated Sepharose, because of the more stable linkage between the support and the ligand [22]. When using 10 mg IgG/ml Sepharose (at pH 7.8 $\pm$ 0.2), the coupling efficiency was 80 $\pm$ 5% (n = 5). Columns containing 0.7 ml of Sepharose anti-clenbuterol were applied in the experiments described below.

Desorption of analytes bound to the immobilized antibodies may be achieved by either non-specific elution or by competitive elution with an excess of a related compound. The competitor should preferably have a longer retention time on the analytical column, to allow separation from the analyte.

The cross-reactions of the antibodies against clenbuterol with a number of β-agonistic drugs are all below 10% (Table II), necessitating relatively large amounts for competitive desorption of clenbuterol. Further, their retention times are all shorter than that of clenbuterol (Table II). Hence, a non-specific desorption step is preferred here. In this instance, the requirement for reconcentration of desorbed analyte on a C_{18}-bonded silica precolumn limits the possibilities for non-specific elution. Although clenbuterol is a basic compound that is protonated at pH < 9.5, it can be successfully retained on an RP-18 precolumn from an aqueous solution at pH 3.5. Using this precolumn within the column-switching system, in every run it is switched on-line with the analytical column during the analytical separation using a mixture of acetonitrile and SDS–acetate buffer (pH 3.5) as the mobile phase. SDS is used as an ion-pair reagent to increase the retention time of the polar clenbuterol on the apolar stationary phase. Although the RP-18 precolumn is flushed with water (see step 7 in Table I) prior to the reconcentration of the desorbed analyte, a sufficient amount of SDS apparently remains on the column. Loading up to 10 ml of solutions of clenbuterol in 20 mM citric acid (pH 3.5) or in 0.01 M acetic acid (pH 3.5) onto this precolumn resulted in recoveries higher than 80% compared with small volumes of

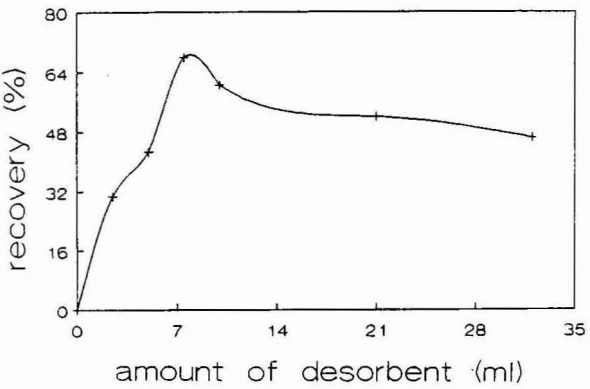

Fig. 4. Recovery of clenbuterol (%) as a function of the amount of desorbent (0.01 *M* acetic acid) in ml.

standard solutions loaded directly onto the analytical column. This positive effect of SDS was confirmed by the low recoveries (10–20%) obtained after loading standard solutions of clenbuterol in 0.01 *M* acetic acid onto a comparable solid-phase extraction column which was never used with this ion-pair reagent. Using dilute acid solutions (pH 3.5) as desorbent, both the immobilized antibodies of the immuno precolumn and the bound clenbuterol will be positively charged, which should facilitate the desorption.

The amount of 7 ml of 0.01 *M* acetic acid needed for the optimum desorption of clenbuterol bound to the immuno precolumn and for the reconcentration onto the RP-18 precolumn was determined by loading 27 ml of a solution of clenbuterol in water (5 ng/ml) onto the system and using different amounts of desorbent (Fig. 4). Under these conditions, loading 27 ml of standard solutions of clenbuterol in water of concentration 1, 2 and 3 ng/ml onto the immuno precolumn resulted in a final recovery of 69 $\pm$ 8% (n = 4), compared with small injections directly onto the analytical column. However, the concentrations of clenbuterol in urine will be calculated by a comparison with standard solutions of clenbuterol loaded onto the same system, which should compensate for these losses. After the desorption of the immuno precolumn with 0.01 *M* acetic acid, this column was flushed with methanol–water (70:30) as an extra washing step, to exclude cross-contamination between samples. Executing the procedure described in Table I, cross-contamination between a standard with a concentration of 10 ng/ml and a blank sample was $<2\%$.

For quantitative determinations the capacity of the immuno precolumn must be known. To determine this capacity, first 27 ml of standard solutions of clenbuterol in water with increasing concentrations were loaded onto the system. However, surprisingly high concentrations of clenbuterol (up to 2 μg/ml) still resulted in an increasing peak area. Calculated from the amount of antibodies immobilized to the Sepharose, such an amount of clenbuterol could not be bound specifically, not even when all antigen binding sites would have remained available after coupling of the antibodies. Aspecific binding of clenbuterol to the Sepharose was confirmed by the binding of clenbuterol to an immuno precolumn packed with Sepharose-immobilized polyclonal antibodies against the anabolic hormone 17β,19-nortestosterone. The

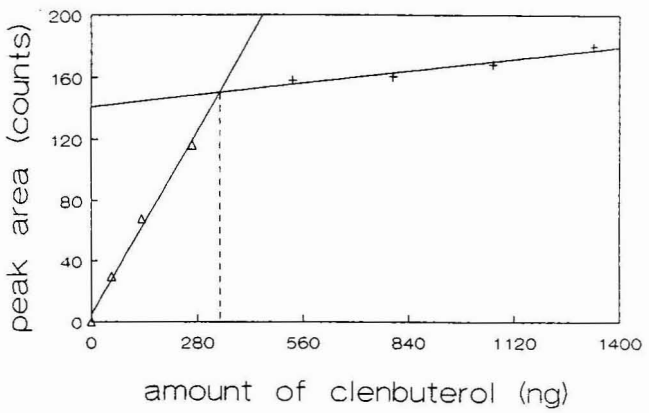

Fig. 5. Peak area as a function of the amount of clenbuterol (ng in 27 ml of standard solution) loaded onto the HPLC system. The total clenbuterol capacity of the immuno precolumn (334 ng) was determined as the intersection of the two straight lines.

aspecific binding to the Sepharose diminishes the degree of selective preconcentration. Hence the latter immuno precolumn was used to explore conditions under which the aspecific binding could be eliminated. Small amounts of organic solutions such as acetonitrile or methanol (up to 10%) added to the sample did not abolish aspecific binding. Dissolving clenbuterol in PBS–buffer was sufficient to eliminate this effect, without affecting the specific binding of clenbuterol to the Sepharose-immobilized antibodies against clenbuterol.

Increasing concentrations of clenbuterol in PBS were loaded onto the system and the peak areas of clenbuterol were measured and plotted against the absolute amount of clenbuterol offered to the system (Fig. 5). The maximum capacity of the immuno precolumn (334 ng) was determined as the intersection of the two straight lines. Comparable experiments with other β-agonistic drugs using the system described here resulted in only very low recoveries of these drugs. As found during experiments using the immunoaffinity column off-line, a number of β-agonistic drugs were bound by this column and could be eluted with 0.01 *M* acetic acid. However, reconcentration of the drugs from this desorbent onto the RP-18 precolumn led to the low recoveries. In off-line experiments, good results were obtained by adjusting the pH of the affinity column eluate to 7.4 prior to reconcentration onto an RP-18 solid-phase extraction column. In our laboratory, this combination was used for the clean-up of urine, feed and tissue samples prior to GC–MS [23]. Hence changes of the system described in this paper have to be made in order to allow the determination of these other β-agonistic drugs.

Urine samples

Urine samples from a blank calf, with and without addition of clenbuterol (5 ng/ml), and urine samples obtained from a calf treated with clenbuterol · HCl (Ventipulmin) were analysed. The only pretreatment was dilution of the sample with an equal volume of PBS–buffer (pH 7:4) and, if necessary, adjustment of the pH to 7.4 $\pm$ 0.2. In total, 27 ml of the diluted sample were loaded onto the immuno-column.

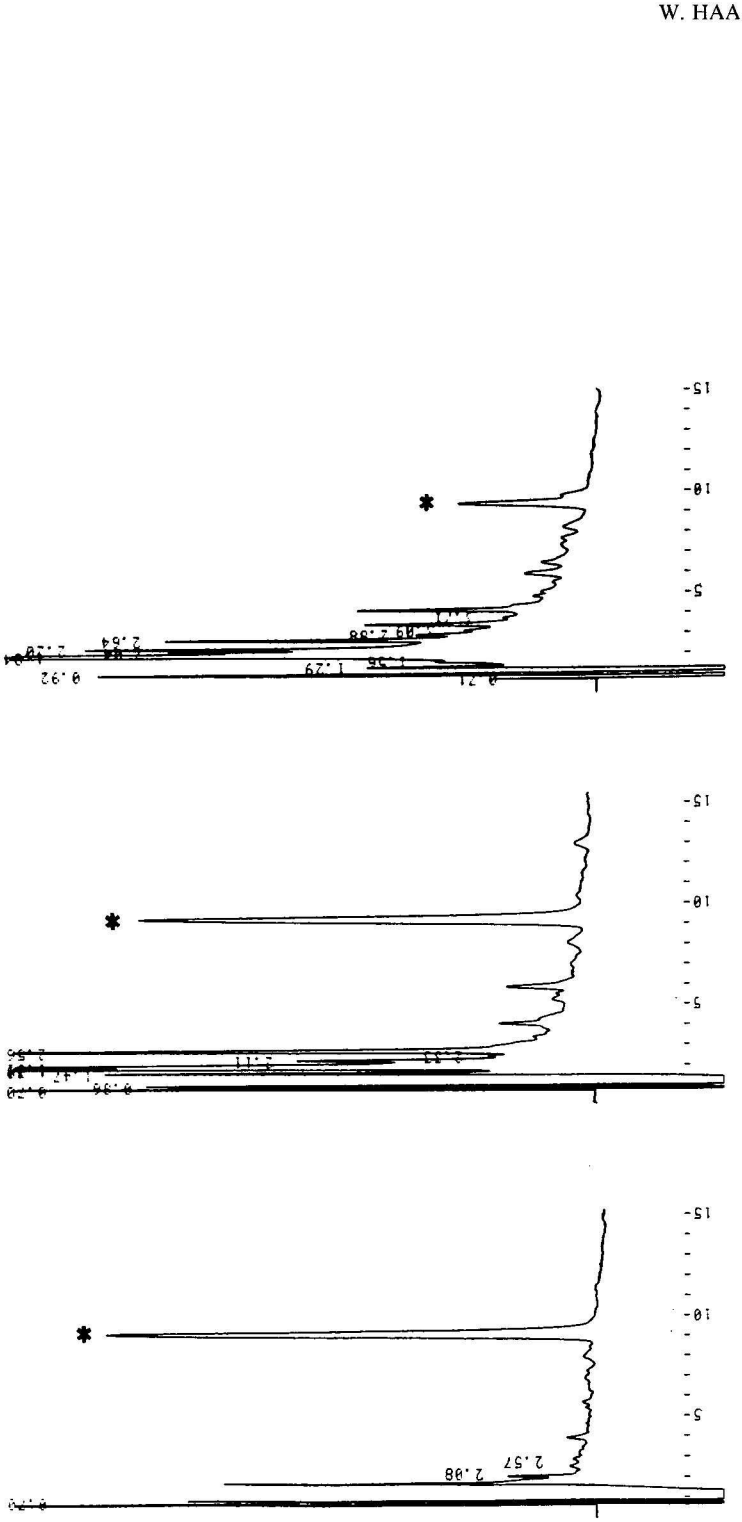

Fig. 6. Chromatograms of (A) a standard solution of clenbuterol (5 ng/ml), (B) a clenbuterol-containing urine sample (26 ng/ml; sample code 14) diluted with PBS (1:4, v/v) and (C) a clenbuterol-containing urine sample (3 ng/ml; sample code 7) diluted with PBS (1:1, v/v). Of the standard solution and dilute urine samples, 27 ml were loaded onto the HPLC system (0.002 a.u.f.s.). The peaks marked with asterisks are clenbuterol. For further conditions, see the text.

As shown in Fig. 6, clenbuterol could be detected clearly and a detection limit of 0.5 ng/ml in urine was achieved. The detection limit may easily be lowered by loading more sample. In addition to clenbuterol, other compounds were detected, most of which eluted between 0 and 5 min. These compounds could be bound non-specifically to the stationary phase backbone, the coupling group or even to the large non-selective surface of the antibodies, or specifically to the immobilized antibodies (unknown cross-reacting compounds). Compared with a standard solution of clenbuterol, (5 ng/ml), the mean recovery of clenbuterol added to the diluted (1:1 with PBS) blank urine sample at the same level was 82% ($n = 5$, S.D. 2%). This could be an effect of the presence of compounds in the urine sample which influence the antigen–antibody interaction. Therefore, for quantitative analyses, a comparison with a calibration graph in urine is preferred.

Clenbuterol was found in urine samples from a calf treated with clenbuterol · HCl (Ventipulmin) at levels between 1 and 5 ng/ml and in two urine samples obtained from the Dutch General Inspection Service. The same urine samples were analysed with an HPLC method using solid-phase extraction [4], and the results are given in Table III. The same urine samples had been analysed 5 months earlier by the latter method and the mean concentration of clenbuterol found at that time was 2.4 times higher, suggesting that the concentration of clenbuterol in urine samples decreases during storage even at temperatures below −20°C. In spite of deviations at low levels of clenbuterol, both methods are suitable for the determination of clenbuterol in urine. However, the advantage of the method presented in this paper is that "off-line" sample preparation is minimized to a dilution only.

The presence of an hydroxyl group in clenbuterol theoretically gives the possibility of conjugation with glucuronic acid or sulphuric acid. Analysis of urine

TABLE III

COMPARISON OF THE CONCENTRATIONS OF CLENBUTEROL FOUND WITH TWO HPLC METHODS USING A DIFFERENT SAMPLE CLEAN-UP

Sample code	Concentration of clenbuterol (ng/ml)	
	Immunoaffinity chromatography on-line with HPLC	HPLC with solid-phase extraction
1	2.2	1.1
2	2.3	1.3
3	2.4	1.2
4	5.9	7.2
5	2.9	1.5
6	2.3	3.8
7	2.7	2.6
8	1.7	1.1
9	1.5	2.0
10	1.6	2.5
11	2.0	2.8
12	1.1	1.9
13	5.0	8.1
14	26.8	27.0

samples from treated calves, with and without enzymatic hydrolysis using *Helix pomatia* juice, showed that hydrolysis did not result in higher concentrations of free clenbuterol.

In fact, the concentrations of clenbuterol were lower after hydrolysis, probably because of degradation of clenbuterol during the 2-h incubation at 37°C.

During the experiments described in this paper, the immuno precolumn has been used for more than 200 runs with standard solutions and samples with no marked decrease in the peak area with the most frequently used highest standard solution of 3 ng/ml.

CONCLUSION

The selectivity of the immuno precolumn, *i.e.*, a column packed with immobilized antibodies against clenbuterol, results in a high clean-up efficiency, allowing the determination of clenbuterol in urine samples at the sub-μg/l level. Such a selective preconcentration in combination with HPLC gives a high probability of excluding false-positive results. By using the immuno precolumn in an automated HPLC system, the required off-line sample pretreatment is limited to a 2-fold dilution with PBS, which leads to a better reproducibility.

The antibodies showed cross-reactions with some other β-agonistic drugs, as determined with an EIA. In experiments using the immunoaffinity column off-line with GC–MS, this resulted in methods for the simultaneous determination of clenbuterol, salbutamol, cimaterol and terbutaline in urine and tissue samples [23]. With the HPLC procedure described here, however, the recoveries of these other β-agonists were low ($< 10\%$). Future research will be focused on the determination of these β-agonistic drugs using the immuno precolumn on-line with a modified HPLC procedure.

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Application de la dérivation par l'isocyanate de phényle à l'analyse chromatographique de molécules phénoliques (antioxydants)

A. BAILLET*, S. RAKOTOMANGA, D. FERRIER et F. PELLERIN

Centre d'Études Pharmaceutiques, Laboratoire de Chimie Analytique, Rue J. B. Clément, 92296 Chatenay-Malabry Cedex (France)

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ABSTRACT

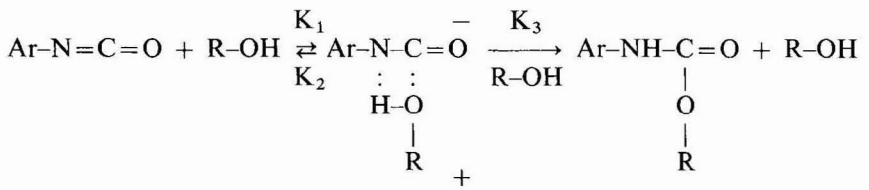
Application of derivatization with phenylisocyanate to chromatography of phenolic compounds (antioxydants)

Phenylisocyanate reactivity towards compounds with mobile hydrogen (active hydrogen) has been applied to phenolic antioxidants, usually found in plastic polymers. The first part of this work studies the kinetics and the reaction mechanism in order to optimize its parameters. This method was then applied to the reversed-phase liquid chromatographic analysis of several phenolic antioxidants. The phenylcarbamates formed are detectable at 230 nm and the detection limit is lowered by a factor 5–10, depending on the compound, compared to direct detection at 280 nm.

INTRODUCTION

Les uréthanes et les urées substituées, issus de la réaction d'addition entre l'isocyanate de phényle et les composés hydroxylés ou aminés, sont des dérivés stables et présentent un coefficient d'absorbance élévé dans le domaine de l'ultra-violet. Cette réaction a donc été appliquée à la détection et au dosage par chromatographie en phase liquide, d'amines aliphatiques [1,2], d'alcools [3–5], d'eau [6] et de sucres [7].

Baker et Holdsworth [8,9] ont été parmi les premiers à étudier le mécanisme d'additon des isocyanates d'aryle sur les alcools et ont déterminé l'ordre de la cinétique réactionnelle (ordre 2):



Plus récemment, Satchell et Satchell [10] ont explicité les mécanismes de réaction

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entre les composés à hydrogène actif (alcools, acides, amines, eau ...) et le groupement carboxyle électrophile des isocyanates.

L'objet de ce travail a pour but le développement d'une méthode sensible pour la détermination de traces d'antioxydants phénoliques utilisés dans les matériaux plastiques: Irganox 1010, Irganox 1076, Ionox 330 et Goodrite 3114 (Fig. 1). Le travail comprend deux parties: la première consiste en l'étude de la cinétique et du mécanisme de la réaction qui aboutit, dans la deuxième partie, à la détermination des paramètres de la dérivation fixant les conditions optimales en vue de l'analyse chromatographique des dérivés formés.

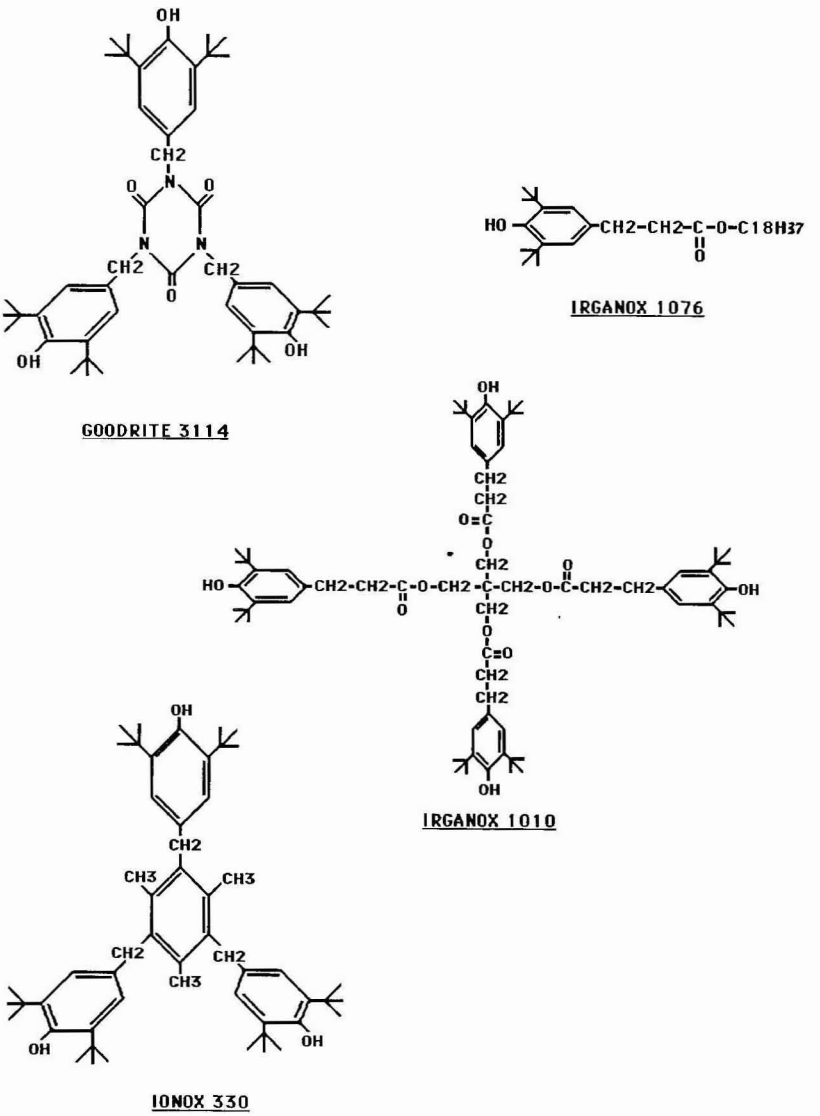

Fig. 1. Formules chimiques des antioxydants phénoliques étudiés.

PARTIE EXPÉRIMENTALE

Appareillage

L'ensemble chromatographique est constitué d'une pompe Chromatem 380 (Touzart et Matignon, Vitry sur Seine, France) équipée d'un injecteur à boucle Rheodyne de 20 μl (Touzart et Matignon), d'un détecteur spectrophotométrique UV Shimatzu SPD 2 (Touzart et Matignon) et d'un enregistreur Kipp & Zonen BD-40 (Cunow, Cergy St. Christophe, France).

La colonne chromatographique (250 mm × 4 mm I.D.) est remplie de silice greffée octadecyle LiChrosorb RP-18 (Merck, Nogent sur Marne, France) de 7 μm de diamètre nominal.

La structure des phénylcarbamates d'aryle formés est analysée à l'aide d'un spectrophotomètre UV SP8-100 (Pye-Unicam, Cambridge, Angleterre) et d'un spectrophotomètre IR 295 (Perkin Elmer, ZI de Courtabœuf, France).

Réactifs

La phase éluante est un mélange d'acétonitrile (R.S. pour HPLC, Carlo-Erba, Milan, Italie), d'eau bidistillée filtrée sur filtre de 0.22 μm de porosité (Millipore, St. Quentin en Yvelines, France) et de tétrahydrofuranne (J. T. Baker Chemicals, Deventer, Pays-Bas).

L'isocyanate de phényle a été obtenu chez Prolabo (Paris, France).

Les antioxydants phénoliques ont été fournis par Ciba-Geigy (Rueil Malmaison, France) pour le Goodrite 3114, l'Irganox 1010 l'Irganox 1076 et l'Ionox 330.

Synthèse des phénylcarbamates d'aryle témoins

Dans des tubes scellés de 10 ml, mettre en contact chacun des antioxydants phénoliques en solution dans l'acétonitrile avec l'isocyanate de phényle, dans un rapport molaire 1: x dans lequel:

$x = 1.1$ pour les composés monophénoliques (Irganox 1076);

$x = 3.3$ pour les composés triphénoliques (Goodrite 3114–Ionox 330);

$x = 4.4$ pour les composés tétraphénoliques (Irganox 1010).

Laisser en contact pendant 12 h au bain marie à 50°C. Evaporer sous pression réduite jusqu'à siccité. Laver le résidu sec par 10 ml d'heptane puis sécher à l'étuve à 50°C. Reprendre le résidu sec par l'acétonitrile jusqu'à dissolution complète. Filtrer la solution ainsi obtenue sur filtre papier et concentrer à l'aide d'un évaporateur rotatif sous pression réduite. Les phénylcarbamates d'aryle précipitent.

La formation des groupements phénylcarbamates a été vérifiée par spectrophotomètrie UV et IR ainsi que par chromatographie en phase liquide: (i) disparition du maximum d'absorption UV à 280 nm caractéristique du noyau phénolique; (ii) disparition de la bande de vibration des hydroxyles phénoliques à 3650 cm^{-1} et apparition de nouvelles bandes de vibration des fonctions C=O et NH respectivement vers 1600–1700 cm^{-1} et 3400 cm^{-1}; (iii) diminution du temps de rétention chromatographique par rapport à celui du composé initial.

Étude de la dérivation

Dans une fiole jaugée de 25 ml, introduire 25 mg d'un antioxydant phénolique puis 24.5 ml d'acétonitrile. Après 10 min dans un bain marie thermostaté à 25°C (pour

TABLEAU I

COMPOSITION DE LA PHASE MOBILE EN FONCTION DE L'ANTIOXYDANT ÉTUDIÉ

Antioxydant	Composition de la phase mobile			Débit (ml/min)
	Acétonitrile (%)	Eau (%)	Tétrahydrofuranne (%)	
Irganox 1076	75	0	25	1.2
Goodrite 3114	95	5	0	1.8
Ionox 330	75	5	20	1.4
Irganox 1010	75	5	20	1.5

l'Irganox 1010, l'Ionox 330 et l'Irganox 1076) ou régulé à 50°C (pour le Goodrite 3114), ajouter 0.5 ml d'isocyanate de phényle puis replacer immédiatement la fiole au bain marie et déclencher le chronomètre. A chacun des temps fixés, prélever 0.5 ml du milieu réactionnel et l'introduire dans un tube à hémolyse contenant 0.1 ml de méthanol pour arrêter la réaction et détruire l'excés d'isocyanate de phényle. La solution est ainsi stabilisée et peut être conservée à +4°C pendant plusieurs jours.

Conditions chromatographiques

Les solutions ainsi obtenues sont injectées directement dans le système chromatographique, afin de suivre la cinétique de dérivation. La colonne chromatographique est maintenue à température ambiante. L'élution s'effectue en mode isocratique à l'aide d'une phase mobile dont la composition quantitative et le débit dépendent de chacun des antioxydants phénoliques étudiés et sont regroupés dans le Tableau I.

Les longueurs d'onde du spectrophotomètre sont fixées à 280 et 230 nm afin de suivre simultanément la disparition de l'antioxydant phénolique et la formation du ou des phénylcarbamates d'aryle formés.

RÉSULTATS ET DISCUSSION

Cinétique de la dérivation

La cinétique de dérivation a tout d'abord été étudiée sur le composé le plus simple, monophénolique, l'Irganox 1076 (Fig. 2A). En présence d'un large excès de réactif, l'apparition du dérivé se fait à partir du composé initial selon une loi exponentielle. A température ambiante, la réaction se développe lentement et elle est totale au bout de 65 min de contact.

Plusieurs molécules polyphénoliques ont été ensuite étudiées:

Le Goodrite 3114. Il possède un noyau isocyanurate sur lequel sont greffés trois noyaux phénoliques substitués. La cinétique de dérivation est présentée dans Fig. 2B. L'apparition du produit final est précédée de la formation de deux composés intermédiaires mono- et di-dérivés. La réaction est totale au bout de 95 min de contact à température ambiante.

L'Ionox 330. La formule chimique est proche de celle du Goodrite 3114, le noyau cyanurate étant remplacé par un noyau triméthylbenzène. Lors de la dérivation, il

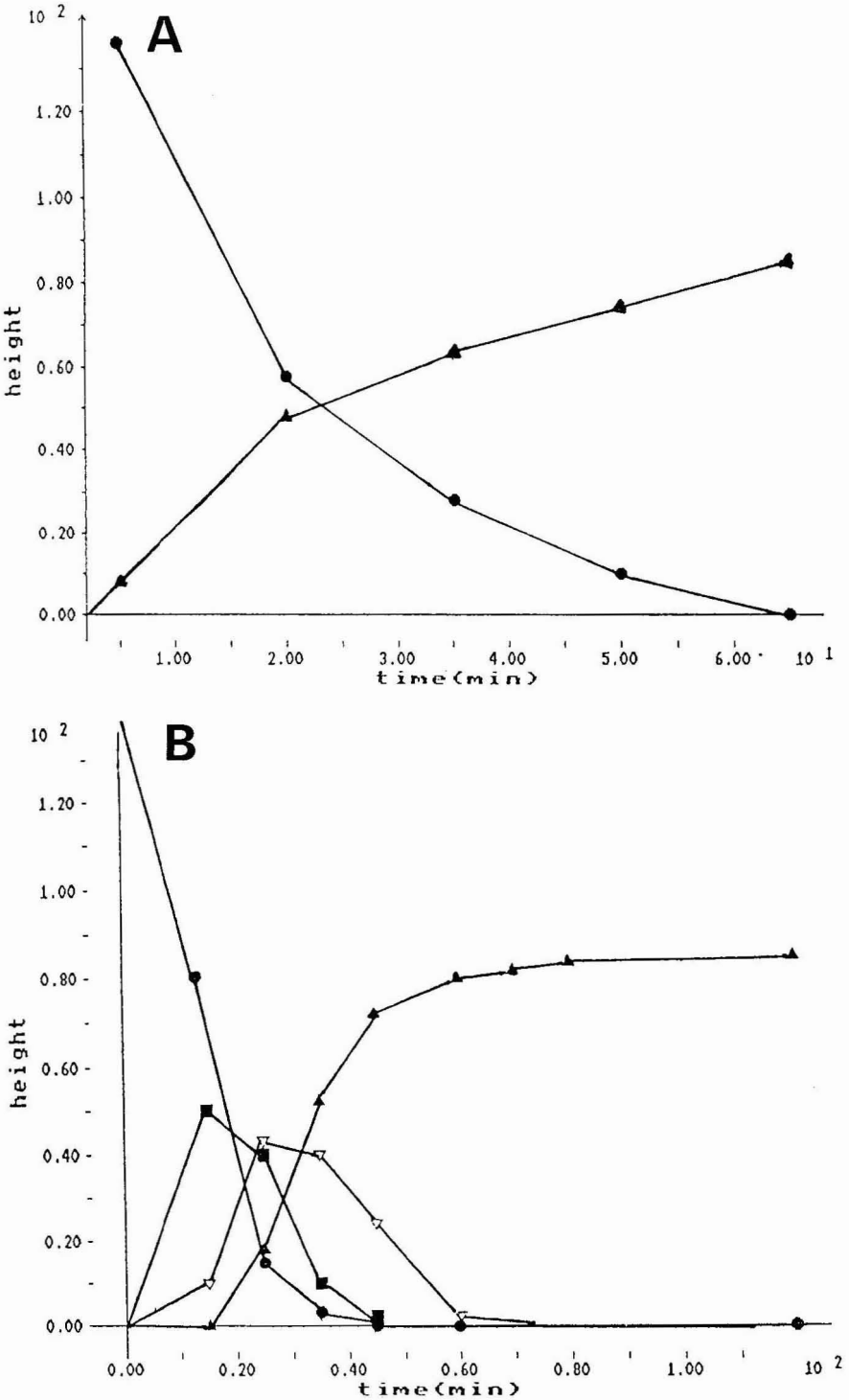

Fig. 2.

(Continué sur la p. 342)

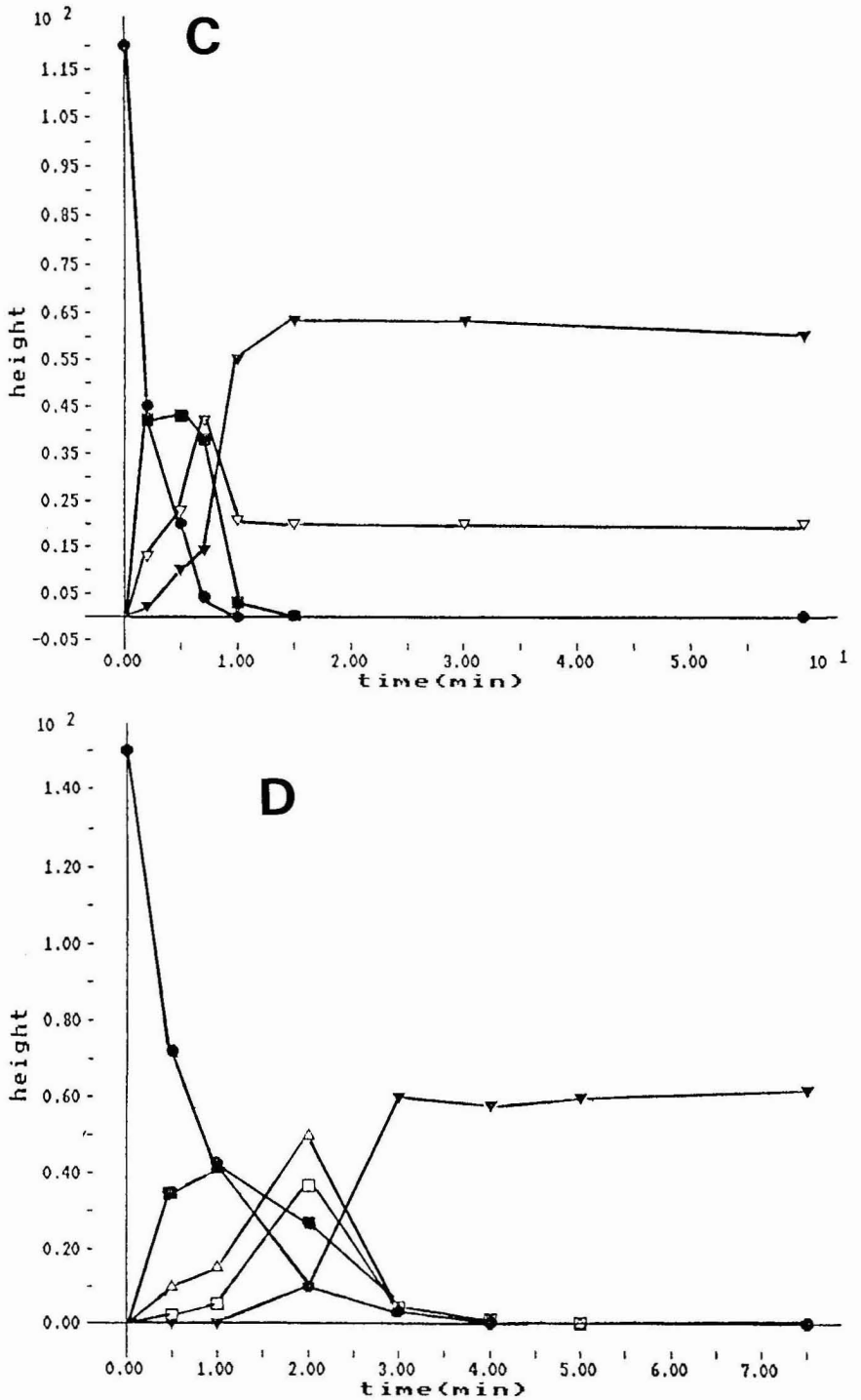

Fig. 2. Cinétique de la réaction de dérivation des antioxydants à 280 nm. (A) ● = Irganox 1076; ▲ = Irganox 1076 dérivé. (B) ● = Goodrite 3114; ■ = Goodrite 3114 mono-dérivé; ▽ = Goodrite 3114 di-dérivé; ▲ = Goodrite 3114 tri-dérivé. (C) ● = Ionox 330; ■ = Ionox 330 mono-dérivé; ▽ = Ionox 330 di-dérivé; ▼ = Ionox 330 tri-dérivé. (D) ● = Irganox 1010; ■ = Irganox 1010 mono-dérivé; ▼ = Irganox 1010 di-dérivé; □ = Irganox 1010 tri-dérivé; △ = Irganox 1010 tétra-dérivé.

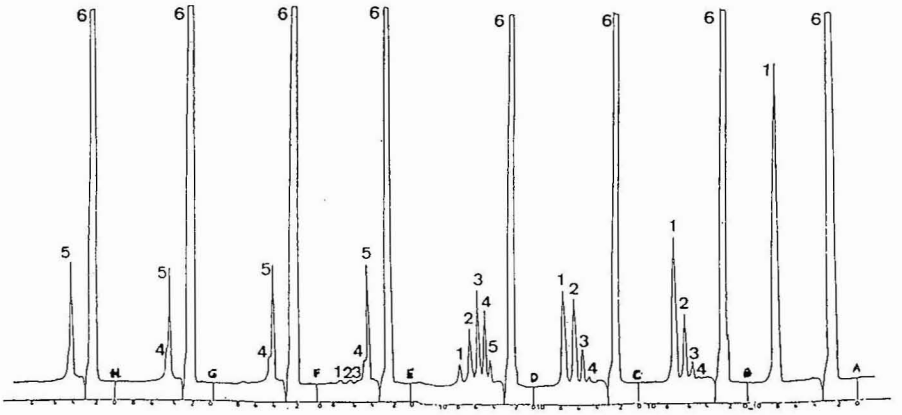

Fig. 3. Chromatogramme des dérivés formés successivement au cours de la réaction entre l'Irganox 1010 et l'isocyanate de phényle. Phase stationnaire: colonne C_{18} 250 × 4 mm I.D.; phase mobile: acétonitrile–tétrahydrofuranne–eau distillée (75:20:5, v/v/v); débit 1.5 ml/min, 280 nm, attenuation 0.32, papier 5 mm/min. Temps de réaction: (A) 0 min; (B) 0.5 min; (C) 1 min; (D) 2 min; (E) 3 min; (F) 4 min; (G) 5 min; (H) 7.5 min. Pics: 1 = Irganox 1010; 2 = Irganox 1010 mono-dérivé; 3 = Irganox 1010 di-dérivé; 4 = Irganox 1010 tri-dérivé; 5 = Irganox 1010 tétra-dérivé; 6 = réactif en excès.

apparait également deux composés intermédiaires mono- et di-dérivés. Cependant la réaction n'est pas totale à 25°C; il se produit un équilibre entre les formes di- et tri-dérivées et la réaction n'évolue plus après 15 min de contact (Fig. 2C).

L'Irganox 1010. Il est caractérisé par quatre groupements phénoliques. En moins de 5 min, le rendement maximal de la dérivation est atteint à température ambiante. Trois composés intermédiaires mono-, di- et tri-derivés sont formés avant d'aboutir au composé final (Figs. 2D et 3).

Les cinétiques de dérivation ont été déterminées à température ambiante afin d'en définir toutes les étapes; les résultats montrent que les composés dérivés sur l'ensemble des groupements phénoliques peuvent être obtenus à 25°C après un temps de contact plus ou moins important selon la structure des composés étudiés.

Mécanisme de la dérivation

Deux facteurs régissent la cinétique de dérivation des composés phénoliques dérivés du di-*tert.*-butyl-3′,5′-hydroxy-4′-benzène: facteur stérique et facteur électronique.

Pour les effets stériques considérés isolément, plus l'encombrement de l'hydrogène est grand (plus les groupements alkyles en *ortho* sont volumineux), plus la réaction est lente.

La contribution de l'effet électronique des groupements alkyles situés en *ortho*, en raison de leur effet inducteur + 1, se traduit par une mobilité accrue de l'hydrogène du groupement phénolique. La vitesse de la réaction est d'autant plus rapide que l'effet électrodonneur est plus important.

Dans le cas des antioxydants phénoliques utilisés dans les matériaux plastiques, tous possèdent deux groupements *tert.*-butyl en position 3′ et 5′ et donc un encombrement stérique équivalent. Seul l'effet électronique du substituant en position 1′ (en *para* du groupement phénolique) peut modifier la cinétique de la réaction.

L'isocyanate de phényle réagit avec l'Irganox 1076 selon une cinétique de 2ème ordre (Fig. 2A), probablement selon un mécanisme similaire à celui proposé par MacFarland et Gaskins [11].

Dans le cas des antioxydants polyphénoliques, l'isocyanate de phényle dérive successivement toutes les fonctions phénoliques. Ceci explique l'apparition de composés intermédiaires. La vitesse de la réaction évolue différemment selon la structure de l'antioxydant. En effet, pour le Goodrite 3114, il existe deux effets électroniques antagonistes; d'une part, les groupements *tert.*-butyl favorisent le départ de l'hydrogène du groupement phénolique, d'autre part, le noyau isocyanurate, par les effets électroattracteurs du carbonyle et de l'azote, tend à l'effet inverse. Au contraire, pour l'Ionox 330, les groupements *tert.*-butyl ainsi que le noyau triméthylbenzène favorisent la réactivité de l'hydrogène mobile. Cependant la réaction n'est pas totale à 25°C; un équilibre se forme entre les deux formes di- et tri-dérivées, qui peut être déplacé en faveur de la formation du composé terminal par augmentation de la température de contact.

Pour l'Irganox 1010, l'effet électronique est très intense car le substituant en *para* du groupement phénolique est important, ce qui augmente la mobilité de l'hydrogène du groupement phénolique et explique la cinétique rapide de la dérivation.

Analyse chromatographique et détection

Afin d'homogénéiser les conditions opératoires d'une analyse de routine tout en assurant une dérivation totale des antioxydants susceptibles d'être présents dans un matériau plastique, nous avons fixé les conditions de la dérivation à: 0.5 mg/ml d'antioxydant + 0.5 ml d'isocyanate de phényle; température 70°C; temps de contact 30 min; addition de méthanol 0.5 ml.

L'analyse chromatographique, par comparaison avec les temps de rétention des substances témoins, montre que les conditions de dérivation conduisent à la formation des composés totalement dérivés.

Les conditions chromatographiques proposées ont été mises en œuvre pour le suivi de dérivation mais elles sont tout à fait adéquates à l'analyse chromatographique des antioxydants après dérivation complète. Les produits secondairement formés dans le milieu réactionnel (issus des réactions de l'isocyanate de phényle avec le méthanol et les traces d'eau présentes dans les solvants utilisés) sont plus polaires et de masses

TABLEAU II

QUANTITÉ MINIMALE DÉTECTABLE DES ANTIOXYDANTS ÉTUDIÉS

Antioxydant	Détection		
	Avant dérivation	Après dérivation	
	280 nm (ng)	280 nm (ng)	230 nm (ng)
Irganox 1076	50	80	3
Goodrite 3114	10	16	1
Ionox 330	6	12	0.4
Irganox 1010	8	20	1

moléculaires plus faibles que les antioxydants étudiés dérivés ou non; ces produits apparaisent sous forme d'un pic important au début du chromatogramme et n'altèrent donc pas la spécificité de l'analyse.

Les antioxydants phénoliques présentent deux maxima d'absorption en UV situés vers 230 et 280 nm. Après dérivation, les phénylcarbamates d'aryle correspondants présentent un maximum d'absorption situé vers 230 nm, avec augmentation du coefficient d'absorbance. En effet, si la dérivation n'améliore pas la selectivité de la détection, elle augmente la sensibilité d'un facteur 5–15 selon l'antioxydant considéré (Tableau II).

Applications

La technique analytique décrite, du fait de sa sensibilité et de sa fiabilité de mise en œuvre, trouve des applications dans la recherche de traces d'antioxydants phénoliques. A titre d'exemple, l'Irganox 1010 a été identifié dans du polyéthylène haute densité qui constitue le conditionnement primaire de nombreuses solutions

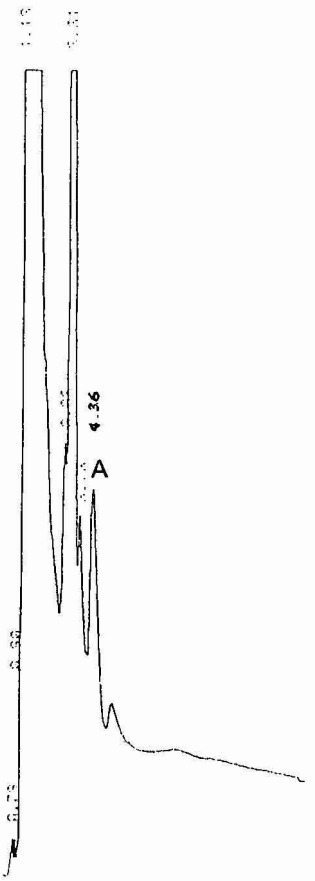

Fig. 4. Application à la recherche d'Irganox 1010 dans un conditionnement en polyéthylène haute densité. Conditions opératoires *cf.* texte. (A) Irganox 1010 tétra-dérivé.

d'intérêt pharmaceutique: après dissolution du matériau plastique par l'hexane au bain marie bouillant à reflux et reprécipitation par le méthanol, le surnageant contenant les additifs est filtré sur filtre papier et la solution obtenue est évaporée à sec. Le résidu est repris par 1 ml d'acétonitrile. La dérivation est ensuite réalisée selon les conditions opératoires précédemment décrites; le chromatogramme obtenu (Fig. 4) met en évidence l'Irganox 1010 tetradérivé correspondant à une teneur en Irganox 1010 voisine de 260 μg par g de matériau. Ainsi le procédé permet de déceler des traces d'antioxydants phénoliques migrant des conditionnements plastiques vers les produits pharmaceutiques ou cosmétiques (interactions contenant–contenu).

En outre, l'étude de la cinétique de dérivation permet, en examinant le nombre de dérivés intermédiairement formés, de définir le nombre de groupements phénoliques présents après traitement chimique ou par irradiation des matériaux plastiques [12]: cette technique peut donc apporter une contribution à l'étude du mécanisme d'action des antioxydants phénoliques en tant qu'agents de protection.

CONCLUSION

L'isocyanate de phényle, d'un emploi facile malgré une certaine toxicité, s'est ainsi révélé un réactif de dérivation particulièrement intéressant. Ce réactif, couplé aux techniques chromatographiques, est applicable à l'étude de nombreuses molécules: de façon similaire aux travaux entrepris sur les antioxydants phénoliques, la connaissance de la cinétique réactionelle de l'isocyanate de phényle avec d'autres molécules peut permettre de moduler les paramètres entrant dans la réaction de dérivation en vue de diverses applications dont la détermination de groupements fonctionnels (en étudiant le nombre de dérivés intermédiairement formés) et le dosage chromatographique avec amélioration de la détection (ceci nécessitant soit une dérivation complète, soit la formation d'un dérivé majoritaire).

RÉSUMÉ

La réactivité de l'isocyanate de phényle vis à vis de composés à hydrogène mobile a été utilisée sur les antioxydants phénoliques habituellement rencontrés dans les matériaux plastiques. La première partie de ce travail consiste en l'étude de la cinétique et du mécanisme de la réaction, afin d'en optimiser les paramètres. Cette méthode a ensuite été appliquée à l'analyse des antioxydants phénoliques par chromatographie en phase liquide à polarité de phases inversée. Les phénylcarbamates d'aryle obtenus sont détectables à 230 nm et la quantité minimale détectable est diminuée, selon les composés étudiés, d'un facteur 5 à 15 par rapport à la détection directe à 280 nm.

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Dynamic impregnation of silica stationary phases for the argentation chromatography of lipids

K. AITZETMÜLLER* and LIRENY A. GUARALDO GONCALVES[a]
Institute of Chemistry and Physics, Federal Centre for Lipid Research, Piusallee 68-76, Münster (F.R.G.)
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ABSTRACT

Homogeneous silver-impregnated thin-layer chromatographic (TLC) plates were obtained in a dynamic manner by chromatographing an acetonitrile solution of silver nitrate on ready-made silica TLC plates. Plates obtained in this manner are very reproducible. Separations according to the number of double bonds in the individual triglycerides on dynamically impregnated plates are equal to or better than those obtained on silver nitrate TLC plates prepared in the conventional manner. The plates are easier to handle, and savings in silver nitrate result in comparison with the dipping procedure. It is possible to impregnate a plate simultaneously with both phloxin and silver in one run, the immobilized phloxin then serving as a fluorescence detection agent for a subsequent triglyceride separation and for semi-quantitative densitometry. Silica high-performance liquid chromatographic columns can also be impregnated with silver nitrate in a similar dynamic manner.

INTRODUCTION

Argentation thin-layer chromatography (TLC) is a well established and important method for the separation of unsaturated lipid species such as triglycerides, wax esters, fatty acid methyl esters and steroids [1–10]. There are hundreds of papers describing this technique, its selectivity and its applications with lipids, and a number of reviews have appeared [3].

Usually, the silver nitrate ($AgNO_3$)-containing silica plates used for this technique are prepared in the laboratory by spreading an aqueous (or aqueous–alcoholic) slurry of silica on glass plates [1–5]. The $AgNO_3$ is dissolved in the solvent mixture used to prepare the slurry, and the amount of $AgNO_3$ used is calculated as, *e.g.*, 5, 10 or 15% by weight based on the weight of silica used. Plates prepared in this way are ready for use after drying and activation.

As $AgNO_3$–silica plates prepared manually in this traditional way are often less than ideal with respect to homogeneity, resolving power and mechanical stability, various other methods of impregnating the more homogeneous and standardized commercially available ready-made pre-coated silica TLC plates have been sought.

[a] Present address: Universidade Estadual de Campinas–F.E.A., Caixa Postal 6091, 13.083 Campinas SP, Brasil.

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It has been reported [6–10] that good-quality $AgNO_3$–silica TLC plates can be produced from commercially available ready-made TLC plates simply by dipping the whole plate in a solution of $AgNO_3$ in water, ethanol, methanol or acetonitrile. The plate is dipped into the solution for about 20–100 s, then quickly removed, allowed to dry and activated. A similar procedure was used in the authors' laboratory during the 1970s [11].

Another procedure is to spray pre-coated silica plates evenly with a solution of $AgNO_3$ in water or in other solvents [12,13].

In previous work, the best results were achieved by dipping pre-coated Merck TLC plates into a 10–20% solution of $AgNO_3$ in acetonitrile for about 30 s. In our hands, plates produced in this way gave better separation results than manually prepared plates [11]. However, the plates still exhibited a number of minor disadvantages, so that other ways of preparing silver-containing plates were sought.

Another impregnation procedure has been in use for some years that avoids most of these disadvantages, and it is described in detail in this paper.

EXPERIMENTAL

Dynamic impregnation of TLC plates

Ready-made TLC plates are impregnated with $AgNO_3$ in a dynamic and reproducible way as follows. We use a TLC tank containing a small separate rectangular vessel (*ca.* 1 cm × 21 cm × 1 cm), the latter containing a 10–20% solution of $AgNO_3$ in acetonitrile (Fig. 1). (The tank may contain a piece of cardboard in another separate chamber or vessel with pure acetonitrile to saturate the tank volume

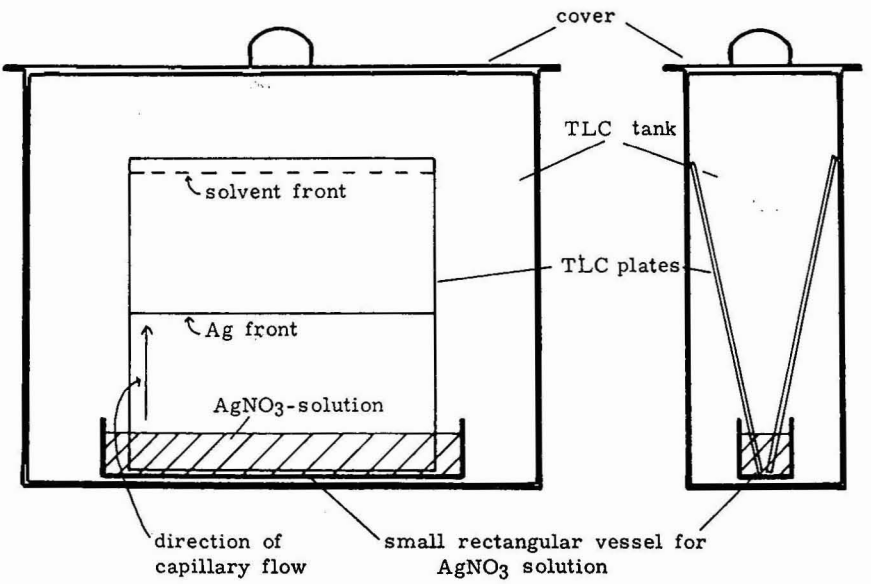

Fig. 1. Schematic diagram of equipment used for dynamic impregnation of commercial ready-made silica TLC plates.

with acetonitrile vapour. However, we prefer to impregnate the plates without chamber saturation.) The ready-made silica TLC plate is then placed in the small vessel containing the silver solution, which is allowed to rise by capillary forces as in ascending chromatography, displacing the air in the silica layer as it moves. Some time after the acetonitrile solvent front has reached the top of the plate, the plate is removed from the tank and allowed to dry. Because of the toxicity of acetonitrile vapour, the whole operation is carried out under a hood.

Silver is adsorbed from the acetonitrile solution by the silica on the plate and therefore the silver does not move all the way with the solvent front (Fig. 1). Plates prepared in this dynamic way will therefore contain $AgNO_3$ only on the lower two thirds to three quarters of the plate, and there is a separate "silver front" which is usually clearly visible. Although gloves must still be used when handling acetonitrile, the dry plates can safely be handled without gloves if the operator takes care to touch the plates only at the silver-free top.

In normal use, TLC plates are often cleaned or pre-washed (*e.g.*, by ascending chloroform–methanol) before applying the samples, to remove dirt, lipids and other organic material adsorbed from the air and packaging materials. With this type of dynamic impregnation, pre-washing of the plates is often not necessary because organic material is displaced by the acetonitrile solvent front. After detection, this displaced material is often seen near the solvent front.

Silver determination

For the determination of the silver distribution on the plates, impregnated plates were divided into narrow horizontal bands and the silica was scraped off and analysed for its silver content by atomic absorption spectrometry and/or potentiometric titration.

Thin-layer chromatography

TLC of triglyceridic fats was carried out as usual. Many solvent systems have been described, and for the best results in double-bond (DB) separations of triglycerides mobile phases containing benzene or toluene have often been used in the past [1–5]. Chlorinated solvents such as chloroform or dichloromethane and diethyl ether can also be used, often to achieve special effects, and always in mixtures with an excess of aliphatic hydrocarbon (hexane, heptane, isooctane or light petroleum). In this work, good results were obtained using hexane–toluene–diethyl ether (42:50:8, v/v/v) as the mobile phase for separations of triglycerides with 0–3 *cis* double bonds. For the more highly unsaturated triglycerides, more polar mobile phases, including mixtures containing increasing proportions of chloroform and/or ethanol, were used.

Spotting of the samples is best done in the form of narrow linear bands of length 2–10 cm using automatic equipment (Linomat IV, Camag). The amounts of sample used range from a few micrograms per spot up to 50 mg per plate. Bands of separated triglycerides can be made visible either by charring or by spraying with a fluorescent indicator, *e.g.*, 2′,7′-dichlorofluorescein [1–5,14] or phloxin [15,16] (see below). For the identification of the separated zones, palm oil or an interesterification mixture of triolein and tripalmitin was often used and was spotted on one side of the plate. The migration distances or R_F values of their major triglycerides (PPP, POP, PPO, POO and OOO) are then compared with those of the sample triglycerides. One has to take

into account, however, that overloading of major triglycerides, particularly in semi-preparative applications, may move that particular band higher up the plate.

Dynamic impregnation with both silver and phloxin

Phloxin (C.I. 45405) or Phloxine B (C.I. 45410) can also be used as an indirect fluorescence detection agent for lipids on silver-impregnated plates. Hammond [15,16] preferred phloxin for densitometric scans. We have now found that it is possible to impregnate ready-made commercial TLC plates simultaneously with both silver and phloxin in one ascending run. For this, we use a solution of 10% $AgNO_3$ plus 0.08% phloxin in acetonitrile.

After spotting triglyceride samples, silver/phloxin-impregnated plates are developed normally. The solvents regularly used for triglyceride argentation TLC do not move phloxin, and the separated triglyceride bands are immediately visible under UV light, after removing the plate and air-drying it under a hood. The technique described here for phloxin does not work with 2′,7′-dichlorofluorescein. The phloxin present also permits indirect fluorescence densitometry of triglycerides [15–17].

In situ impregnation of high-performance liquid chromatographic (HPLC) columns

For the impregnation of silica HPLC columns with silver, we use the following procedure. A conventional silica HPLC column is mounted and heptane is pumped through it continuously. A large, 2-ml loop valve (Specac, Glasgow, U.K.) is mounted between pump and the injector, using wide-bore capillaries. The 2-ml loop is filled with a 10% solution of $AgNO_3$ in acetonitrile and this is injected onto the column as a 2-ml plug of immiscible acetonitrile solution within a stream of flowing heptane. The acetonitrile is pumped through the column, which is then rinsed by pumping heptane for another 15–30 min. The whole operation may be repeated. After this, the column must be reactivated by pumping heptane–diethyl ether mixtures until all the adsorbed acetonitrile is removed and the retention and separation of a test mixture (preferably oleic and elaidic acid methyl esters) is stable.

RESULTS AND DISCUSSION

Dynamically impregnated TLC plates

Among the disadvantages of the earlier dipping procedures were the following: (a) air was often trapped in the silica layer during the dipping process, leading to unequal wetting in parts of the plate; (b) the dipping, run-off and drying process could lead to inhomogeneous deposition of $AgNO_3$, including crystallization effects; (c) the dipping as such was not easy, as vessels of suitable size and material (to withstand both the solvents and the $AgNO_3$) were difficult to find, contamination occurred all over the hood or bench and gloves had to be used all the time to avoid contact with $AgNO_3$ whenever the plate was touched; and (d) relatively large volumes of solvent and large amounts of expensive $AgNO_3$ had to be used or were wasted.

With the dynamic procedure described here, it was of course of primary interest whether or not an even distribution of silver along the plate could be achieved by ascending impregnation. This could be expected if the solvent used does not readily displace silver from the active sites on the silica, so that an eventual saturation of active sites with silver would occur. Previous attempts with ascending impregnation using

aqueous solutions of $AgNO_3$ [14] have met with limited success; at least the technique has been neglected after these initial experiments.

The amount of silver on the silica TLC plates was usually *ca.* 11–13% of the weight of the silica when a 15% solution of $AgNO_3$ in acetonitrile was used for ascending impregnation without chamber saturation. Fig. 2 shows the distribution of silver (as percentage of silver calculated on the silica) as determined by potentiometric titration. For this, zones were removed at various intervals from the plates in the analysis of two different plates. The results indicate that silver is indeed fairly evenly and reproducibly distributed, although the level decreases slightly towards the silver front. This fairly even distribution of silver is found because adsorption with saturation of active sites occurs, because there are no air pockets (air is displaced) and because there is no run-off problem (the plate is equally wet, except for the lowest 1 cm in the $AgNO_3$ solution) when the plate is removed from the tank.

The impregnation process takes longer than the dipping or spraying procedures (*ca.* 30–60 min per plate), but is easier to carry out and requires much smaller volumes of $AgNO_3$ solution. Waste of expensive $AgNO_3$ and contamination of the workplace with silver is minimized. The plates can be stored dry in the dark and their mechanical stability is excellent. Results achieved with plates of this type are at least as good as those obtained with plates impregnated by dipping, and usually much better than those with laboratory-made plates prepared by spreading a slurry on glass. It should be kept in mind, however, that the silver ends at two thirds to three quarters of the height of the plate and that above the "silver front" no further separation due to the presence of silver can take place.

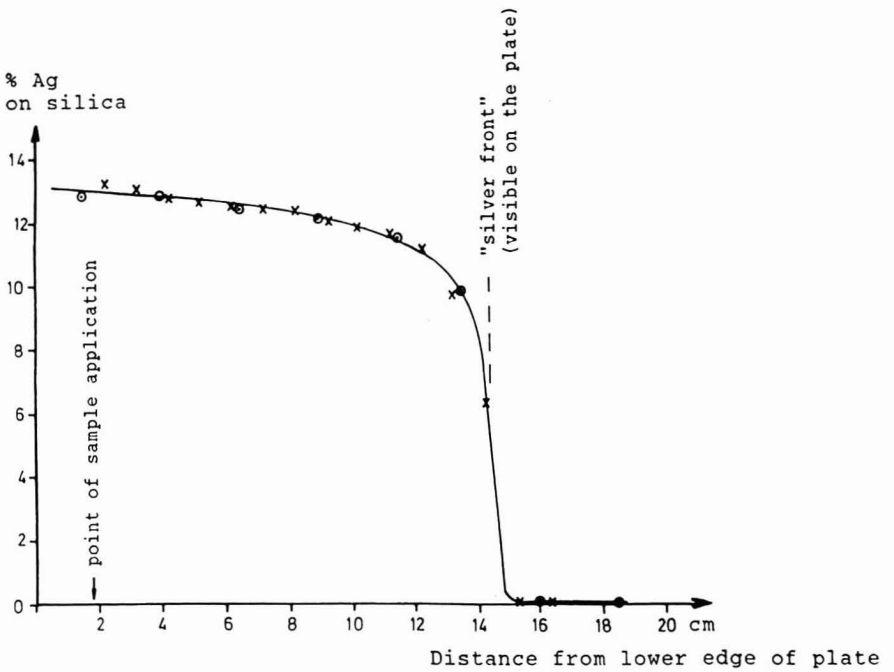

Fig. 2. Curve showing distribution of silver in two dynamically impregnated plates (○, plate 1; ×, plate 2) and relation of silver front to solvent front, as determined by experiment.

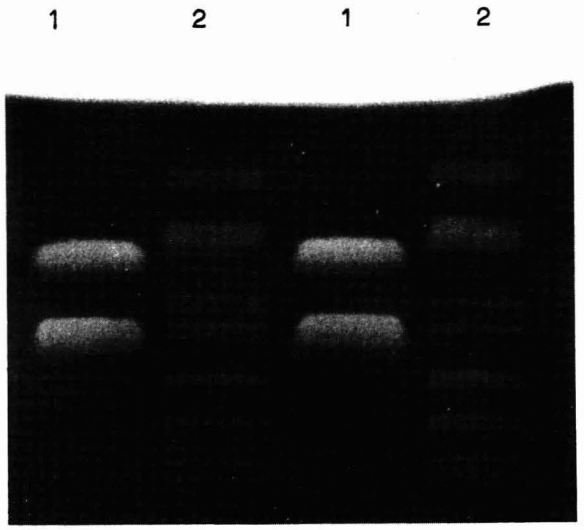

Fig. 3. Separations of elaidic (top) and oleic acid methyl esters (lane 1) and of a triglyceride test mixture (lane 2). The triglyceride test mixture consisted essentially of (from the top) SSS + PPP; PEP + PPE; SOS + POP; SSO + PPO; EEE; SOO + POO; and OOO (S = stearic, P = palmitic, O = oleic and E = elaidic acid residues). Total lipid applied: 100 μg per 3-cm band. Solvent: toluene–hexane (85:15, v/v), developed to the top of the plate. Detection with 2′,7′-dichlorofluorescein.

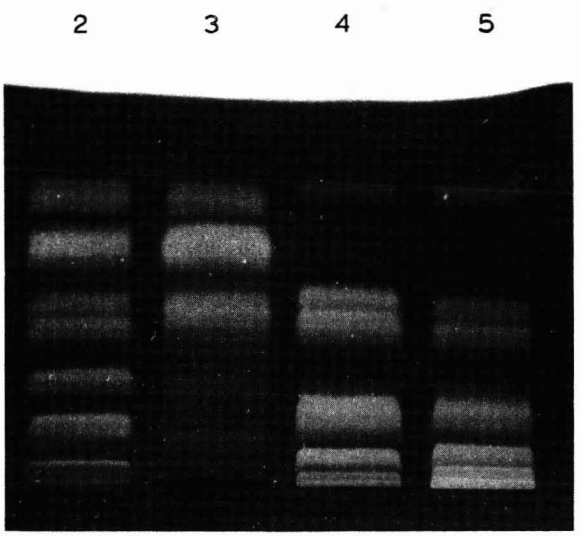

Fig. 4. Separation of various triglyceride mixtures and fat compositions. Experimental details as in Fig. 3. Lanes: 2 = as lane 2 in Fig. 3; 3 = elaidinized fraction of a palm oil; 4 = lower melting fraction of an interesterified mixture (*ca.* 2:1) of olive oil and hardened sunflower oil; 5 = interesterified mixture (*ca.* 3:1) of rapeseed oil and hardened sunflower oil.

With a number of mobile phases, the movement of triglyceride bands on the silver-containing part of the plate is faster than that on the silver-free part of the plate near the top. In some benzene- or toluene-based mobile phases [18], triglycerides will be retarded or may even stop moving when they reach the silver-free part of the plate, so that the best procedures for triglyceride separations are those where saturated triglycerides such as tristearin or tripalmitin, as the fastest moving triglyceride in most mixtures, migrate to a distance just below the "silver front". The difference in mobility between silver-containing and silver-free areas of the plate is less pronounced when more polar mobile phase modifiers are used, such as diethyl ether, acetone or tetrahydrofuran in hexane. Thus, with these partially impregnated plates and with a proper choice of solvents, special effects can be achieved [18]. The use of partially impregnated plates for two-dimensional TLC has been described [14].

The "silver front" can be seen easily with the naked eye on both wet and dry plates. After spraying with lipid reagents such as 2′,7′-dichlorofluorescein, the area above the "silver front" appears yellow and the silver-containing area of the plate is reddish pink. Figs. 3 and 4 show examples of triglyceride separations by degree of unsaturation.

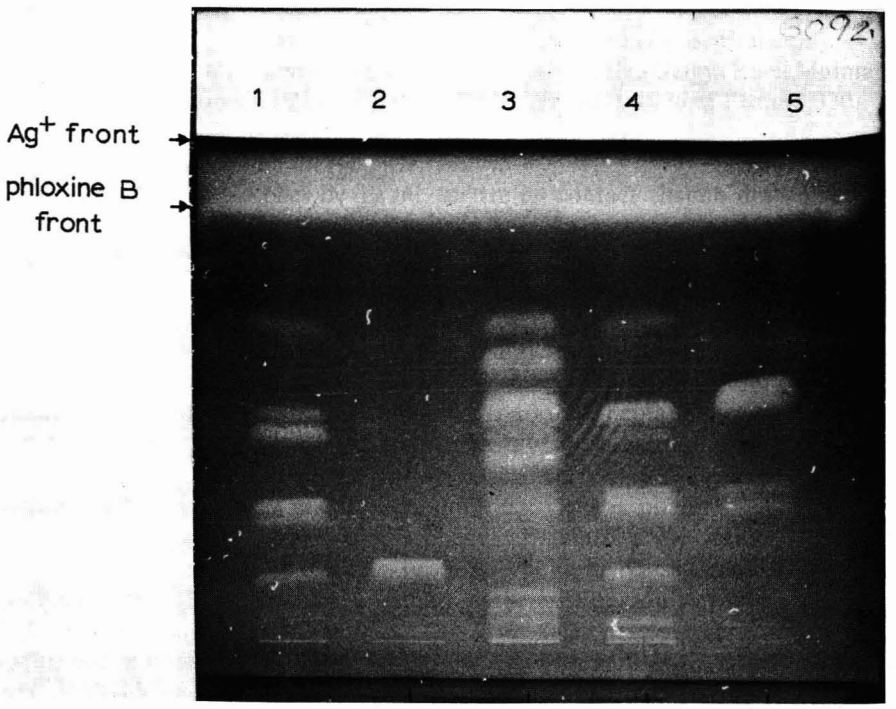

Fig. 5. Silver–phloxin plate, impregnated by ascending chromatography with a solution of 10% $AgNO_3$ plus 0.08% phloxin in acetonitrile. Mobile phase for the triglyceride separation: hexane–toluene–diethyl ether (42:50:8, v/v/v); solvent migration until the silver front. Both the "silver front" and the "phloxin front" are clearly visible. Lanes: 1 = interesterified mixture of triolein and tripalmitin; 2 = triolein; 3 = partially hardened palm oil; 4 = commercial palm oil; 5 = cocoa butter. Total lipid applied: 100 μg per 2-cm Linomat band.

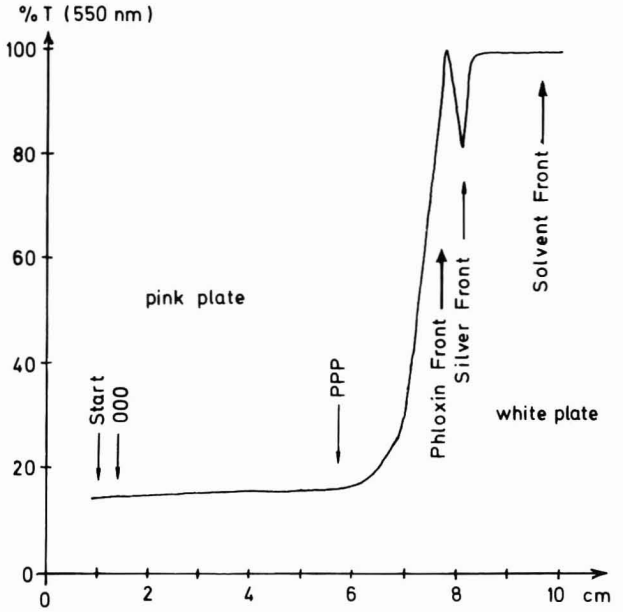

Fig. 6. Dynamic simultaneous impregnation of a TLC plate with silver and phloxin in one run. Phloxin distribution across the plate, as measured by reflectance densitometry in visible light at 550 nm. There is a "phloxin front" visible just below the silver front.

Plates impregnated with silver and phloxin

Impregnation with $AgNO_3$ and phloxin has the added advantage that no separate spraying procedure is needed and that the phloxin is evenly distributed across the lower half of the plate (as is the silver).

With dynamically (silver–phloxin)-impregnated plates, a clearly visible "phloxin front" is found about 1 cm behind the "silver front", which in turn is a few centimetres below the solvent front (Fig. 5). The phloxin distribution on the plate can be measured in UV or visible light, and indeed it can be continuously measured by scanning a lipid-free lane of the plate using visible light reflectance densitometry (Fig. 6). The phloxin distribution across the plate shows a pattern similar to the silver distribution.

Semi-quantitative work using densitometry of induced fluorescence on plates of this type will be described in a subsequent paper [17].

First results with dynamically impregnated silver HPLC columns

Commercial ready-made silica HPLC columns usually have much higher plate numbers than laboratory-made $AgNO_3$–silica columns, where the $AgNO_3$ is first deposited on the silica and then a slurry of $AgNO_3$–silica is pumped into the empty column. If "dynamic impregnation" works equally well on columns as on plates, then it should be possible to prepare $AgNO_3$-containing columns of good quality in simple, clean operation.

Initially, we thought that plugging of the column and frits by precipitated $AgNO_3$ from the end zones of the acetonitrile solvent plug would be a problem. However, this was not the case and only once was a narrow-bore capillary between the

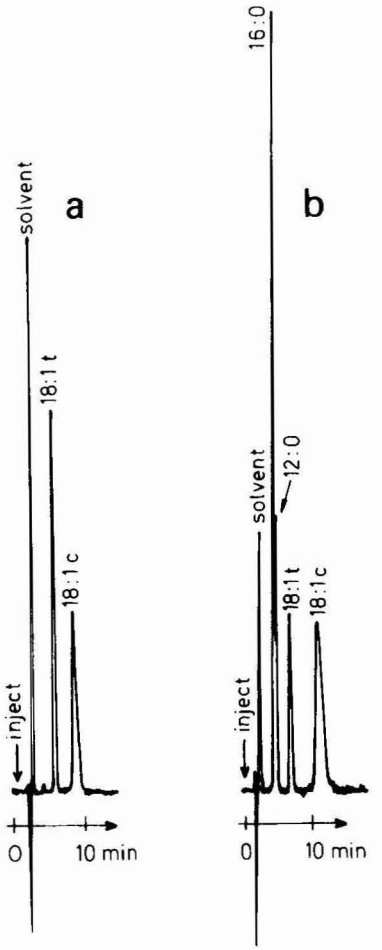

Fig. 7. Separation of saturated and *cis*- and *trans*-monoenoic fatty acid methyl esters on a dynamically impregnated $AgNO_3$–silica HPLC column. Conditions: 150 × 4.6 mm I.D. 5-μm silica column, dynamically impregnated (see text); isocratic mobile phase, (a) 2% diethyl ether in heptane and (b) 1.6% diethyl ether in heptane; flow-rate, 1.5 ml/min; detection, refractive index.

injector and column blocked. (We suggest removing the injector and using wide-bore capillaries only for the impregnation step.)

Initial experiments showed useful *cis–trans* resolution from columns impregnated in this way (Fig. 7). Work is in progress to determine if this technique can be developed further as a useful alternative to the conventional preparation of $AgNO_3$–silica HPLC columns, and to find the optimum conditions for dynamic silver impregnation and column reactivation. In earlier work by others [19] it was found that glass columns filled with a slurry of silica could also be impregnated by percolating $AgNO_3$ solution through the slurry column.

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Note

Comparative study of some columns for direct determination of carbofuran by gas–liquid chromatography with nitrogen-specific detection

CONCEPCION F. LING and GONZALO P. MELIAN

Departamento de Química Analítica, Escuela Técnica Superior de Ingenieros Industriales, Tafira Baja s/n, 35194 Las Palmas de Gran Canaria (Spain)

FERNANDO JIMENEZ-CONDE

Servicio Agrícola, Caja Insular de Ahorros de Gran Canaria, P.O. Box 854, 35080 Las Palmas de Gran Canaria (Spain)

and

EUGENIO REVILLA*

Departamento de Química Agrícola, Geología y Geoquímica, Universidad Autónoma de Madrid, 28049 Madrid (Spain)

(First received December 6th, 1989; revised manuscript received June 7th, 1990)

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuranyl-N-methyl carbamate) is a broad-spectrum pesticide used for the control of insects, mites and nematodes in a variety of edible crops. Its determination may be carried out by several methods which involve gas–liquid chromatography (GLC).

Direct determination of carbofuran may be accomplished by using microcolumetric detection [1], electrolytic conductivity detection [2] and nitrogen-specific detection [3,4]. Several methods make use of suitable derivatives for GLC, because these products are usually more sensitive and chromatographically stable. For this purpose, several procedures involving hydrolysis followed by trichloroacetylation of the phenolic moiety [5], trifluoracetylation [6], thiophosphorylation [7] and mesylate derivatization [8] have been proposed, using electron-capture or flame photometric detection. The disadvantages of derivatization methods are that they are time consuming and increase the chances of errors due to extra sample manipulation.

Direct GLC of carbofuran may be of use for residue control in fruits or for controlling concentrations in irrigation water or nutrient solutions. For these reasons, the suitability of three GLC columns for the direct determination of carbofuran were tested for their efficiency and also to establish the extent of thermal degradation, if any.

EXPERIMENTAL

Instrumentation

Nitrogen-specific GLC was performed on a Perkin-Elmer gas chromatograph

0021-9673/90/$03.50

equipped with a Perkin-Elmer 2B N–P thermionic detector run in the nitrogen mode at 220°C, a Sigma 10B integrator and a Model 550 terminal, using nitrogen as carrier gas. The chromatographic conditions were as follows: injector temperature, 220°C; column temperature, 170°C; hydrogen pressure, 10 p.s.i.; and air pressure, 10 p.s.i. Three columns were tested: 2% OV-101 on Chromosorb WP (100–120 mesh), 122 cm × 2 mm I.D., used by Leppert *et al.* [9] for the determination of carbofuran residues with nitrogen-specific detection; 5% OV-101 on Chromosorb WP (80–100 mesh), 122 cm × 2 mm I.D., used by Nelsen and Cook [4] for direct determination of carbofuran with nitrogen-specific detection; and 1.5% SP-2250 + 1.95% SP-2401 (100–120 mesh) (Supelco, Bellefonte, PA, U.S.A.), 200 cm × 4 mm I.D., described by Technokroma (Barcelona, Spain) for general analyses for pesticides [10].

Reagents

A standard of carbofuran (99.6% purity) was obtained from FMC (Middleport, NY, U.S.A.). A standard of phosphamidon (95% purity) was obtained from PolyScience (Niles, IL, U.S.A.) and used for internal standard calibration. Ethyl acetate and benzene were used for preparing standard solutions of carbofuran and phosphamidon, respectively.

RESULTS AND DISCUSSION

In order to check the linearity of the GLC response, the external standard procedure was used for all columns. In each instance, calibration graphs were obtained by injecting five different volumes of a solution of carbofuran in ethyl acetate (8.52 mg dm^{-3}). Table I shows the nitrogen flow-rate used in the analyses, the mean peak areas corresponding to three injections of 3 μl and their standard deviations, the correlation coefficients for the calibration graph and the retention times for carbofuran. The results indicate that the 2% OV-101 column shows a higher response for a 3-μl injection and a higher correlation coefficient than the other two columns for the calibration graphs. Therefore, the sensitivity of the system using 2% OV-101 on a Chromosorb WP column for the direct determination of carbofuran was checked in

TABLE I

COMPARATIVE BEHAVIOUR OF COLUMNS USED FOR DIRECT DETERMINATION OF CARBOFURAN

Parameter	Column		
	2% OV-101	5% OV-101	1.5% SP-2250 + 1.95% SP-2401
N_2 flow-rate (cm^3 min^{-1})	30	28	35
Mean peak area for 3-μl injections	28.2210	6.5100	8.6126
Standard deviation for 3-μl injections	0.4222	0.0195	0.0788
Correlation coefficient	0.9995	0.9835	0.9575
Retention time (min)	2.62	7.67	5.30

TABLE II

CORRELATION COEFFICIENTS FOR DIRECT DETERMINATION OF CARBOFURAN BY THE EXTERNAL STANDARD PROCEDURE WITH 2% OV-101 ON A CHROMOSORB WP (80–100 MESH) COLUMN

Range of concentration (mg dm^{-3})	Correlation coefficient
8.52–1.70	0.9948
1.70–0.34	0.9645

TABLE III

CORRELATION COEFFICIENTS FOR DIRECT DETERMINATION OF CARBOFURAN BY THE INTERNAL STANDARD PROCEDURE WITH PHOSPHAMIDON USING 2% OV-101 ON A CHROMOSORB WP (80–100 MESH) COLUMN

Range of carbofuran concentration (mg dm^{-3})	Phosphamidon (mg dm^{-3})	Correlation coefficient
43.03–8.60	7.12	0.9943
8.60–1.72	2.37	0.9958
1.72–0.34	0.19	0.9930

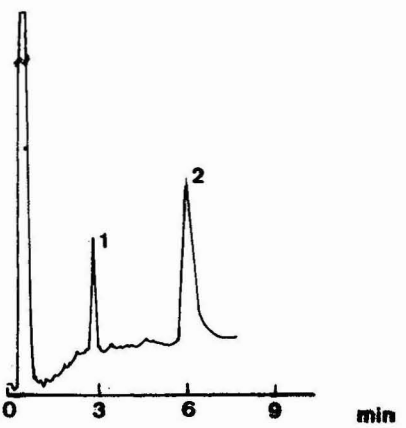

Fig. 1. Chromatographic separation of carbofuran (peak 1) and phosphamidon (peak 2) with 2% OV-101 on a Chromosorb WP column.

the range 8.52–0.34 mg dm^{-3}. Table II gives the correlation coefficients obtained in these experiments for two ranges of concentration.

A similar study was made using phosphamidon as an internal standard. Table III gives the correlation coefficients for three different ranges of carbofuran concentration, with the concentration of phosphamidon used in each instance. High correlation coefficients were obtained for all three ranges of concentration. Fig.

TABLE IV

MEASURED VALUES, RELATED STATISTICAL PARAMETERS AND LIMITS OF DETECTION FOR DIRECT DETERMINATION OF CARBOFURAN WITH 2% OV-101 ON A CHROMOSORB WP COLUMN USING EXTERNAL AND INTERNAL STANDARD PROCEDURES

Parameter	External standard procedure	Internal standard procedure
Amount of carbofuran (mg dm^{-3})	0.34	0.34
Analytical values (mg dm^{-3})	0.39	0.31
	0.34	0.33
	0.37	0.34
	0.34	0.32
Mean value (mg dm^{-3})	0.36	0.32
Standard deviation	0.0252	0.0129
Relative standard deviation (%)	7.00	3.97
Limit of detection (mg dm^{-3})	0.12	0.05

1 shows a chromatogram corresponding to the separation of carbofuran (peak 1) and phosphamidon (peak 2), without noticeable interferences.

Table IV gives the limits of detection for both methods of calibration (external and internal standard procedures) using 2% OV-101 on a Chromosorb WP column and samples containing 0.34 mg dm^{-3} of carbofuran. A lower limit of detection was obtained when the internal standard procedure was used.

These studies show that GLC with 2% OV-101 on a Chromosorb WP column with nitrogen-specific detection may be a good choice for the direct determination of carbofuran using external or internal standard procedures.

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Note

Determination of residual acrylamide monomer in solution and emulsion polymers by column-switching high-performance liquid chromatography

AMY M. TSENG
Nalco Chemical Company, 1 Nalco Center, Naperville, IL 60563-1198 (U.S.A.)
(Received May 29th, 1990)

Acrylamide monomer is used in the manufacture of many polymeric products employed for water treatment applications. Such products may contain residual acrylamide monomers, which because of its toxicity, must be monitored routinely to ensure product safety and to meet government regulations. Currently, most methods used for monitoring manufacturing processes are limited in detection capability to about 0.01% (100 ppm) residual acrylamide [1–9]. As acrylamide levels are likely to come under closer scrutiny in the near future, a lower detection limit will probably be required. New quality control procedures will not only have to be simple and easily automated, but will also have to be capable of determining very low levels of acrylamide without interferences from other components in the sample. Numerous methods for determination of trace levels of acrylamide in water have been reported in literature [10–17]. However, most of these methods require time-consuming sample preparation and are subject to interferences from polymer sample matrices. They are not readily applicable to trace level acrylamide monitoring in polymeric systems. Freshour *et al.* [18] has reported a relatively simple procedure using column switching for determination of trace levels of acrylamide in tissue culture. With modification, this column-switching technique has been adapted for use on polymeric samples. The modified method consists of simple dilution or extraction–precipitation of polymer followed by high-performance liquid chromatographic (HPLC) analysis using UV detection at 210 nm. Detection limits of 5 ppm and 0.5 ppm can be obtained in emulsion polymers and solution polymers, respectively. This paper describes the detailed procedure and validation data for residual acrylamide determinations in both solution and emulsion polymers.

EXPERIMENTAL

Reagents and materials

The following materials and reagents were used: acrylamide (99+%), electrophoresis grade, Gold Label (Aldrich, Milwaukee, WI, U.S.A.), concentrated sulfuric

acid (J. T. Baker, Phillipsburg, NJ, U.S.A.), acetonitrile, methanol and tetrahydrofuran (glass-distilled HPLC grade, Burdick & Jackson, Muskegon, MI, U.S.A.). Water was purified using a Milli-Q water-purification system (Millipore, Bedford, MA, U.S.A.). All solvents for HPLC analysis were filtered through 0.45-μm Durapore filter paper (Millipore) and vacuum degassed before use. All polymer samples analyzed were prepared in-house and are representative of the types of samples used in typical water treatment applications.

Standard solutions

Stock standard solution of acrylamide was prepared by dissolving a known weight of acrylamide in water at a concentration of about 100 ppm. A set of standard solutions were prepared by diluting aliquots of the stock solution with water and 1 ml of acetonitrile in 100-ml volumetric flasks. The concentration range of the working standard solutions was 1.0 ppm to 0.005 ppm.

Sample preparation

Water treatment polymers containing acrylamide can be classified according to their manufacturing process as either solution polymers or emulsion (latex) polymers. The sample preparation for solution polymers was simple dilution of about 1.0 g of polymer product with 1 ml of acetonitrile and water in a 100-ml volumetric flask. These solutions were then mixed well and filtered through 0.45-μm Millipore Millex-HV filter units prior to HPLC analysis.

Emulsion polymers were further classified according to type as either non-ionic, anionic or cationic emulsion polymers. Basic sample preparation for emulsion polymers consisted of initial polymer precipitation with an organic solvent followed by dilution of the supernatant with water. Because the cationic and anionic polymers behaved quite differently in organic solvent matrices, *different* organic solvents were used to precipitate the emulsion polymers to ensure formation of a fine precipitate which would prevent entrapment of the residual monomers. Acetonitrile was used for the precipitation of cationic emulsion polymers, whereas acetonitrile–methanol (50:50, v/v) was used for non-ionic and anionic emulsion polymers. In addition, if the emulsions were prepared with polymeric surfactants, acetonitrile–tetrahydrofuran (50:50, v/v) was used for precipitation of both cationic and anionic polymers.

Precipitation was performed by adding about 1.0 g of latex polymer dropwise through a 20 gauge sterile disposable needle into 10 ml of appropriate solvent with continuous stirring. A fine powder of the polymer was formed. The sample was then capped and stirred for an additional 30 min. The precipitated polymer was allowed to settle and 1 ml of the supernatant was further diluted with water to 100 ml in a volumetric flask. The diluted supernatant generally turned cloudy at this point due to the presence of oil and surfactants. An aliquot of this solution was filtered through 0.45-μm Millex-HV filter, and the clear filtrate was used for HPLC analysis.

Instrumentation and chromatography

The HPLC system consisted of two Model M6000A pumps (Waters Assoc., Milford, MA, U.S.A.), two Model SPD-6AV UV–VIS spectrophotometric detectors (Shimadzu, Wood Dale, IL, U.S.A.), one corrosion-resistant six-port switching valve with air actuator (Valco, Houston, TX, U.S.A.; part No. AC6WHC), and two

columns. The first column was an RCM-100 radial compression module with a 10-μm 10 cm × 8 mm Radial-Pak C_{18} column and Guard-Pak Resolve C_{18} (Waters Assoc.). The second column was a Bio-Rad HPLC fast acid analysis column, 10 cm × 7.8 mm (Bio-Rad, Richmond, CA, U.S.A.). A Micromeritics Model 725 Autosampler (Alcott Chromatography, Norcross, GA, U.S.A.) with a 100-μl loop was used for sample injection. Fig. 1 shows the complete instrument set-up. The switching valve was plumbed as described by Freshour *et al.* [18], using heart cut switching technique. Column switching time, chromatographic data collection, and integration were controlled by a P. E. Nelson Analytical Data System Model 4430 (P. E. Nelson Analytical, CA, U.S.A.). The mobile phase for both columns was 0.02 *M* sulphuric acid. A flow-rate of 1.0 ml/min was used for the first column while 0.6 ml/min was used for the second column. The UV detectors were operated at 210 nm with sensitivity of 0.002 a.u.f.s. for monitoring the effluent from the second column. The UV detector at the end of the first column was used solely for the purpose of establishing the column-switching time. After this valve-switching time has been established, only one UV detector is needed for analysis. Quantitation was based on the peak area of the sample and external standard calibration.

RESULTS AND DISCUSSION

Determinations of residual acrylamide in polymeric systems are commonly performed using single-column HPLC techniques. Oligomers, surfactants, additives

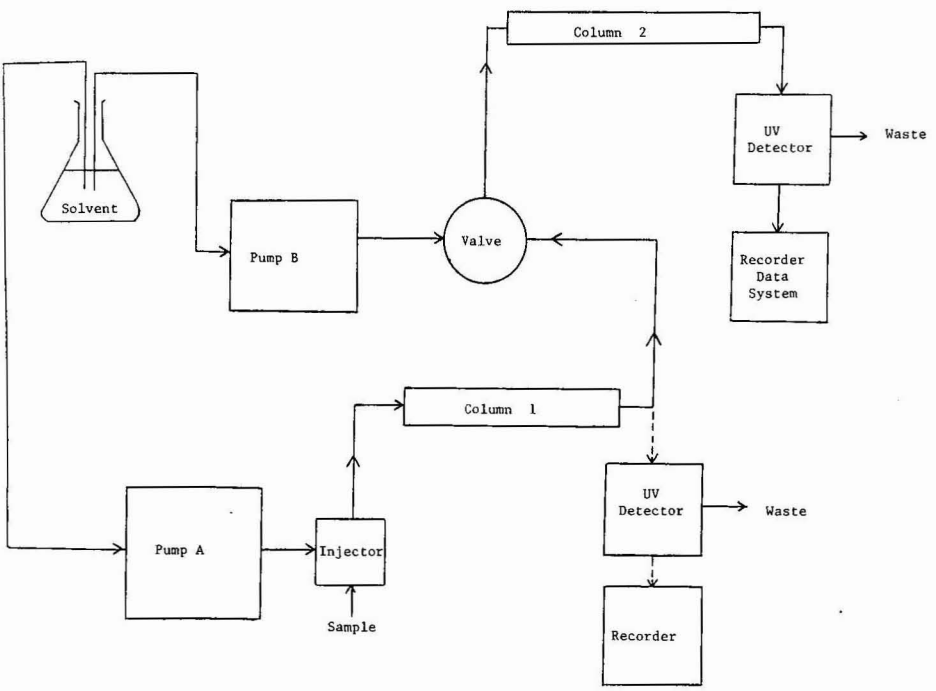

Fig. 1. A block diagram of column-switching system set-up. Pump A: 1.0 ml/min; pump B: 0.6 ml/min; column 1: RCM-100 Radial-Pak C_{18}; column 2: Bio-Rad fast acid analysis column.

and oils frequently interfered with the analysis, particularly when extremely low levels residual acrylamide are being determined. These interferences can be minimized using a two-column separation. This column-switching technique initially separates residual acrylamide on a reversed-phase C_{18} column; the acrylamide peak is then switched onto a second column (fast acid column) for further separation by adsorption chromatography. By combining the two separation techniques (reversed-phase and adsorption) most of the interfering materials in the polymeric sample can be eliminated. Acrylamide can now be determined directly and accurately in both solution and latex polymeric systems at very low levels. Fig. 2 shows a typical chromatogram.

The limit of detection for acrylamide in our current instrumental set up is 0.005 ppm at a 95% confidence level, which corresponds to a detection limit of 5 ppm and 0.5 ppm in emulsion and solution polymer products, respectively. Reproducibility data for residual acrylamide determination are presented in Table I. Percent relative standard deviations range from 0.4 to 5.5% for residual acrylamide levels from 2.5 to 650 ppm.

Spike recovery studies were performed on both solution and latex polymers. An appropriate aliquot of acrylamide standard solution was spiked into the polymer sample directly. The spiked sample was then treated as an unknown and analyzed using the column-switching procedure. Recovery data are shown in Table II. Better than 70% recovery was observed at acrylamide levels ranging from 0.4–200 ppm.

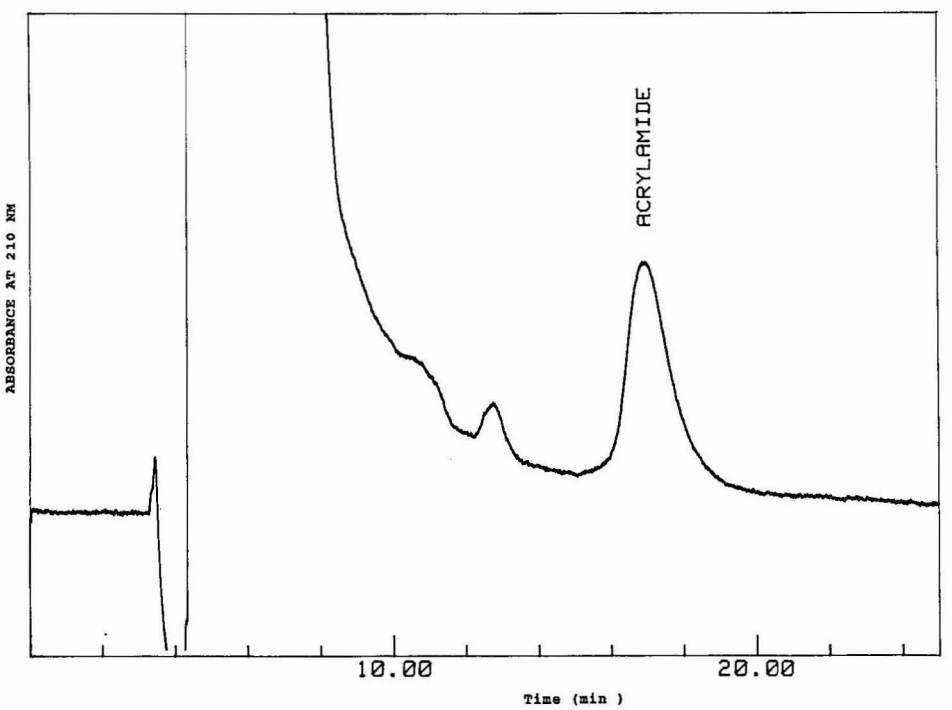

Fig. 2. Typical chromatogram of acrylamide analysis. 0.02 ppm acrylamide in a diluted sample solution. HPLC conditions as described under Experimental.

TABLE I

REPRODUCIBILITY STUDIES OF THE PRECIPITATION–COLUMN-SWITCHING PROCEDURE IN A POLYMERIC SYSTEM

Analysis	Acrylamide in polymers (ppm)					
	Solution polymers		Emulsion polymers			
	Sample A	Sample B	Anionic		Cationic	
			Sample C	Sample D	Sample E	Sample F
1	8.8	2.5	5.6	641	15.3	125
2	8.8	2.5	5.7	642	15.6	120
3	8.8	2.5	5.8	643	15.1	126
4	8.7	2.5	5.1	638	14.8	124
5	8.8	2.7	5.9	645	15.6	113
Mean	8.76	2.54	5.62	642	15.28	121.7
S.D.	0.055	0.089	0.31	2.59	0.34	5.37
Relative S.D. (%)	0.62	3.5	5.5	0.40	2.2	4.4

TABLE II

DATA OF THE SPIKE RECOVERY STUDIES

Sample type	Acrylamide spiked (ppm)	Acrylamide found (ppm)	Recovery (%)
Anionic latex	(A) 4.7	4.2	89.4
	(B) 5.0	4.7	94.0
	(C) 8.6	7.0	81.4
	(D) 12.2	10.0	82.0
	(E) 16.5	14.3	86.7
Cationic latex[a]	(A) 9.3	7.0	75.3
	(B) 16.6	12.0	72.3
	(C) 21.3	16.8	78.9
	(D) 49.6	35.3	71.2
	(E) 93.4	75.2	80.5
Cationic latex[b]	(A) 69.8	69.0	98.8
	(B) 144.0	126.3	87.7
Cationic latex[b]	(A) 100.0	100.0	100.0
	(B) 200.0	198.4	99.2
Non-ionic latex	(A) 220.0	202.0	91.8
	(B) 129.0	114.0	88.4
Solution polymer	(A) 19.5	19.5	100.0
	(B) 7.6	7.8	102.6
	(C) 3.4	3.5	102.9
	(D) 0.8	0.8	100.0
	(E) 0.4	0.5	125.0

[a] This sample contained polymeric surfactants.
[b] These emulsions contained common non-polymeric surfactants.

This method has been used in our laboratory for more than two years. With heavy usage, slowly eluting substances such as surfactants, oligomers and additives will build up on the guard column and the C_{18} column. Consequently, the guard column must be changed and the C_{18} column cleaned periodically. Our experience indicated that with a daily load analysis of about 30 samples, the C_{18} guard column has to be changed once a month. However, the fast acid guard column can last as long as six months without change. After each guard column or analytical column change, the valve switching time must be re-established by running standard solutions.

CONCLUSION

The column-switching HPLC method described has been shown to provide good separation of residual acrylamide from matrix interferences in both solution and emulsion polymeric systems. This allows very low residual acrylamide determination in such samples. The procedure for sample preparation and analysis is relatively simple, precise, and accurate and can be easily automated for routine analysis of residual acrylamide in polymer products.

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Note

Quantitative determination of free volatile fatty acids from dairy products on a Nukol capillary column

LUCIANO CECCON

Università di Trieste, Dipartimento di Economia e Merceologia delle Risorse Naturali e della Produzione, Via Valerio 6, 34127 Trieste (Italy)

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Free volatile fatty acids (FVFA) in dairy products are produced in only small amounts by lipolytic processes, and are derived substantially from amino acid degradations and lactose fermentation. For this reason, FVFA concentrations show a trend that varies progressively during ripening. As a consequence, the qualitative and quantitative evaluation of these components could both contribute to the characterization of various kinds of cheeses and enable one to relate chemical composition to ripening level for each type of product [1–11].

Determination of FVFA is usually carried out by gas chromatography (GC). In early studies, methods employing packed columns were proposed for the determination of the analytes either in the free form, by utilizing stationary phases such as silicone oil–stearic acid mixtures [12–16], Tween 80 [15,17,18], diethylene glycol adipate (DEGA)–H_3PO_4 [19,20], free fatty acid phase (FFAP) [21–23], Porapak Q–H_3PO_4 [24–27], diethylene glycol succinate (DEGS)–H_3PO_4 [28], NPGA–H_3PO_4 [29,30], Chromosorb 101 [31–33] or SP-1200–H_3PO_4 [34–36], or after conversion into the corresponding methyl [37–40], butyl [41,42], decyl [41], benzyl [43,44], phenacyl [41] or *p*-bromobenzyl [45] esters. More recently, methods employing capillary columns have been proposed [46–49].

In this paper, a method for the determination of C_2–C_8 free carboxylic acids in dairy products is described, involving use of a Nukol capillary column and crotonic acid as the internal standard.

EXPERIMENTAL

Chemicals

Acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, crotonic, caproic and caprylic acids were purchased from Fluka and were all standards for gas chromatography (>99% purity, except isovaleric acid, which was >98%). Isocaproic acid was obtained from Sigma and was *ca.* 99% pure.

The corresponding sodium salts were prepared by neutralization of acetone solutions of the free acids with 0.1 *M* methanolic sodium hydroxide, dried in a rotary

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vacuum evaporator at 40°C, powdered in a mortar, transferred to screw-stoppered vials and kept in a desiccator over phosphorus pentoxide.

Formic acid was obtained both from BDH (90% pure) and from Carlo Erba (99% pure). Lactic, pyruvic and 2-oxobutyric acids were obtained from Fluka.

Preparation of standard solutions

A stock standard FVFA solution was prepared by accurately weighing *ca.* 500 mg each of acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, caproic and caprylic acids in a 25-ml glass-stoppered volumetric flask and diluting to the mark with dichloromethane. By successive 1:10 dilutions with dichloromethane, the working standard FVFA solutions were prepared. Stock and working standard crotonic acid (internal standard) solutions used were prepared in a similar manner. The final concentrations of each component for all the standard solutions prepared are given in Table I.

Gas chromatography

A Carlo Erba HRGC 5300 Mega gas chromatograph equipped with a flame ionization detector was used. A Nukol fused-silica capillary column (15 m × 0.53 mm I.D.) with a film thickness of 0.5 μm (Supelco) was employed. The column temperature was programmed from 100 to 180°C at 10°C/min. Cold on-column injection was used. The detector temperature was 200°C, carrier gas (hydrogen) flow-rate 15 ml/min (set at 100°C), detector attenuation 10 and sample size 1 μl. Peak areas were determined with a Carlo Erba Mega integrator.

Calibration

A 5-ml volume of working standard FVFA solution and 5 ml of working standard crotonic acid solution were transferred into a 20-ml glass-capped tube over anhydrous sodium sulphate and 1 μl was injected immediately into the column.

Various combinations of working standard FVFA and crotonic acid solutions allowed amounts of each acid from 10^{-6} down to 10^{-9} g to be injected and 100:1, 10:1,

TABLE I

FINAL CONCENTRATIONS OF EACH COMPONENT IN THE STANDARD SOLUTIONS PREPARED

Standard solution	Concentration of each component (mg/l)
Stock FVFA solution	20 000
Working FVFA solution 1	2000
Working FVFA solution 2	200
Working FVFA solution 3	20
Working FVFA solution 4	2
Stock crotonic acid solution	20 000
Working crotonic acid solution 1	2000
Working crotonic acid solution 2	200
Working crotonic acid solution 3	20
Working crotonic acid solution 4	2

1:1, 1:10 and 1:100 ratios between the amount of each FVFA and the amount of crotonic acid to be obtained. Five analyses were performed for each determination.

Conversion of the sodium salts into the corresponding free acids

Standard FVFA sodium salts suspension (SSS). Amounts of acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, crotonic, caproic and caprylic acid sodium salts (corresponding to about 10 mg each of free acid) were accurately weighed in a 25-ml glass-stoppered volumetric flask, diluted to the mark with dichloromethane and 20 μl of phosphoric acid (Merck, 85%) were added. The flask was kept on a magnetic stirrer overnight, then the solids were allowed to settle.

1-Dodecanol solution. A 6.3-mg amount of 1-dodecanol (Fluka, >99% pure) was accurately weighed in a 25-ml glass-stoppered volumetric flask and diluted to the mark with dichloromethane.

Procedure. A 500-μl volume of clear solution from the SSS and 500 μl of the 1-dodecanol solution were transferred into a 5-ml glass-capped tube over anhydrous sodium sulphate, and 1 μl of the solution obtained was injected immediately into the column.

Both the SSS and the 1-dodecanol solutions were diluted again to the mark with dichloromethane, and to the SSS 20 μl of phosphoric acid were added, the flask was kept on a magnetic stirrer overnight and, after the solids had settled, the procedure was repeated from the beginning. With successive 20-μl additions in this manner, amounts of phosphoric acid in the range up to 120 μl were examined. Five analyses were performed for each determination.

Determination of FVFA in cheese

A 100-g amount of a 15 day-old Montasio cheese was steam distilled as described elsewhere [10,11]. The distillate was neutralized with 0.1 *M* sodium hydroxide solution, using phenolphthalein as the indicator, and mixed with 5 ml of a 250 mg/l solution of sodium crotonate. The sodium salts were dried on a rotary vacuum evaporator at 40°C. The dry salts (*ca.* 130 mg) were converted into the corresponding free acids by adding 10 ml of a solution of phosphoric acid in dichloromethane (20 mg/l); the solution obtained was transferred into a 20-ml glass-capped tube over anhydrous sodium sulphate, and 1 μl was injected immediately into the column.

RESULTS AND DISCUSSION

One of the most widely used methods for the determination of FVFA in dairy products involves preliminary recovery from the matrix by steam distillation, followed by GC separation [1,5,7,9–11]. However, relatively high distillate volumes have to be collected in order to achieve a quantitative yield of all the compounds of interest [10,11]. As a consequence, a solution in which FVFA are present at lower concentrations than in the starting product is usually obtained by steam distillation. For this reason, the distillate has to be neutralized and water eliminated, thus obtaining the salts of the corresponding acids. By adding a strong acid, the salts are reconverted into the free acids before GC separation.

Recently, a wide-bore Nukol column has become commercially available and seems very attractive for the determination of FVFA in dairy products. A wide-bore

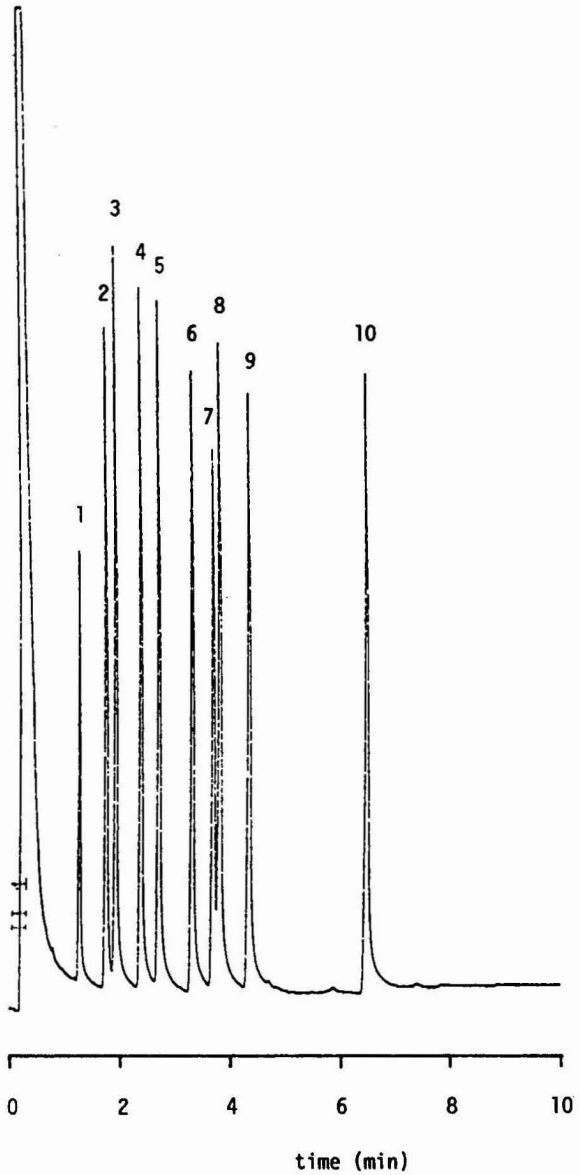

Fig. 1. GC separation on a Nukol capillary column of a synthetic mixture of volatile fatty acids. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = *n*-butyric acid; 5 = isovaleric acid; 6 = *n*-valeric acid; 7 = crotonic acid; 8 = isocaproic acid; 9 = caproic acid; 10 = caprylic acid.

column has the advantages of both a packed and a capillary column (high capacity, high sensitivity, short analysis time, high resolving power). It is therefore possible to inject a mixture in which the components are present in amounts that are notably different from one another (a situation typical of FVFA recovered from dairy products), and to obtain an excellent separation in a short time.

As an example, Fig. 1 shows the separation of a synthetic mixture of all the C_2–C_8 FVFA usually present in dairy products, with the addition of crotonic acid as a suitable internal standard. The responses of the individual FVFA are linear over a wide range of injected amounts (see Fig. 2).

FVFA are present in cheeses in amounts usually ranging from about 100 to about 0.01 mg per 100 g. It is therefore possible to use an amount of internal standard of 1 mg per 100 g of product for the determination of all the compounds of interest, by utilizing a calibration graph of the type shown in Fig. 3.

Formic acid has been suggested for the conversion of the salts into the corresponding free acids [9,50–54]. However, it was not possible to use formic acid here because, unexpectedly, the injection of a solution of formic acid in dichloromethane into the Nukol column produced a peak that interferes with, and may obscure, the propionic acid peak. The phenomenon was observed both on utilizing formic acid from different commercial sources and on passing the solution of formic acid even over a large excess of anhydrous sodium sulphate. In contrast, the injection of a solution of phosphoric acid in dichloromethane gave a chromatogram completely free from

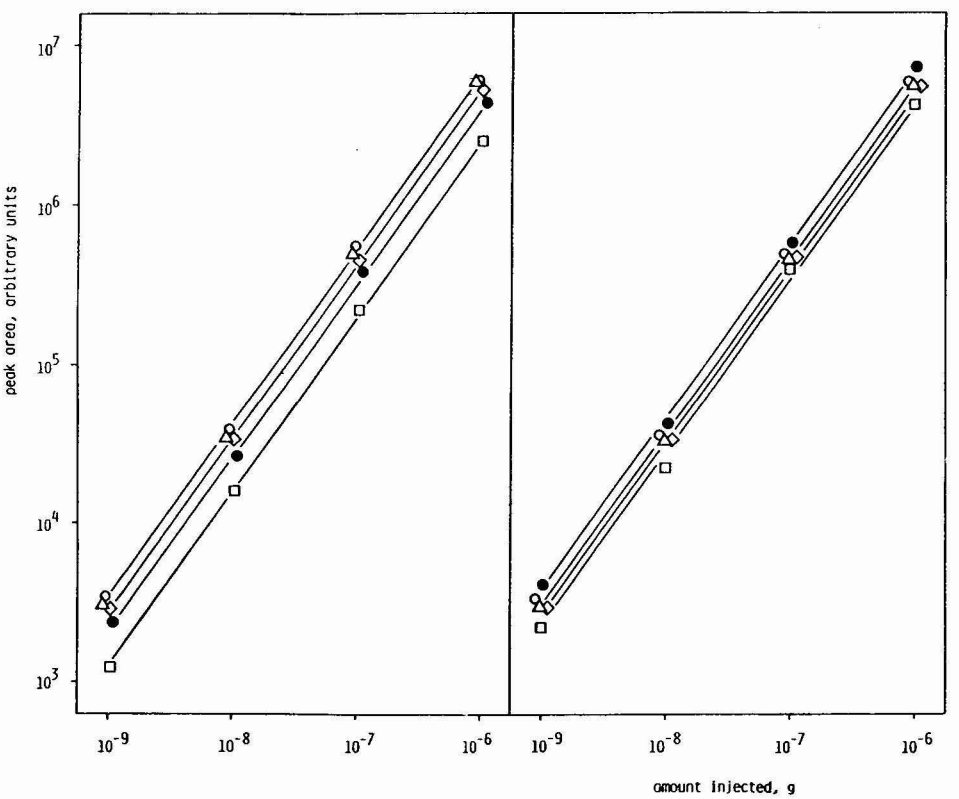

Fig. 2. Calibration graph of amount of each volatile fatty acid injected *versus* detector response (average of five determinations). Left: □ = acetic acid; ● = propionic acid; ◇ = isobutyric acid; ○ = isocaproic acid; △ = caproic acid. Right: ◇ = *n*-butyric acid; ○ = isovaleric acid; △ = *n*-valeric acid; □ = crotonic acid; ● = caprylic acid.

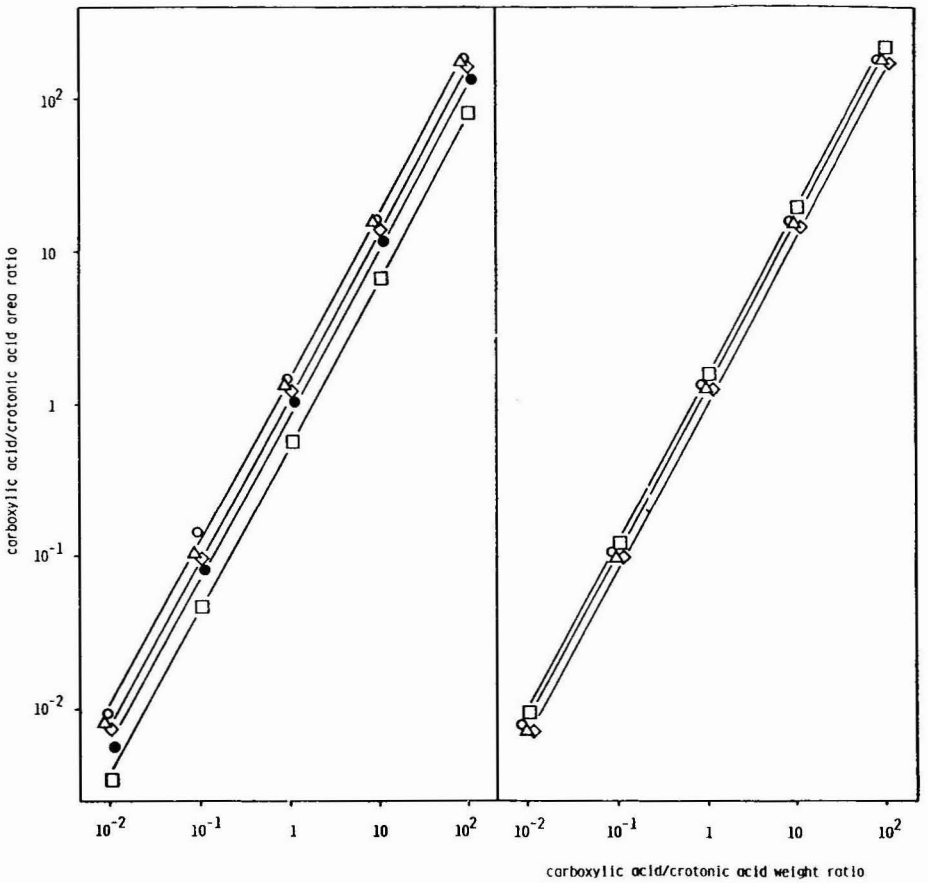

Fig. 3. Calibration graph of peak-area ratio *versus* weight ratio for each volatile fatty acid with respect to crotonic acid as the internal standard (average of five determinations). Left: □ = acetic acid; ● = propionic acid; ◇ = isobutyric acid; ○ = isocaproic acid; △ = caproic acid. Right: ◇ = *n*-butyric acid; ○ = isovaleric acid; △ = *n*-valeric acid; □ = caprylic acid.

interfering peaks. The addition of phosphoric acid to the salts in order to obtain the FVFA sample to be injected was been reported previously [20,22,26,27,55–59]. The possibility of using phosphoric acid for the conversion of the salts into the corresponding free acids was then investigated. In particular, the best ratio between the amount of salts and the amount of phosphoric acid was studied, in order to obtain a quantitative yield of all the acids of interest. For this purpose, increasing amounts of phosphoric acid were added to a synthetic mixture of sodium salts; the conversion of the salts into the corresponding free acids was evaluated by utilizing 1-dodecanol as an internal standard. The free carboxylic acid-to-1-dodecanol peak-area ratios were used to evaluate the completeness of the conversion.

The results obtained, presented in Fig. 4, show that a 1:2 (v/w) ratio of phosphoric acid to the salts is sufficient to obtain a complete conversion into the free

acids; an amount of phosphoric acid even twice the minimum necessary gives rise to no undesirable effects.

It is interesting that the addition of phosphoric acid leads to the conversion first of the branched-chain, then to the straight-chain and finally to the unsaturated acids.

The method was applied to the determination of FVFA in a Montasio cheese (Fig. 5). An excellent separation of the C_2–C_8 FVFA was obtained even with respect to lactic acid, which is recovered by steam distillation from dairy products together with the volatile acids of interest. Oxo acids do not interfere, as they are not eluted from the Nukol column, as was verified by injecting pure standards of pyruvic and 2-oxobutyric acids.

No ghosting effect was observed on the Nukol column on injecting a 5 g/l solution of phosphoric acid in dichloromethane even just after a mixture of 1 g/l of each FVFA.

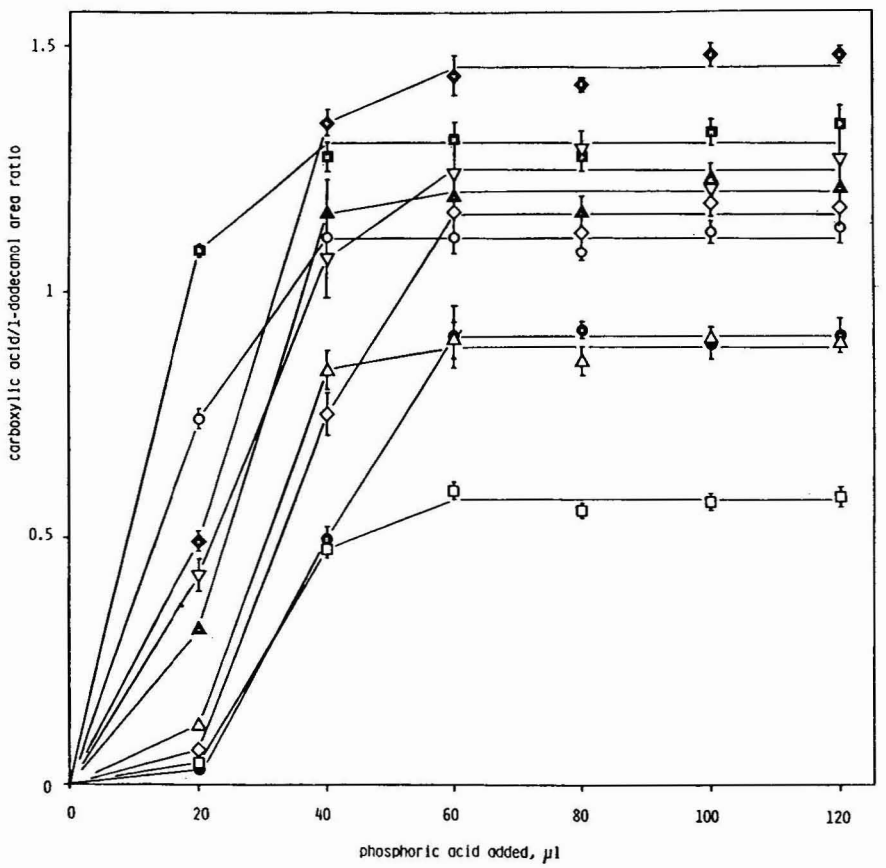

Fig. 4. Evaluation of the conversion of sodium salts into the corresponding free acids by addition of phosphoric acid, using 1-dodecanol as the internal standard (average of five determinations ± S.D.). □ = Acetic acid; △ = propionic acid; ○ = isobutyric acid; ◇ = *n*-butyric acid; ◘ = isovaleric acid; ▲ = *n*-valeric acid; ● = crotonic acid; ◆ = caproic acid; ▽ = caprylic acid.

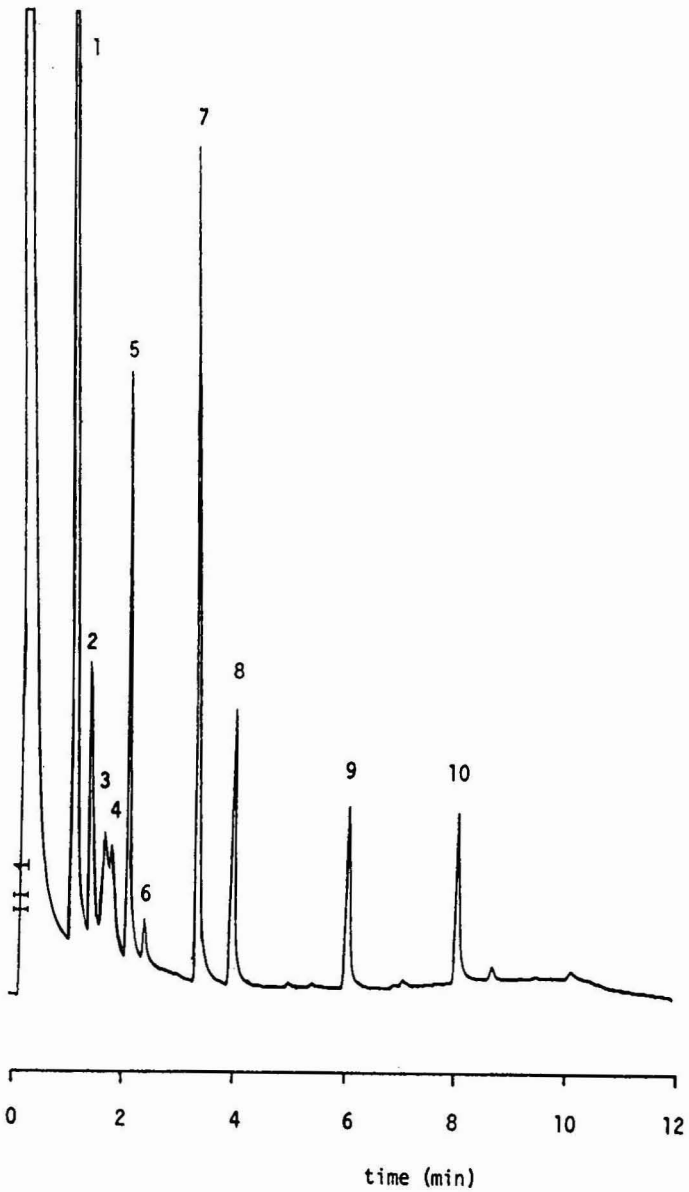

Fig. 5. GC separation on Nukol capillary column of free volatile fatty acids recovered from a Montasio cheese, with the addition of crotonic acid as the internal standard. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = unknown; 5 = *n*-butyric acid; 6 = isovaleric acid; 7 = crotonic acid; 8 = caproic acid; 9 = caprylic acid; 10 = lactic acid.

No apparent sign of deterioration of the column performance, such as tailing or the appearance of spurious peaks, was observed after 6 months of continuous use.

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CHROM. 22 612

Note

Purification of yeast alcohol dehydrogenase by Congo red affinity chromatography

A. H. SCHMIDT and H. WOMBACHER*
TFH Berlin, FB Chemie und Biotechnik, Luxemburger Strasse 10, D-1000 Berlin 65 (F.R.G.)
(First received October 24th, 1989; revised manuscript received March 14th, 1990)

The successful purification of several dehydrogenases by dye ligand affinity chromatography has been described previously [1,2] and the design and application of biomimetic dyes in affinity chromatography has been discussed in detail [3,4]. However, the theoretical basis of the interactions of aromatic dye molecules with enzymes containing the dinucleotide fold is in part still undefined. Thus, a more empirical approach to finding dyes suitable for the affinity chromatography of alcohol dehydrogenase (E.C. 1.1.1.1) (ADH), a widely used enzyme in biochemistry and medicine, seemed to be justified and useful.

In addition to linking an adenyl-containing ligand to a Sepharose 4B matrix, different dyes were also tested as ligands in order to purify ADH. A comparison of different types of affinity chromatography showed purifications of 885-fold by Congo red (azo dye), 827-fold by toluidine blue (phenothiazine dye), 379-fold by safranin (phenazine dye) and 424-fold when AMP was covalently linked also to cyanogen bromide-activated Sepharose 4B via adipic dihydrazide as a spacer after periodate cleavage of the ribose ring. The ADH extracted from yeast with toluene–plasmolyse [5], precipitated with ammonium sulphate at 60% saturation and reprecipitated, then chromatographed on Congo red–Sepharose 4B, dialysed and crystallized, showed homogeneity of preparation when subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

EXPERIMENTAL AND RESULTS

Materials

Congo Red dye was obtained from Fluka (Neu-Ulm, F.R.G.) and Sepharose 4B from Pharmacia (Sweden). Toluidine blue and safranin dyes and all other chemicals (analytical-reagent grade) were purchased from Merck (F.R.G.). Yeast (*Saccharomyces cerevisiae*) was obtained from Uniferm (F.R.G.).

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Preparation of dye ligand–Sepharose 4B

A 20-ml volume of settled Sepharose 4B was activated with cyanogen bromide [6]. A 200-mg amount of the dye, e.g., Congo red, used as the ligand was dissolved in 60 ml of 0.05 mol/l phosphate buffer (pH 8.0) and coupled to the gel immediately after cyanogen bromide activation. The mixture of activated Sepharose and Congo red was gently stirred overnight at 4°C. The excess of the dye was removed by washing consecutively with 20 ml each of water, 0.2 mol/l acetic acid, 3 mol/l sodium chloride solution, 1% (w/v) Triton X-100 and ethanol–water (1:1, w/v). This washing cycle removed the uncoupled ligand and ensured that no free ligand remained bound in any way to the immobilized material. The coupling efficiency was determined spectrophotometrically at 500 nm in a suspension of glycerol. For efficient adsorption a capacity of 5 μmol of Congo Red per ml of settled gel was found to be useful. The other ligands were attached using the same procedure.

Preparation of AMP ligand–Sepharose 4B

For AMP–Sepharose, adipic dihydrazide was used as a spacer, which linked the cyanogen bromide-activated Sepharose to the ribosyl part of adenosine monophosphate after periodate treatment [7].

In detail, 10 ml of cyanogen bromide-activated Sepharose, prepared as described above, were suspended in 15 ml of a 0.1 mol/l sodium carbonate solution containing 1.5 g of adipic dihydrazide and stirred overnight at 4°C. The spacer-linked Sepharose was washed with 200 ml of 0.2 mol/l sodium chloride solution and 1000 ml of distilled water and subsequently with 100 ml of 0.1 mol/l sodium acetate solution. Separately, 60 mg of AMP were dissolved in 0.7 ml of water and neutralized dropwise with 1 mol/l sodium hydroxide solution. After the addition of 18 mg of sodium

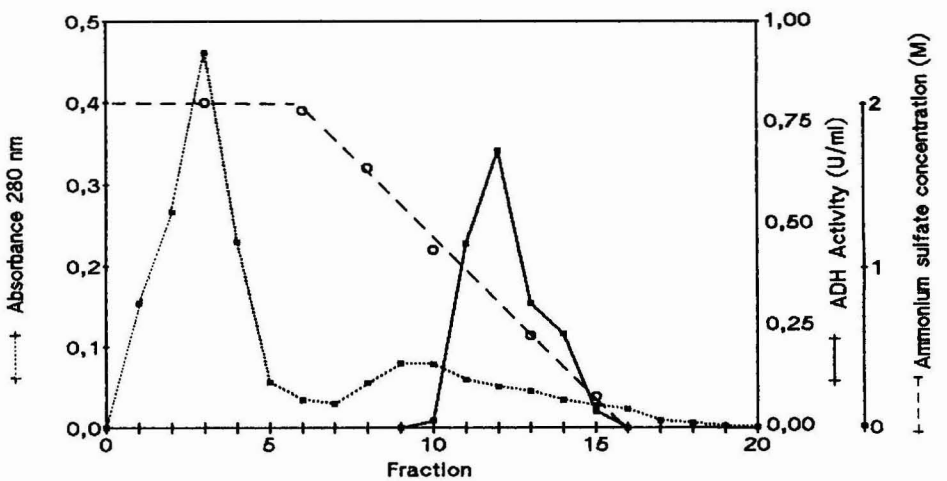

Fig. 1. Affinity chromatography of alcohol dehydrogenase from yeast extract on Congo red–Sepharose. A sample of yeast extract (12.1 U ADH) was applied to a column of Congo red–Sepharose equilibrated with 0.05 mol/l phosphate buffer (pH 8) containing 2 mol/l ammonium sulphate. Non-adsorbed protein was washed off with the same buffer and the enzyme was eluted with an ammonium sulphate gradient from 2 to 0 mol/l in 0.05 mol/l phosphate buffer (pH 8).

TABLE I

PURIFICATION OF ALCOHOL DEHYDROGENASE FROM BAKER'S YEAST

Fraction	Specific activity (U/mg)[a]	Yield (%)	Degree of purification (-fold)[b]
Yeast extract	0.15	100	1
Ammonium sulphate (60% saturated) precipitate	1.88	72	12.5
Ammonium sulphate, reprecipitated	7.92	66	53
Chromatographed on			
Congo red–Sepharose	132.8	11	885
Toluidine blue–Sepharose	124.0	10	827
Safranin–Sepharose	56.8	28	379
AMP–Sepharose	63.5	13	424

[a] 1 unit (U) = 1 μmol NADH formed/min. The enzyme activity was measured at 20°C in 15 mmol/l glycine pyrophosphate buffer (pH 8.8) containing 0.5 mol/l ethanol, 2 mmol/l NAD, 75 mmol/l semicarbazide and 1 mmol/l gluthathione, with a slightly modified method [9]. Protein (mg) was determined by the Lowry method [10] using bovine serum albumin as the standard.

[b] Increase in specific activity with respect to the start.

periodate the mixture was incubated at 0°C for 1 h. Finally, the spacer-linked Sepharose, suspended in 25 ml of 0,1 mol/l sodium acetate solution, was added to the periodate-treated nucleotide and the mixture was stirred at 4°C for 3h. Thereafter the material was collected and washed with 500 ml of 2 mol/l sodium chloride solution and 1000 ml of water.

Preparation of yeast extract

In accordance with the toluene–plasmolyse method [5], the yeast extract was prepared from 252 g of baker's yeast and precipitated with ammonium sulphate at 60% saturation. The precipitated material was redissolved and reprecipitated, then the reprecipitated material was dissolved again in 0.05 mol/l phosphate buffer (pH 8) containing 2 mol/l ammonium sulphate for the chromatographic step.

Affinity chromatography

The dye ligand–Sepharose 4B, prepared as described, was used in small Polyprep columns (Bio-Rad Labs, Richmond, CA, U.S.A.) and equilibrated with 2 mol/l ammonium sulphate in 0.05 mol/l phosphate buffer (pH 8). After equilibration, the ADH material was loaded on the column and incubated for 30 min. The high ammonium sulphate concentration increased the binding of ADH, mainly because of hydrophobic interactions. After washing with the equilibration buffer, a linearly decreasing ammonium sulphate gradient was used to elute the ADH. The elution rate was 30 ml/h and 3-ml fractions were collected (Fig. 1). A comparison of the efficiency of the dye ligand and AMP ligand chromatography is shown in Table I.

DISCUSSION

The binding of the dye or AMP ligand–Sepharose to the enzyme ADH is interpreted as mimicking by the ligands of the coenzyme NAD. The selectivity of ADH binding is limited because only the adenine subsite of NAD seems to be relevant for binding, as discussed previously [2], and the strength of affinity is mainly dependent on the hydrophobic interactions, as evidenced by the conditions of binding and elution of the enzyme.

The affinity chromatography using Congo red or Toluidine blue as the ligand led to a fairly high degree of ADH purification (about 850-fold). However, an even higher specific activity has been reported previously [8] using a recrystallized preparation of ADH from yeast after ethanol and ammonium sulphate precipitation, resulting in about a 40-fold purification. This discrepancy may be attributed to the high specific activity of the crude yeast-cell extract at the start of the preparation. However, the ADH preparation recrystallized after Congo red affinity chromatography also showed only a single band on SDS-PAGE and obviously no co-purification of proteolytic activities occurred, as the enzyme is stable at least 6 months.

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Note

Rapid high-performance liquid chromatographic analysis of phytotoxins from *Phoma lingam*[a]

M. SOLEDADE C. PEDRAS*
Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan S7N 0W9 (Canada)
and
GINETTE SÉGUIN-SWARTZ
Agriculture Canada Research Station, Saskatoon, Saskatchewan S7N 0X2 (Canada)
(First received May 17th, 1990; revised manuscript received August 13th, 1990)

Phoma lingam (Tode ex Fr.) Desm., [perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.] is the causative agent of blackleg disease of the rapeseed/canola (*Brassica napus* and *B. campestris*) oilseeds. The phytotoxins produced by virulent isolates of *P. lingam* were recently reported [1]. The metabolite profiles of several fungal isolates were analyzed and correlated with the virulence of the pathogen [1]. There is a good indication that only virulent isolates of *P. lingam* produce phytotoxins; however, to establish such a correlation, a large number of fungal isolates should be screened. If the production of phytotoxic compounds proves to be a characteristic of virulent isolates, it may be used as a chemotaxonomical marker of particular strains of *P. lingam*.

In order to carry on a wide screening programme, it is essential to have a rapid and reliable analytical method for the detection of phytotoxins. In this note we report a high-performance liquid chromatography (HPLC) method for the rapid analysis of phytotoxins produced by *P. lingam*.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Spectra-Physics solvent delivery system, Model SP8700, equipped with pump and injector, and a Kratos Analytical Instruments absorbance detector (variable wavelength), Model Spectroflow 773. Chromatograms and retention times were recorded on a Hewlett-Packard integrator, Model 3392A. The column used was a Partisil PXS 5/25 (5-μm particle size silica, 25 cm × 4.6 mm

[a] N.R.C.C. No. 32 448.

0021-9673/90/$03.50

I.D.; Whatman, Clifton, NJ, U.S.A.), equipped with a guard column filled with the same stationary phase.

Reagents

The organic solvents (Table I) were HPLC grade and were degassed during operation through continuous bubbling of a stream of helium.

TABLE I

RETENTION TIME OF SIRODESMIN PL (MINIMUM DETECTABLE AMOUNT 0.1 μg) UNDER DIFFERENT EXPERIMENTAL CONDITIONS [COLUMN PARTISIL PXS 5/25 (WHATMAN); ISOCRATIC ELUTION; 235 nm]

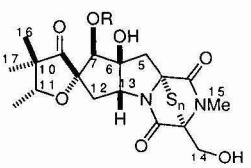
Solvent system	Flow-rate (ml/min)	Retention time (min)
(A) Hexane–2-propanol (90:10)	2.0	6.8
(B) Hexane–2-propanol (92:8)	2.0	8.4
(C) Dichloromethane–2-propanol (94:6)	2.0	2.4
(D) Dichloromethane–2-propanol (97:3)	2.0	3.0
(E) Dichloromethane–2-propanol (98:2)	2.0	4.7
(F) Dichloromethane–ethyl acetate (50:50)	2.0	3.5
(G) Ethyl acetate–hexane (60:40)	2.0	5.0

Preparation of extracts

The isolates of *P. lingam* (obtained from G. A. Petrie, Agriculture Canada Research Station, Saskatoon, Canada) were grown in liquid minimal medium supplemented with thiamine [1]. Initially the fungus was grown in still culture for three weeks, because the phytotoxicity of the culture filtrate was highest at that stage. Subsequently, one to four weeks old still cultures and five to seven days old shake cultures were investigated. The liquid cultures were filtered through cheesecloth, and the broth was freeze-dried. The freeze-dried broth was diluted with distilled water to one tenth of the initial volume and extracted four times (separatory funnel) with an equivalent volume of ethyl acetate (yields from 20–200 mg of crude extract per liter of broth) [1]. The crude broth extracts were subjected to a preliminary "clean-up" by filtering ethyl acetate solutions (100 mg/ml) through a mini-silica gel column (Pasteur pipette containing silica gel about 4 cm high) eluted with ethyl acetate (10 ml). After evaporation of the ethyl acetate, the extracts were dissolved in dichloromethane and/or in the mobile solvent system and filtered through a cotton plug (2.5 mg/ml; injection volume 1–5 μl).

Preparation of natural standards

The naturally occurring phytotoxins sirodesmin PL, deacetylsirodesmin PL, sirodesmins H, J and K, and phomalirazine (structures in Fig. 1) were isolated from the crude broth extracts of *P. lingam* liquid cultures and purified as previously described [1]. Solutions of these compounds prepared in dichloromethane and filtered through a cotton plug were used as natural standards.



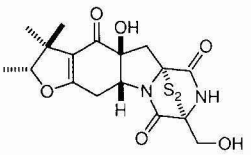
1 Sirodesmin PL R=Ac; n=2

2 Deacetylsirodesmin PL R=H; n=2

3 Sirodesmin H R=Ac; n=1

4 Sirodesmin J R= Ac; n=3

5 Sirodesmin K R= Ac; n=4



6 Phomalirazine

Fig. 1. Chemical structures of phytotoxins from *Phoma lingam*. Ac = Acetyl; Me = methyl.

RESULTS AND DISCUSSION

The chemical structures of the putative phytotoxins isolated from *P. lingam* extracts [1] are shown in Fig. 1. Sirodesmins H, J and K are unstable in methanol and other polar solvents [1,2], therefore a normal-phase column was used in the HPLC analysis. Table I lists several of the solvent systems tried with a Partisil PXS column. Compounds **1** and **4** showed identical retention times in systems A and B (Table I); similarly compounds **2** and **3** were indistinguishable in systems F and G. In dichloromethane–2-propanol (systems C–E) each phytotoxin (**1–6**) had a different retention time and could be readily differentiated. The minimum detectable amount for each phytotoxin was *ca.* 0.1 μg (experimental conditions on Table II).

The chromatograms of Fig. 2 represent extracts of the virulent isolates "Leroy" and "FRA 88" (three-week-old cultures) and were obtained under identical conditions. Sirodesmin PL (**1**) is the major component of the "Leroy" extract. The components of the "FRA 88" extract are deacetylsirodesmin PL (**2**), and sirodesmins PL, J and K (**1, 4, 5**). Two other virulent isolates examined showed chromatographic pro-

TABLE II

RETENTION TIMES OF PHYTOTOXINS OF *PHOMA LINGAM*; UV DETECTION SET AT 235 nm; COLUMN PARTISIL PXS 5/25 (WHATMAN); SYSTEMS A AND D

Phytotoxin	Retention time (min)	
	A	D
Sirodesmin PL (**1**)	6.8	2.4
Deacetylsirodesmin PL (**2**)	6.4	3.8
Sirodesmin H (**3**)	13.5	4.0
Sirodesmin J (**4**)	6.8	3.4
Sirodesmin K (**5**)	8.5	6.4
Phomalirazine (**6**)	10.9	16.6

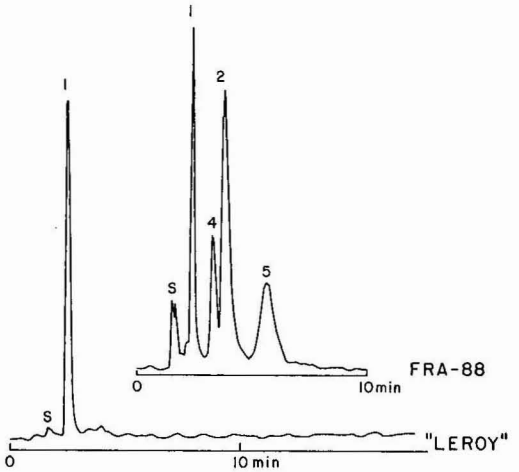


Fig. 2. HPLC chromatograms of extracts of two virulent isolates ("Leroy" and "FRA 88") of *Phoma lingam*. Peak numbers refer to structures in Fig. 1 and S to solvent peak. For experimental conditions see Table II, system C.

files identical to the isolate "Leroy". No phytotoxins (or other UV-absorbing compounds) were detected in five avirulent isolates. The limit of detection of phytotoxins per g of extract (dry material) was *ca.* 0.1%.

Three-week-old cultures are impractical (slow growth) to be used in a wide screening programme. To find a faster and yet characteristic fungal growth stage, the chromatographic profiles of still and shake cultures of different ages were compared. The metabolite profiles of seven-day-old shake cultures and three-week-old still cultures were similar. Thus, seven-day-old shake cultures are more convenient to carry out a rapid screening of a large number of fungal isolates.

Considering the worldwide occurrence and importance of the disease caused by *P. lingam* it is of interest to differentiate virulent isolates from avirulent ones with reliable methods, which at present are insufficient [3]. The simplicity of the HPLC method reported here, coupled with the use of seven-day-old shake cultures, will allow a rapid and reliable screening of isolates of *P. lingam*.

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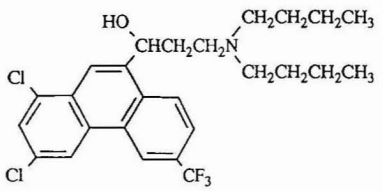
CHROM. 22 738

Letter to the Editor

Fluorescence detection of the enantiomers of halofantrine at picomole levels using chiral high-performance liquid chromatography

Sir,

1 - (1,3 - Dichloro - 6 - trifluoromethyl - 9 - phenanthryl) - 3 -N,N -dibutylamino-propan-1-ol (halofantrine, **1**) is an effective antimalarial agent currently marketed by SmithKline Beecham as the hydrochloride salt under the trade name of Halfan. In a previous report [1] we described a chiral high-performance liquid chromatography (HPLC) procedure for the resolution of the optical isomers of halofantrine using a Pirkle type chiral stationary phase. We have now found that fluorescence detection is far superior to UV absorbance in the analysis of very low levels of **1**.



(1)

Prior to chromatography studies fluorescence spectra of halofantrine free base were acquired in the mobile phase using a Perkin-Elmer LS-4 fluorescence spectrometer. The excitation and emission scans were performed at a scan speed of 30 nm min^{-1} and were acquired using a Nelson Analytical 960 Series analog-to-digital (A/D) interface sampling at 100 Hz for high resolution. The fluorescence excitation and emission spectra of **1** are shown in Fig. 1. As expected, the excitation spectra between 230 and 350 nm bear much resemblance to the UV spectra reported in ref. 1. The emission spectrum in Fig. 1b shows a well-defined pair of bands of approximately equal intensity at 355.2 and 371. 4 nm. The first emission band was chosen for the chiral analysis of **1**.

The HPLC method to separate the optical isomers of halofantrine was developed on a Perkin-Elmer Series 4 liquid chromatograph, equipped with a Perkin-Elmer ISS-100 autoinjector. UV detection was performed using an Applied Biosystems 783A absorbance detector operating at 260 nm, connected in series to a Perkin-Elmer LS-4 fluorescence spectrophotometer operating at an excitation

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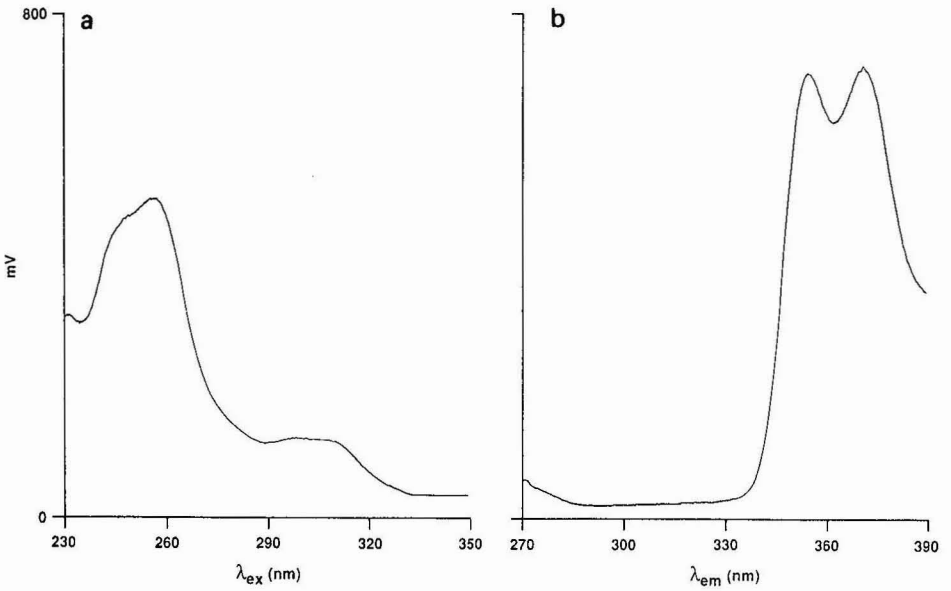

Fig. 1. Fluorescence excitation (a) and emission (b) spectra of halofantrine.

wavelength of 256 nm and an emission wavelength of 358 nm. HPLC data acquisition was performed using a Nelson Analytical 960 Series A/D interface sampling at 2 Hz.

The chiral column (250 mm × 4.9 mm I.D.) was of the Pirkle type and the chiral stationary phase consisted of L-N-(3,5-dinitrobenzyl)leucine covalently bonded to a 3-aminopropyl silica support (particle size 5 μm). This column supplied by Hichrom was operated at -10°C. The best separation of the optical isomers was achieved using *n*-hexane–chloroform–propan-2-ol (containing 1% triethylamine) in the ratio of 95:5:5 (v/v/v) flowing at a rate of 0.2 ml min^{-1}.

Fig. 2. shows a comparison of the resolution of the optical isomers of **1** (605 pmol injected) using both UV absorbance and fluorescence detection. The first and second eluted molecules have been assigned (+) and (−) as shown previously [1] by polarimetric analysis of isolated fractions. The much higher sensitivity of fluorescence compared to UV absorbance detection is clear from Fig. 2 where both methods of detection are displayed on an absolute scale. In fact, the fluorescence detector could be increased by a factor of 100 higher than that shown. The sensitivity of fluorescence detection is demonstrated in Fig. 3 when 2 fmol of halofantrine were injected and each of the optical isomers could be detected with ease.

Analysis of the linearity of the fluorescence response of the detector for injections of halofantrine in the range 10 μmol to 10 pmol showed linear behaviour in obeying eqn. 1 as derived by Scott [2]

$$y = AC^r \tag{1}$$

where y is the fluorescence response and C is the concentration of halofantrine; A is an arbitrary constant and r is the linearity index. A plot of log (response) *vs.* log

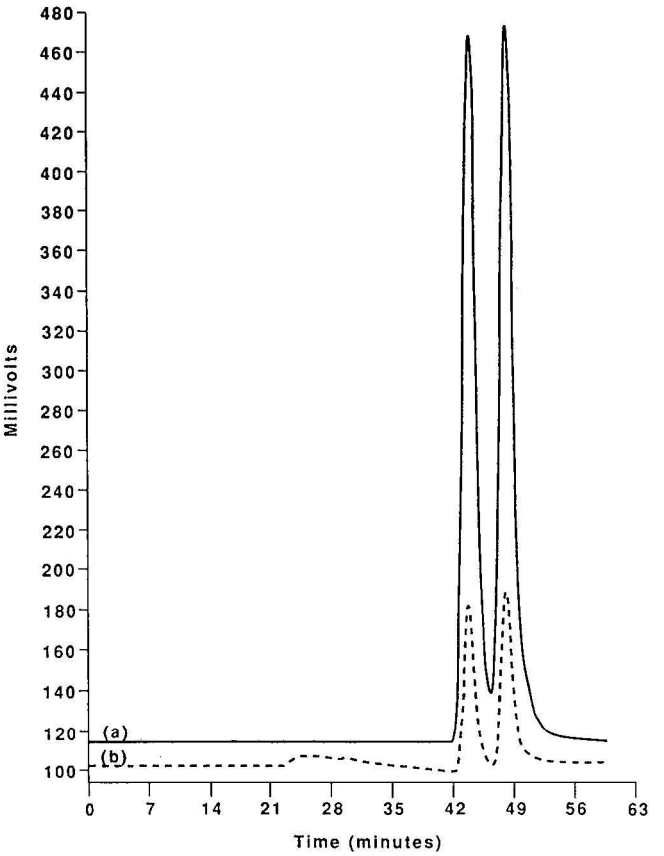

Fig. 2. Enantiomeric separation of the optical isomers of halofantrine using (a) fluorescence and (b) UV absorbance detection.

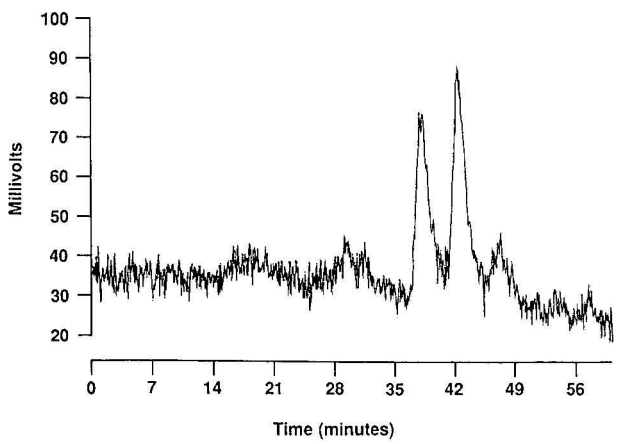

Fig. 3. Fluorescence detection of the enantiomers of halofantrine at 2.2 fmol of halofantrine injected.

[halofantrine] gave values of 1.132 ± 0.53 and 20.374 ± 0.574 for r and log A, respectively [3]. This value obtained for r is higher than that described by Scott as the definition of ideal linearity of a detector, however, it has been mentioned by this author that if the linearity index of the detector is known the use of calibration curves for quantitative analysis is possible.

The fluorescence method of detection outlined can be applied to study any physiological differences between the two enantiomers of **1** at very low concentration levels and can be used in pharmacokinetic, pharmacodynamic and metabolic studies. Moreover, this method of detection can be applied to studies utilising other methods of separation such as reversed phase HPLC, which will not necessarily involve the chirality of **1**.

SmithKline Beecham Pharmaceuticals, PATRIC CAMILLERI
The Frythe, Welwyn, Herts. AL6 9R (U.K.) CHRISTOPHER J. THORPE

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Errata

J. Chromatogr., 498 (1990) 205–211
Page 209, caption of Fig. 3: "2 = 2,3-DBA" should read "2 = hydrogencarbonate" and "4 = HHQ" should read "4 = 2,3-DBA".
Page 211, line 5: "*o*-quinone" should read "*p*-quinone".

J. Chromatogr., 514 (1990) 227–239
Page 229, section *Derivatization*, 5th line, "$Na_2Br_1O_4$" should read "$Na_2Br_7O_4$"

PUBLICATION SCHEDULE FOR 1990

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 513, pp. 413–416. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

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