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Automatic Methods of Analysis

by **M. VALCÁRCEL** *and* **M.D. LUQUE DE CASTRO,**
Department of Analytical Chemistry, University of Córdoba, Córdoba, Spain

(Techniques and Instrumentation in Analytical Chemistry, 9)

This new book gives a comprehensive overview of the state of the art of the automation of laboratory processes in analytical chemistry. The topics have been chosen according to such criteria as the degree of consolidation, scope of application and most promising trends.

The book begins with the basic principles behind the automation of laboratory processes, then describes automatic systems for sampling and sample treatment. In the second part the principal types of analysers are discussed: continuous, batch and robotic. The third part is devoted to the automation of analytical instrumentation: spectroscopic, electroanalytical and chromatographic techniques and titrators. The last part presents examples of the application of automation to clinical chemistry, environmental pollution monitoring and industrial process control.

The text is supplemented by 290 figures and 800 literature references. It is written primarily for those directly involved in laboratory work or responsible for industrial planning and control, research centres, etc. It will also be useful to analytical chemists wishing to update their knowledge in this area, and will be of especial interest to scientists directly related to environmental sciences or clinical chemistry.

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POLARITY OF OLIGOOXYETHYLENE DERIVATIVES OF ALCOHOLS, THIO ALCOHOLS AND ALKYLAMINES AS MEASURED BY GAS CHROMATOGRAPHY

ADAM VOELKEL
Technical University of Poznań, Institute of Chemical Technology and Engineering, Pl.M. Skłodowskiej-Curie 2, 60-965 Poznań (Poland)
(Received April 19th, 1988)

SUMMARY

The polarities of oligooxyethylene derivatives of alcohols, thio alcohols and alkylamines were determined by gas chromatography. Relationships between empirical, thermodynamic and dispersive force polarity parameters are discussed. The influence of the structures of the compounds [lengths of oligooxyethylene and hydrocarbon chains, heteroatom linked to the alkyl group(s), distribution of carbon atoms in alkyl chains and the type of the group terminating the molecule] was examined. The average structural increments of the considered parameters for characteristic structural fragments were calculated and used in the prediction of the polarities of compounds from their formulae.

INTRODUCTION

The polarity of pure model non-ionic compounds exhibiting surface activity and used as surfactants and metal extractants has been extensively examined[1–10]. Oligooxyethylene derivatives of alcohols, thio alcohols and alkylamines have been used as liquid stationary phases in polarity measurements[1]. Their retention indices and thermodynamic parameters in solution on different standard stationary phases have also been reported[11,12].

The aim of this work was to determine the polarities of a large number of oligooxyethylene derivatives of alcohols, thio alcohols and alkylamines and to correlate these polarities with their structures. Relationships between the empirical, thermodynamic and dispersive force polarity parameters were determined and are discussed.

EXPERIMENTAL

Sixty-one test compounds containing oligooxyethylene chains were used as liquid stationary phases in a gas chromatographic (GC) column. The structures of the compounds examined are as follows:

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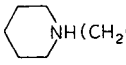
		R	R'	n
I:	$RO(CH_2CH_2O)_nR'$	C_4H_9, C_6H_{13}, C_8H_{17}, $C_{10}H_{21}$ or $C_{12}H_{25}$	H or CH_3	1–4
II:	$RR'N(CH_2CH_2O)_nH$	C_6H_{13}, C_8H_{17}, $C_{10}H_{21}$, $C_{12}H_{25}$ or $C_{14}H_{29}$	H, CH_3 or C_4H_9	1–5
III:	$RS(CH_2CH_2O)_nH$	C_6H_{13}, C_8H_{17}, $C_{10}H_{21}$ or $C_{12}H_{25}$		1–4
IV:	$RO(CH_2CH_2O)_nCH_2CH_2Cl$	C_6H_{13}, C_8H_{17} or $C_{10}H_{21}$		0–2
V:				1–3

Information on the synthesis of these compounds and the conditions of the GC measurements were reported previously[1,4,5]. The empirical and thermodynamic polarity parameters were calculated as in the earlier work[4,5] and dispersive force parameters according to the procedure given previously[13]. Increments of the considered polarity parameters for characteristic fragments were calculated according to the procedure described by Szymanowski *et al.*[14].

RESULTS AND DISCUSSION

The empirical and thermodynamic polarity parameters calculated for the compounds examined are given in Tables I and II, while dispersive force parameters are presented in Table III. As previously, relationships between the polarity parameters were examined. Higher values of the polarity parameters were obtained when ethanol was used as the polar solute in comparison with methanol. The following linear relationships were obtained:

$$I_R^{C_2H_5OH} = 58.7 + 0.9857\, I_R^{CH_3OH}; \qquad r = 0.9862$$
$$PI^{C_2H_5OH} = 29.97 + 0.8069\, PI^{CH_3OH}; \qquad r = 0.9826$$
$$\rho^{C_2H_5OH} = 0.6869 + 1.2602\, \rho^{CH_3OH}; \qquad r = 0.9842$$
$$\Delta G_s^m(OH)^{C_2H_5OH} = -0.5956 + 0.8597\, \Delta G_s^m(OH)^{CH_3OH}; \qquad r = 0.9698$$
$$\Delta G_s^m(C{=}O)^{MPK} = -0.5442 + 0.9943\, \Delta G_s^m(C{=}O)^{MEK}; \qquad r = 0.9552$$

where I_R = retention index; PI = polarity index; ρ = coefficient ρ, relative retention of polar solute and *n*-hexane; $\Delta G_s^m(OH)$, $\Delta G_s^m(C{=}O)$ = partial molal Gibbs free energy of solution of hydroxyl and carbonyl group, respectively; these parameters were estimated for ethanol (*e.g.*, $I_R^{C_2H_5OH}$) and methanol used as polar solutes; MPK and MEK denote 2-pentanone and 2-butanone, respectively. The regression coefficients in the above equations are different to those reported for other homologues series. However, such relationships allow the values of polarity parameters to be calculated for ethanol when only data for methanol are available[15,16].

An accepted measure of stationary phase polarity is the sum of the first five McReynolds constants, $\sum_{i=1}^{5} \Delta I_i$. Generally, approximately linear relationships exist between the examined polarity parameters and $\sum_{i=1}^{5} \Delta I_i$ (Table IV), similarly to results obtained earlier[4–6,13].

TABLE I

POLARITY PARAMETERS

Group of compounds	*R*	*R'*	*n*	*Solute*	I_R		*PI*		ρ	
					70°C	*90°C*	*70°C*	*90°C*	*70°C*	*90°C*
I	C_4H_9	H	2	CH_3OH	746	708	104.0	97.4	3.33	2.20
				C_2H_5OH	808	769	113.0	107.5	5.59	3.46
	C_4H_9	H	3	CH_3OH	767	751	107.0	105.1	3.96	3.10
				C_2H_5OH	822	805	115.0	112.6	6.18	4.62
	C_6H_{13}	H	1	CH_3OH	657	632	90.1	87.3	1.47	1,21
				C_2H_5OH	723	708	104.0	101.8	3.00	2.46
	C_6H_{13}	H	2	CH_3OH	686	671	93.2	89.9	2.02	1.55
				C_2H_5OH	750	739	106.2	103.3	3.60	3.01
	C_6H_{13}	H	3	CH_3OH	716	691	98.7	94.6	2.57	2.01
				C_2H_5OH	775	756	108.6	105.9	4.34	3.30
	C_6H_{13}	H	4	CH_3OH	727	704	101.2	97.1	2.89	2.19
				C_2H_5OH	783	766	109.7	107.4	4.59	3.48
	C_6H_{13}	CH_3	3	CH_3OH	597	567	70.7	58.7	0.97	0.77
				C_2H_5OH	661	642	88.4	83.7	1.71	1.40
	C_8H_{17}	H	1	CH_3OH	617	593	77.4	72.2	1.17	0.93
				C_2H_5OH	683	663	93.6	92.1	2.14	1.93
	C_8H_{17}	H	2	CH_3OH	644	629	83.6	79.1	1.42	1.12
				C_2H_5OH	709	700	97.6	93.2	2.63	2.11
	C_8H_{17}	H	3	CH_3OH	682	654	92.2	85.8	1.96	1.49
				C_2H_5OH	731	714	101.5	98.4	2.94	2.34
	C_8H_{17}	H	4	CH_3OH	700	684	96.1	93.1	2.33	1.91
				C_2H_5OH	753	736	105.2	102.6	3.67	2.85
	C_8H_{17}	CH_3	3	CH_3OH	578	560	63.5	55.4	0.82	0.73
				C_2H_5OH	643	627	84.0	79.6	1.47	1.24
	$C_{10}H_{21}$	H	1	CH_3OH	581	565	69.3	57.2	0.73	0.51
				C_2H_5OH	648	638	87.8	80.3	1.46	1.02
	$C_{10}H_{21}$	H	2	CH_3OH	611	575	75.2	62.1	1.10	0.82
				C_2H_5OH	678	648	92.0	85.2	2.00	1.47
	$C_{10}H_{21}$	H	3	CH_3OH	649	620	82.6	79.2	1.63	1.36
				C_2H_5OH	699	671	95.7	89.7	2.54	2.27
	$C_{10}H_{21}$	CH_3	3	CH_3OH	569	543	59.8	45.7	0.98	0.62
				C_2H_5OH	627	607	80.0	73.8	1.28	1.05
	$C_{12}H_{25}$	H	3	CH_3OH	615	597	75.0	61.0	1.28	0.93
				C_2H_5OH	659	638	89.7	74.2	2.07	1.71
II	C_6H_{13}	H	1	CH_3OH	760	733	108.1	101.1	3.07	2.73
				C_2H_5OH	789	759	112.3	107.6	5.21	4.88
	C_6H_{13}	H	2	CH_3OH	811	802	113.9	112.1	5.50	5.32
				C_2H_5OH	844	875	118.1	122.0	7.26	7.26
	C_6H_{13}	H	3	CH_3OH	857	817	119.6	116.0	8.01	6.58
				C_2H_5OH	881	836	122.8	121.6	10.48	9.63
	C_6H_{13}	H	4	CH_3OH	908	890	125.3	124.2	11.11	7.73
				C_2H_5OH	945	942	128.4	127.5	14.25	9.98
	C_8H_{17}	H	1	CH_3OH	710	694	98.5	95.5	2.67	2.15
				C_2H_5OH	749	742	105.0	104.1	3.74	3.15
	C_8H_{17}	H	2	CH_3OH	753	742	106.3	101.1	3.77	3.32
				C_2H_5OH	800	792	111.2	102.1	6.26	5.77
	C_8H_{17}	H	3	CH_3OH	807	805	113.9	111.3	5.77	5.14
				C_2H_5OH	852	843	118.4	113.0	8.35	8.41

(Continued on p. 294)

TABLE I (*continued*)

Group of compounds	*R*	*R'*	*n*	*Solute*	I_R		*PI*		*ρ*	
					70°C	*90°C*	*70°C*	*90°C*	*70°C*	*90°C*
	$C_{10}H_{21}$	H	1	CH_3OH	688	669	97.5	93.3	2.70	2.68
				C_2H_5OH	740	733	103.6	101.2	2.98	2.82
	$C_{10}H_{21}$	H	2	CH_3OH	725	715	104.8	104.2	3.34	2.94
				C_2H_5OH	786	776	110.1	108.8	4.45	3.69
	$C_{10}H_{21}$	H	3	CH_3OH	769	732	111.3	109.2	4.00	3.21
				C_2H_5OH	825	778	115.6	113.7	5.63	4.86
	$C_{10}H_{21}$	H	4	CH_3OH	811	780	117.5	109.6	4.60	3.68
				C_2H_5OH	874	811	122.0	113.8	6.86	4.60
	$C_{12}H_{25}$	H	1	CH_3OH	661	655	88.2	86.8	1.69	1.53
				C_2H_5OH	712	708	98.6	98.0	2.63	2.32
	$C_{12}H_{25}$	H	2	CH_3OH	693	664	99.8	98.1	2.73	2.47
				C_2H_5OH	757	747	106.4	102.8	3.84	3.44
	$C_{14}H_{29}$	H	1	CH_3OH	649	644	85.7	84.5	1.54	1.43
				C_2H_5OH	698	693	98.3	95.4	2.39	2.13
	C_4H_9	C_4H_9	1	CH_3OH	720	708	102.0	98.3	2.62	1.77
				C_2H_5OH	767	763	106.8	104.4	4.55	2.43
	C_4H_9	C_4H_9	2	CH_3OH	765	759	106.2	103.1	4.47	4.24
				C_2H_5OH	815	809	113.0	108.7	6.75	5.98
	C_4H_9	C_4H_9	3	CH_3OH	817	813	114.0	109.6	6.34	6.11
				C_2H_5OH	860	851	118.0	109.8	8.79	6.63
	C_4H_9	C_4H_9	4	CH_3OH	861	856	119.9	116.2	7.97	6.91
				C_2H_5OH	906	897	124.1	121.1	9.80	8.57
	C_4H_9	C_4H_9	5	CH_3OH	920	901	125.6	122.5	9.96	8.11
				C_2H_5OH	947	928	127.9	126.1	11.90	9.77
	$C_{10}H_{21}$	CH_3	1	CH_3OH	667	645	90.8	80.3	2.01	1.44
				C_2H_5OH	724	694	101.8	97.2	2.71	2.14
	$C_{10}H_{21}$	CH_3	2	CH_3OH	700	695	98.1	94.3	2.84	1.98
				C_2H_5OH	718	703	107.5	103.9	3.91	2.22
	$C_{10}H_{21}$	CH_3	3	CH_3OH	729	709	103.6	98.4	3.61	2.34
				C_2H_5OH	764	757	110.1	106.3	5.06	3.37
III	C_6H_{13}	–	1	CH_3OH	640	599	86.2	81.1	1.45	1.33
				C_2H_5OH	720	700	95.3	90.3	2.91	2.77
	C_6H_{13}	–	2	CH_3OH	674	648	91.5	86.3	1.75	1.62
				C_2H_5OH	745	741	99.1	95.5	3.33	3.08
	C_6H_{13}	–	3	CH_3OH	708	700	94.6	90.8	2.03	1.95
				C_2H_5OH	771	761	105.2	103.2	3.76	3.59
	C_6H_{13}	–	4	CH_3OH	743	737	99.1	93.7	2.45	2.30
				C_2H_5OH	780	773	109.6	107.3	4.38	4.17
	C_8H_{17}	–	1	CH_3OH	611	583	81.2	75.8	1.19	1.08
				C_2H_5OH	660	654	88.7	84.7	2.62	2.53
	C_8H_{17}	–	2	CH_3OH	653	621	86.2	83.3	1.54	1.46
				C_2H_5OH	682	670	93.8	90.2	3.00	2.87
	C_8H_{17}	–	3	CH_3OH	689	649	91.3	85.6	1.88	1.50
				C_2H_5OH	723	680	99.6	92.7	3.55	1.93
	C_8H_{17}	–	4	CH_3OH	723	687	97.2	85.9	2.36	1.91
				C_2H_5OH	740	725	105.5	103.8	4.10	3.42
	$C_{10}H_{21}$	–	1	CH_3OH	583	514	74.2	72.6	1.05	1.02
				C_2H_5OH	626	580	85.6	82.5	2.36	1.92
	$C_{10}H_{21}$	–	2	CH_3OH	629	600	79.8	75.5	1.27	1.19
				C_2H_5OH	653	633	90.0	86.8	2.56	2.41

TABLE I (*continued*)

Group of compounds	*R*	*R'*	*n*	*Solute*	I_R		*PI*		*ρ*	
					70°C	*90°C*	*70°C*	*90°C*	*70°C*	*90°C*
	$C_{10}H_{21}$	–	3	CH_3OH	646	622	84.4	80.3	1.47	1.36
				C_2H_5OH	680	667	95.0	92.2	2.83	2.77
	$C_{12}H_{25}$	–	1	CH_3OH	568	570	70.3	59.7	0.98	0.79
				C_2H_5OH	669	642	82.9	83.8	1.86	1.40
	$C_{12}H_{25}$	–	2	CH_3OH	606	579	73.5	63.5	1.06	0.84
				C_2H_5OH	636	642	82.9	83.8	1.97	1.51
IV	C_6H_{13}	–	2	CH_3OH	681	610	84.3	76.4	1.85	1.88
				C_2H_5OH	734	715	91.4	89.0	2.33	2.40
	C_8H_{17}	–	1	CH_3OH	612	618	61.9	60.8	1.47	1.43
				C_2H_5OH	672	665	71.0	67.5	1.79	1.76
	C_8H_{17}	–	2	CH_3OH	672	663	80.7	72.3	1.78	1.72
				C_2H_5OH	701	705	81.9	77.1	2.01	2.04
	$C_{10}H_{21}$	–	0	CH_3OH	530	520	47.5	45.5	0.54	0.50
				C_2H_5OH	547	538	58.7	57.2	0.63	0.55
	$C_{10}H_{21}$	–	1	CH_3OH	541	558	53.6	54.1	0.60	0.72
				C_2H_5OH	583	575	65.4	61.7	0.86	0.82
	$C_{10}H_{21}$	–	2	CH_3OH	609	555	65.5	62.7	1.08	0.71
				C_2H_5OH	648	605	76.4	73.1	1.52	1.04
V	–	–	1	CH_3OH	811	807	113.2	111.4	5.16	4.93
				C_2H_5OH	851	843	118.3	117.2	7.10	6.57
	–	–	2	CH_3OH	833	824	118.1	117.2	6.20	4.80
				C_2H_5OH	903	896	121.3	119.4	9.16	8.33
	–	–	3	CH_3OH	857	853	122.3	120.1	7.30	7.01
				C_2H_5OH	949	944	124.7	122.3	12.13	9.98

TABLE II

POLARITY PARAMETERS

Group of compounds	*R*	*R'*	*n*	$\Delta G_s^m(OH)$ *(kJ/mol)*		$\Delta G_s^m(C=O)$ *(kJ/mol)*		$\sum_{i=1}^{5} \Delta I_i$
				CH_3OH	*C_2H_5OH*	*MEK**	*MPK**	
I	C_4H_9	H	2	−11.5	−10.9	− 9.5	− 8.9	1427
	C_4H_9	H	3	−12.0	−11.5	−10.0	− 9.5	1577
	C_6H_{13}	H	1	−10.6	− 9.9	− 8.7	− 7.9	1071
	C_6H_{13}	H	2	−11.1	−10.3	− 9.1	− 8.4	1163
	C_6H_{13}	H	3	−11.4	−10.8	− 9.4	− 8.7	1270
	C_6H_{13}	H	4	−11.8	−11.2	− 9.8	− 9.2	1374
	C_6H_{13}	CH_3	3	− 9.9	− 9.1	− 8.0	− 7.1	931
	C_8H_{17}	H	1	− 9.9	− 9.1	− 7.0	− 7.0	933
	C_8H_{17}	H	2	−10.2	− 9.6	− 8.2	− 7.5	1040
	C_8H_{17}	H	3	−10.7	−10.0	− 8.6	− 8.0	1161
	C_8H_{17}	H	4	−11.3	−10.6	− 9.2	− 8.6	1262
	C_8H_{17}	CH_3	3	− 9.1	− 8.5	− 7.1	− 6.5	887
	$C_{10}H_{21}$	H	1	− 9.1	− 8.4	− 7.1	− 6.4	856
	$C_{10}H_{21}$	H	2	− 9.4	− 9.0	− 7.5	− 7.0	947

(Continued on p. 296)

TABLE II (*continued*)

Group of compounds	*R*	*R'*	*n*	$\Delta G_s^m(OH)$ (kJ/mol)		$\Delta G_s^m(C=O)$ (kJ/mol)		$\sum_{i=1}^{5} \Delta I_i$
				CH_3OH	C_2H_5OH	MEK*	MPK*	
	$C_{10}H_{21}$	H	3	−10.0	− 9.4	− 8.1	− 7.5	1038
	$C_{10}H_{21}$	CH_3	3	− 9.2	− 8.6	− 7.3	− 6.7	812
	$C_{12}H_{25}$	H	3	− 8.6	− 8.0	− 6.6	− 6.0	943
II	C_6H_{13}	H	1	−12.0	−10.5	−10.2	−10.0	1455
	C_6H_{13}	H	2	−12.5	−11.1	−10.6	−10.3	1642
	C_6H_{13}	H	3	−13.3	−11.8	−10.9	−10.5	1920
	C_6H_{13}	H	4	−13.8	−12.4	−11.4	−11.0	2024
	C_8H_{17}	H	1	−11.3	− 9.8	− 9.5	− 9.2	1045
	C_8H_{17}	H	2	−12.1	−10.6	−10.0	− 9.8	1270
	C_8H_{17}	H	3	−12.9	−11.4	−10.4	−10.2	1506
	$C_{10}H_{21}$	H	1	−11.2	− 9.3	− 8.8	− 8.5	1099
	$C_{10}H_{21}$	H	2	−11.7	−10.3	− 9.1	− 8.8	1136
	$C_{10}H_{21}$	H	3	−12.2	−10.9	− 9.6	− 9.4	1270
	$C_{10}H_{21}$	H	4	−12.8	−11.7	−10.1	− 9.7	1388
	$C_{12}H_{25}$	H	1	−10.2	− 9.6	− 8.6	− 8.2	888
	$C_{12}H_{25}$	H	2	−10.6	− 9.9	− 8.9	− 8.6	1028
	$C_{14}H_{29}$	H	1	−10.1	− 9.0	− 8.5	− 8.2	794
	C_4H_9	C_4H_9	1	−11.6	−10.3	− 9.8	− 9.6	1080
	C_4H_9	C_4H_9	2	−12.3	−11.1	−10.1	− 9.9	1300
	C_4H_9	C_4H_9	3	−13.0	−11.6	−10.4	−10.4	1513
	C_4H_9	C_4H_9	4	−13.6	−12.7	−10.8	−10.8	1735
	C_4H_9	C_4H_9	5	−14.0	−13.2	−11.1	−11.0	1970
	$C_{10}H_{21}$	CH_3	1	−10.8	− 9.7	− 8.7	− 8.0	935
	$C_{10}H_{21}$	CH_3	2	−11.1	−10.1	− 9.9	− 7.7	1138
	$C_{10}H_{21}$	CH_3	3	−12.1	−10.5	−10.7	− 8.4	1633
III	C_6H_{13}	–	1	−10.1	− 9.2	− 8.2	− 7.3	1032
	C_6H_{13}	–	2	−10.6	− 9.7	− 8.6	− 7.7	1128
	C_6H_{13}	–	3	−10.9	−10.0	− 8.9	− 8.0	1245
	C_6H_{13}	–	4	−11.5	−10.6	− 9.5	− 8.6	1352
	C_8H_{17}	–	1	− 9.3	− 8.7	− 7.2	− 6.7	920
	C_8H_{17}	–	2	− 9.8	− 9.0	− 7.8	− 7.0	1035
	C_8H_{17}	–	3	−10.1	− 9.4	− 8.1	− 7.7	1152
	C_8H_{17}	–	4	−10.9	− 9.6	− 8.9	− 8.2	1249
	$C_{10}H_{21}$	–	1	− 8.9	− 8.3	− 6.8	− 6.1	847
	$C_{10}H_{21}$	–	2	− 9.3	− 8.7	− 7.2	− 6.6	931
	$C_{10}H_{21}$	–	3	− 9.7	− 9.0	− 7.6	− 7.0	1032
	$C_{12}H_{25}$	–	1	− 8.4	− 8.0	− 6.2	− 5.8	788
	$C_{12}H_{25}$	–	2	− 8.8	− 8.4	− 6.7	− 6.3	849
IV	C_6H_{13}	–	2	−10.9	−10.2	− 8.8	− 8.5	1054
	C_8H_{17}	–	1	− 8.9	− 7.9	− 7.4	− 7.1	936
	C_8H_{17}	–	2	− 9.3	− 8.6	− 7.5	− 7.2	1065
	$C_{10}H_{21}$	–	0	− 8.0	− 7.5	− 6.0	− 5.5	773
	$C_{10}H_{21}$	–	1	− 8.5	− 8.2	− 6.7	− 6.5	860
	$C_{10}H_{21}$	–	2	− 9.1	− 8.9	− 7.4	− 7.2	937
V	–	–	1	−11.3	−10.1	−10.6	−10.0	1633
	–	–	2	−11.8	−10.5	−11.0	−10.6	1799
	–	–	3	−12.3	−11.0	−11.3	−10.8	1931

* MEK = 2-Butanone; MPK = 2-pentanone.

TABLE III

DISPERSIVE FORCE PARAMETERS

Group of compounds	*R*	*R'*	*n*	*Criterion A*		*$\Delta G^E(CH_2)$ (J/mol)*			*$\overline{\Delta G^E(CH_2)}$ (J/mol)*	*$\Delta G_s^m(CH_2)$ (kJ/mol)*
				70°C	*90°C*	*A**	*B**	*C**		
I	C_4H_9	H	2	2.280	2.101	201	120	638	320	−2.30
	C_4H_9	H	3	2.210	2.044	217	135	672	341	−2.33
	C_6H_{13}	H	1	2.275	2.139	171	94	573	279	−2.54
	C_6H_{13}	H	2	2.266	2.111	186	109	608	301	−2.50
	C_6H_{13}	H	3	2.265	2.240	205	123	642	323	−2.43
	C_6H_{13}	H	4	2.240	2.063	219	138	677	345	−2.34
	C_6H_{13}	CH_3	3	2.379	2.160	192	109	611	304	−2.48
	C_8H_{17}	H	1	2.334	2.164	157	83	543	261	−2.46
	C_8H_{17}	H	2	2.310	2.120	174	97	575	282	−2.40
	C_8H_{17}	H	3	2.285	2.109	189	112	613	305	−2.34
	C_8H_{17}	H	4	2.265	2.062	206	126	647	326	−2.28
	C_8H_{17}	CH_3	3	2.325	2.170	177	97	581	285	−2.63
	$C_{10}H_{21}$	H	1	2.358	2.200	145	71	514	243	−2.58
	$C_{10}H_{21}$	H	2	2.334	2.178	159	84	548	264	−2.54
	$C_{10}H_{21}$	H	3	2.310	2.150	176	101	585	287	−2.48
	$C_{10}H_{21}$	CH_3	3	2.367	2.201	135	86	551	257	−2.64
	$C_{12}H_{25}$	H	3	2.332	2.208	160	89	553	267	−2.56
II	C_6H_{13}	H	1	2.334	2.133	179	102	594	292	−2.35
	C_6H_{13}	H	2	2.269	2.068	193	116	625	311	−2.22
	C_6H_{13}	H	3	2.217	1.999	211	133	663	336	−2.16
	C_6H_{13}	H	4	2.150	1.914	228	146	698	357	−2.11
	C_8H_{17}	H	1	2.358	2.168	165	90	564	274	−2.36
	C_8H_{17}	H	2	2.295	2.101	182	105	597	295	−2.32
	C_8H_{17}	H	3	2.237	2.033	197	120	635	317	−2.29
	$C_{10}H_{21}$	H	1	2.414	2.242	151	79	535	255	−2.39
	$C_{10}H_{21}$	H	2	2.348	2.186	167	93	569	276	−2.35
	$C_{10}H_{21}$	H	3	2.306	2.116	185	108	604	299	−2.32
	$C_{10}H_{21}$	H	4	2.259	2.066	199	122	636	319	−2.27
	$C_{12}H_{25}$	H	1	2.399	2.263	135	67	505	236	−2.42
	$C_{12}H_{25}$	H	2	2.388	2.213	152	82	540	258	−2.39
	$C_{14}H_{29}$	H	1	2.397	2.257	123	56	475	218	−2.51
	C_4H_9	C_4H_9	1	2.363	2.218	169	91	567	276	−2.31
	C_4H_9	C_4H_9	2	2.321	2.168	183	106	605	298	−2.28
	C_4H_9	C_4H_9	3	2.283	2.104	199	124	637	320	−2.21
	C_4H_9	C_4H_9	4	2.217	2.058	216	135	669	340	−2.14
	C_4H_9	C_4H_9	5	2.150	2.000	233	150	703	362	−2.09
	$C_{10}H_{21}$	CH_3	1	2.402	2.242	146	74	520	247	−2.46
	$C_{10}H_{21}$	CH_3	2	2.351	2.185	162	88	555	268	−2.44
	$C_{10}H_{21}$	CH_3	3	2.311	2.174	175	103	584	287	−2.42
III	C_6H_{13}	H	1	2.334	2.141	169	93	572	278	−2.51
	C_6H_{13}	H	2	2.240	2.100	184	106	603	298	−2.47
	C_6H_{13}	H	3	2.165	2.053	201	122	641	321	−2.42
	C_6H_{13}	H	4	2.080	2.040	218	135	678	344	−2.39
	C_8H_{17}	H	1	2.372	2.160	155	80	543	259	−2.56
	C_8H_{17}	H	2	2.280	2.125	172	96	575	281	−2.52
	C_8H_{17}	H	3	2.220	2.099	188	111	613	304	−2.48
	C_8H_{17}	H	4	2.112	2.093	203	125	645	324	−2.43

(Continued on p. 298)

TABLE III (*continued*)

Group of compounds	*R*	*R'*	*n*	*Criterion A*		$\Delta G^E(CH_2)$ *(J/mol)*			$\overline{\Delta G^E(CH_2)}$ *(J/mol)*	$\Delta G_s^m(CH_2)$ *(kJ/mol)*
				70°C	*90°C*	*A**	*B**	*C**		
	$C_{10}H_{21}$	H	1	2.410	2.188	141	70	512	241	−2.59
	$C_{10}H_{21}$	H	2	2.340	2.150	155	83	543	260	−2.56
	$C_{10}H_{21}$	H	3	2.276	2.130	170	97	581	283	−2.52
	$C_{12}H_{25}$	H	1	2.418	2.182	143	72	484	237	−2.63
	$C_{12}H_{25}$	H	2	2.355	2.168	156	84	517	248	−2.61
IV	C_6H_{13}	–	2	2.296	2.109	197	111	625	311	−2.49
	C_8H_{17}	–	1	2.356	2.164	167	84	556	269	−2.46
	C_8H_{17}	–	2	2.324	2.135	180	100	588	289	−2.43
	$C_{10}H_{21}$	–	0	2.383	2.202	135	60	501	232	−2.62
	$C_{10}H_{21}$	–	1	2.357	2.172	147	70	527	248	−2.58
	$C_{10}H_{21}$	–	2	2.293	2.139	160	87	555	267	−2.53
V	–	–	4	2.243	2.153	188	109	610	302	−2.24
	–	–	1	2.117	2.078	205	123	642	323	−2.10
	–	–	2	2.032	1.995	220	135	677	373	−1.93

* A, B and C denote alkanes, alcohols and ketones, respectively, as solutes.

TABLE IV

RELATIONSHIP BETWEEN THE POLARITY PARAMETERS AND THE SUM OF THE FIRST FIVE McREYNOLDS CONSTANTS

$P = a + b\sum_{i=1}^{5} \Delta I_i$, where P represents any polarity parameter.

Parameter P	*Solute*	*a*	*b*	*r*
I_R	Methanol	390.98	0.2575	0.9479
	Ethanol	443.03	0.2548	0.9384
PI	Methanol	30.707	0.0484	0.9824
	Ethanol	55.447	0.0387	0.9584
ρ	Methanol	−4.888	$6.589 \cdot 10^{-3}$	0.9308
	Ethanol	−5.808	$8.584 \cdot 10^{-3}$	0.9470
Criterion *A*	At 70°C	2.542	$-2.078 \cdot 10^{-4}$	0.8932
	At 90°C	2.376	$-2.022 \cdot 10^{-4}$	0.9175
$\Delta G_s^m(OH)$	Methanol	−6.363	$-3.731 \cdot 10^{-3}$	0.8673
	Ethanol	−5.589	$-3.272 \cdot 10^{-3}$	0.8577
$\Delta G_s^m(C=O)$	2-Butanone	−4.421	$-3.676 \cdot 10^{-3}$	0.8776
	2-Pentanone	−3.643	$-3.837 \cdot 10^{-3}$	0.8770
$\Delta G^E(CH_2)$	Alkanes	95.36	0.0692	0.8593
	Alcohols	30.73	0.0596	0.8700
	Ketones	413.78	0.1474	0.8668
$\overline{\Delta G^E(CH_2)}$		180.03	0.0919	0.8687
$\Delta G_s^m(CH_2)$	–	−2.8918	$4.124 \cdot 10^{-4}$	0.9008

The retention index of alcohol, the polarity index, the coefficient ρ, the partial molar Gibbs free energy of solution per methylene group [$\Delta G_s^m(CH_2)$] and the partial molar excess Gibbs free energy of solution per methylene group [$\overline{\Delta G}^E(CH_2)$] increase with increasing polarity, whereas the criterion A and the partial molal Gibbs free energy of solution of hydroxyl [$\Delta G_s^m(OH)$] and carbonyl [$\Delta G_s^m(C=O)$] groups decrease (Fig. 1). Typical relationships between polarity parameters are presented in Fig. 2 and Table V. Generally, the polarity parameters can be correlated according to approximately linear relationships, although in many instances higher correlation coefficients were obtained for parabolic equations. Lower correlation coefficients were obtained for parameters calculated with lower precision, *i.e.*, for the coefficient ρ and the criterion A.

The inter-parameter relationships presented confirm the suggestion that all the parameters considered properly describe the polarity of the compounds examined. Significant differences are observed when the sensitivity of these parameters to changes

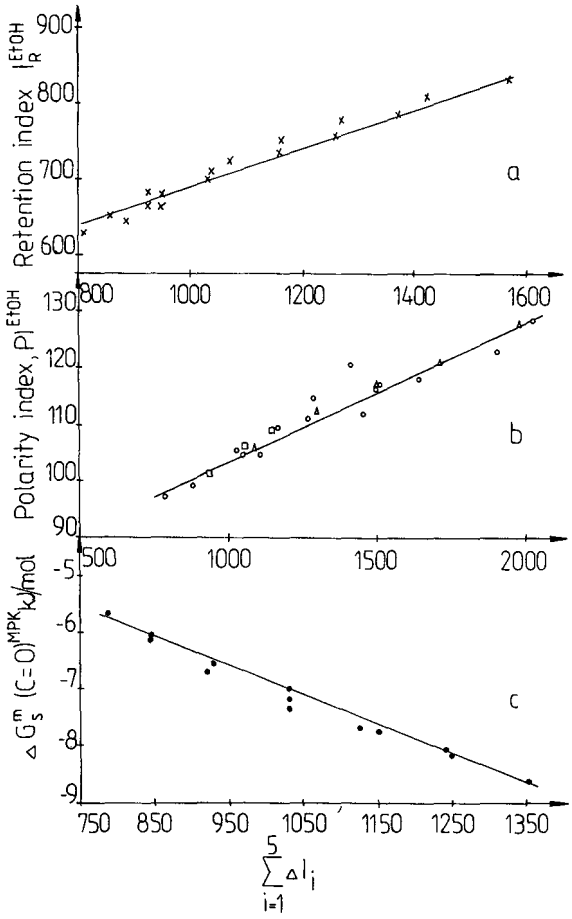


Fig. 1. Relationships between some polarity parameters and the sum of the first five McReynolds constants for oligooxyethylene derivatives of (a) alcohols, I (×), (b) alkylamines, II (○, R′ = H; △, R = R′ = C_4H_9; □, R = $C_{10}H_{21}$, R′ = CH_3), and (c) thio alcohols, III (●). EtOH = Ethanol.

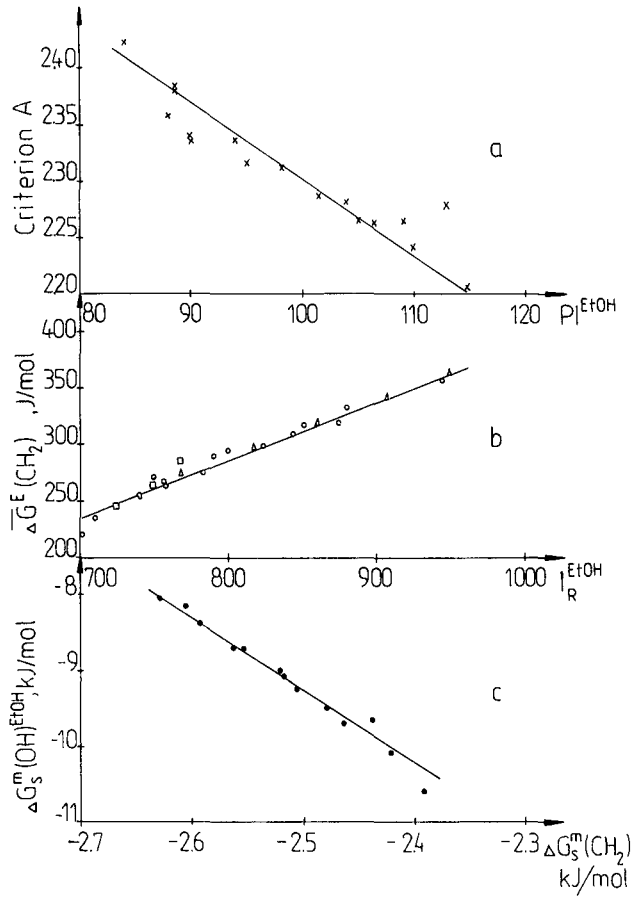


Fig. 2. Relationships between polarity parameters: (a) criterion A *vs.* $P_I^{CH_3OH}$ for I (×); (b) $\overline{\Delta G^E}(CH_2)$ *vs.* $I_R^{C_2H_5OH}$ for II (○, R′ = H; △, R = R′ = C_4H_9; □, R = $C_{10}H_{21}$, R′ = CH_3); (c) $\Delta G_s^m(OH)^{C_2H_5OH}$ *vs.* $\Delta G_s^m(CH_2)$ for III (●).

in the structures of the examined compounds, $[(P_{max} - P_{min})/P_{min}]100\%$, is taken into account (Table VI). The appropriate data for 1,3-bis-(ω-alkoxyoligooxyethylene)-2-propanols are added for comparison. These data show that the criterion A and $\Delta G_s^m(CH_2)$ are less sensitive to changes in the structures of the compounds. The sensitivity of other parameters is higher and comparable to results reported for other groups of compounds[13].

The individual values of $\Delta G^E(CH_2)$ for alkanes, alcohols and ketones used as solutes are significantly different but they all increase with increasing stationary phase polarity.

The structure of the compounds significantly influences their polarities. The length of the oligooxyethylene chain, the length of alkyl group(s), the distribution of carbon atoms in the alkyl chain(s), the presence of different heteroatoms linked to alkyl group(s) and a hydroxy or methoxy group or chlorine atom terminating the molecule influence the polarity.

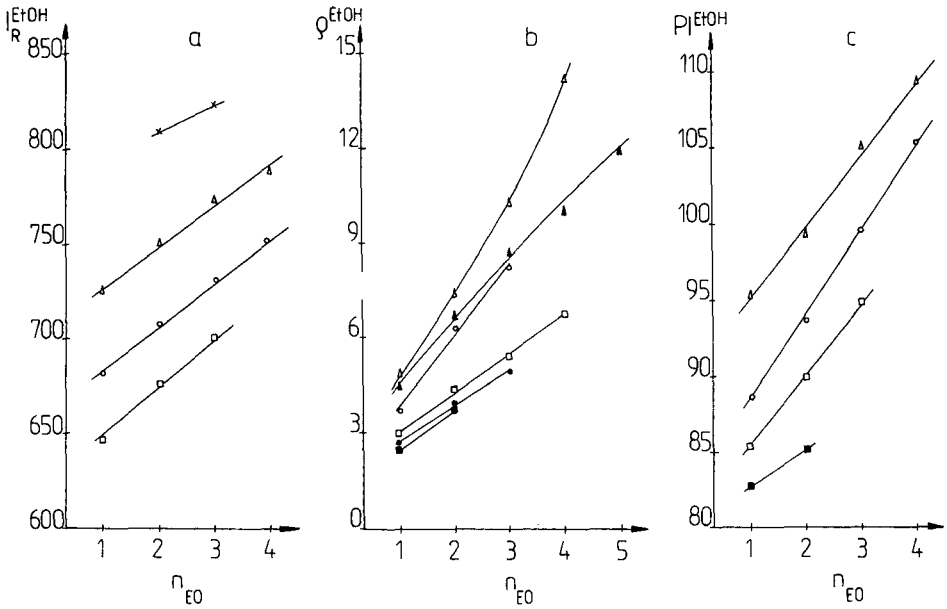


Fig. 3. Influence of the lengths of the oligooxyethylene chain and the alkyl group(s) on polarity parameters: (a) $I_R^{C_2H_5OH}$ *vs.* n_{EO} for I; (b) $\rho^{C_2H_5OH}$ *vs.* n_{EO} for II; (c) $PI^{C_2H_5OH}$ *vs.* n_{EO} for III (×, $R = C_4H_9$; △, $R = C_6H_{13}$; ○, C_8H_{17}; □, $C_{10}H_{21}$; ■, $C_{12}H_{25}$; ▲, $R = R' = C_4H_9$; ●, $R = C_{10}H_{21}$, $R' = CH_3$).

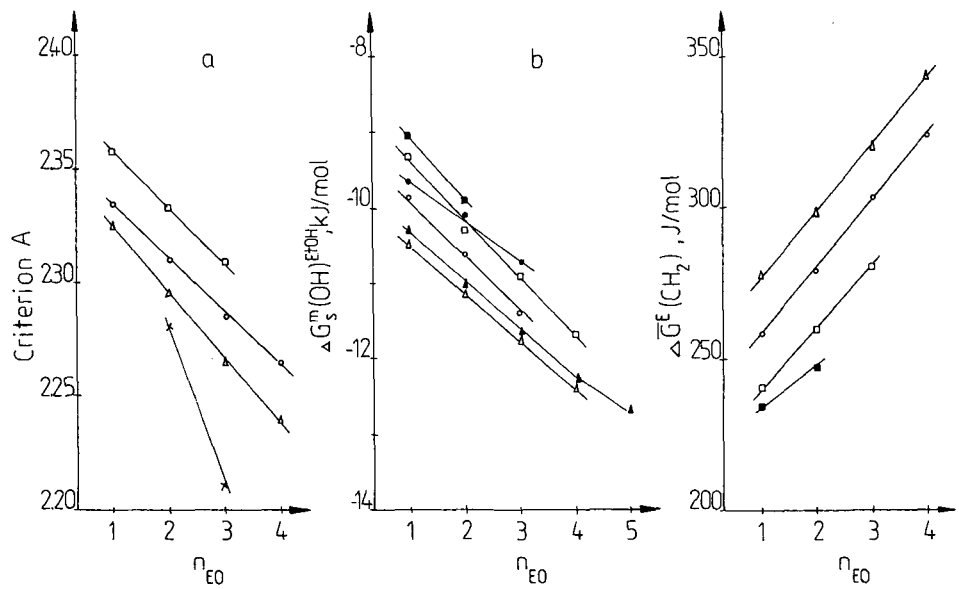


Fig. 4. Influence of the lengths of the oligooxyethylene chain and the alkyl group(s) on some of the thermodynamic and dispersive force parameters: (a) criterion *A* *vs.* n_{EO} for I; (b) $\Delta G_s^m(OH)^{C_2H_5OH}$ *vs.* n_{EO} for II; (c) $\Delta G^E(CH_2)$ *vs.* n_{EO} for III. Symbols as in Fig. 3).

TABLE V

RELATIONSHIPS BETWEEN EMPIRICAL, THERMODYNAMICAL AND DISPERSIVE FORCE POLARITY PARAMETERS

P_y and P_x represent two polarity parameters; between them exists the relationship presented in Table V; *e.g.*, $P_y = I_R$, $P_x = PI$: $P_y = 166.3 + 5.7177\ P_x$.

P_y	P_x	$P_y = a + bP_x$			$P_y = a + bP_x + cP_x^2$			
		a	b	r	a	b	c	r
$I_R^\star$	$PI^\star$	166.3	5.7177	0.9484	821.1	−7.94	0.0693	0.9761
	$\rho^\star$	622.2	28.141	0.9395	564.5	54.12	−1.944	0.9700
	$\Delta G_s^m(OH)^\star$	104.3	−64.97	0.9126	77.92	−70.3	−0.264	0.9126
	$\Delta G_s^m(C=O)^{\star\star}$	272.6	−57.75	0.9305	541.2	10.05	4.127	0.9395
	$\Delta G_s^m(CH_2)$	2075	553.3	0.9332	1038.1	−338.1	−199.9	0.9354
	$\Delta G^E(CH_2)$	142.1	2.088	0.8142	791.7	−2.45	$-7.82 \cdot 10^{-3}$	0.8259
$PI^\star$	$\rho^\star$	82.59	4.282	0.8617	67.11	11.27	−0.523	0.9464
	$\Delta G_s^m(OH)^\star$	−3.57	−10.6	0.9001	−102.6	−30.6	−0.989	0.9117
	$\Delta G_s^m(C=O)^{\star\star}$	25.85	−9.22	0.8954	28.81	−8.47	0.045	0.9354
	$\Delta G_s^m(CH_2)$	300.7	82.98	0.8937	−156.2	−310	−84.11	0.9211
	$\Delta G^E(CH_2)$	9.63	0.317	0.9160	64.49	−0.05	$6.36 \cdot 10^{-1}$	0.9191
$\rho^\star$	$I_R^\star$	3.841	0.012	0.9088	−212.1	0.588	$-3.74 \cdot 10^{-3}$	0.9188
	$PI^\star$	−13.2	0.173	0.8617	27.66	−0.68	$4.32 \cdot 10^{-3}$	0.9638
	$\Delta G_s^m(OH)^\star$	−15.5	−2.01	0.8467	10.31	3.188	0.258	0.8672
	$\Delta G_s^m(C=O)^{\star\star}$	−9.94	−1.75	0.8444	14.01	4.291	0.368	0.9053
	$\Delta G_s^m(CH_2)$	47.82	18.08	0.9132	140.8	98.04	17.11	0.9286
	$\Delta G^E(CH_2)$	−14.5	0.065	0.8646	31.47	−0.26	$5.57 \cdot 10^{-4}$	0.9197
$\Delta G_s^m(OH)^\star$	$I_R^\star$	−9.31	$-7 \cdot 10^{-4}$	0.9056	−17.9	0.022	$-1.51 \cdot 10^{-5}$	0.9135
	$PI^\star$	−2.51	−0.08	0.9001	−9.19	0.071	$-7.42 \cdot 10^{-4}$	0.9173
	$\rho^\star$	−8.31	−0.36	0.8867	−7.23	−0.84	0.0365	0.9059
	$\Delta G_s^m(C=O)^{\star\star}$	−3.38	0.792	0.9092	−3.43	0.778	$-8.43 \cdot 10^{-4}$	0.9092
	$\Delta G_s^m(CH_2)$	−26.1	−6.71	0.8062	10.29	24.51	6.681	0.8222
	$\Delta G^E(CH_2)$	−1.25	−0.03	0.8082	−4.19	−9.27	$-3.54 \cdot 10^{-5}$	0.8294

$\Delta G_s^m(C=O)^{**}$	I_R^*	−8.59	$4.8 \cdot 10^{-4}$	0.9029	−21.9	0.036	$-2.32 \cdot 10^{-5}$	0.9168
	PI^*	−0.62	−0.087	0.8954	−7.99	0.092	$-9.11 \cdot 10^{-4}$	0.9150
	ρ^*	−6.41	−0.408	0.8444	−5.09	−0.99	0.0441	0.9102
	$\Delta G_s^m(OH)^*$	2.1	1.043	0.9092	10.32	2.698	0.0819	0.9176
	$\Delta G^E(CH_2)$	0.803	−0.031	0.8534	1.450	−0.04	$7.79 \cdot 10^{-6}$	0.9535
Criterion	I_R^*	2.802	$-6.81 \cdot 10^{-2}$	0.9065	–	–	–	–
A at 70°C	PI^*	2.663	$-3.64 \cdot 10^{-3}$	0.9251	–	–	–	–
	ρ^*	2.383	−0.0202	0.8997	–	–	–	–
	$\Delta G_s^m(OH)^*$	2.723	0.043	0.9314	–	–	–	–
	$\Delta G_s^m(C=O)^{**}$	2.602	0.0376	0.9282	–	–	–	–
	$\Delta G_s^m(CH_2)$	1.408	−0.369	0.8469	–	–	–	–
	$\Delta G^E(CH_2)$	2.930	$-2.19 \cdot 10^{-3}$	0.8879	–	–	–	–
$\Delta G_s^m(CH_2)$	I_R^*	−3.37	$1.57 \cdot 10^{-3}$	0.9332	–	–	–	–
	PI^*	−3.27	$8.58 \cdot 10^{-3}$	0.9437	–	–	–	–
	ρ^*	−2.61	0.0461	0.9132	–	–	–	–
	$\Delta G_s^m(OH)^*$	−3.36	−0.0971	0.9062	–	–	–	–
	$\Delta G_s^m(C=O)^{**}$	−3.62	−0.0811	0.9156	–	–	–	–
	Criterion A	0.186	−1.1272	0.8449	–	–	–	–
	$\Delta G^E(CH_2)$	−3.31	$3.14 \cdot 10^{-3}$	0.9272	–	–	–	–
$\Delta G^E(CH_2)$	I_R^*	52.64	0.317	0.9142	–	–	–	–
	PI^*	111.7	1.735	0.9460	–	–	–	–
	ρ^*	250.2	8.931	0.9146	–	–	–	–
	$\Delta G_s^m(OH)^*$	62.31	−22.89	0.9282	–	–	–	–
	$\Delta G_s^m(C=O)^{**}$	140.0	−18.23	0.8535	–	–	–	–
	Criterion A	1106	−382.2	0.8934	–	–	–	–
	$\Delta G_s^m(CH_2)$	693.3	168.1	0.9272	–	–	–	–

* Ethanol as solute.
** 2-Pentanone as solute.

TABLE VI

SENSITIVITY OF THE POLARITY PARAMETERS FOR THE COMPOUNDS EXAMINED (A) AND 1,3-BIS(ω-ALKOXYOLIGOOXYETHYLENE)-2-PROPANOLS[5,6,13] (B)

$$\frac{P_{max.} - P_{min.}}{P_{min.}} \cdot 100\%$$

P_{max} and P_{min} are the highest and the lowest values of P polarity parameters for a given group of compounds.

Parameter	*Solute*	*Compounds used as the stationary phase*	
		A	*B*
I_R	Methanol	74	35
	Ethanol	75	28
PI	Methanol	165	79
	Ethanol	120	48
ρ	Methanol	1957	393
	Ethanol	2162	292
$\Delta G_s^m(OH)$	Methanol	76	23
	Ethanol	91	25
$\Delta G_s^m(C=O)$	2-Butanone	105	–
	2-Pentanone	160	22
$\sum_{i=1}^{5} \Delta I_i$	–	331	128
Criterion *A*	–	24	17
$\Delta G_s^m(CH_2)$	–	37	13
$\Delta G^L(CH_2)$	–	86	109

An increase in the length of the oligooxyethylene chain increases the polarities of compounds I–V (Figs. 3 and 4). The regression and correlation coefficients are presented in Table VII. The slopes of the straight lines that describe the polarity *vs.* number of oxyethylene units relationships depend on the length of the alkyl chain, the type of heteroatom linked to the alkyl group and the distribution of the carbon atoms in one or two chains connected with a nitrogen atom. A decrease in the length of the alkyl chain decreases the polarity. Compounds having two short alkyl groups (compounds II, $R = R' = C_4H_9$) are more polar than those with one longer alkyl chain (compounds II, $R = C_8H_{17}$, $R' = H$). This conclusion is consistent with those reported for derivatives of α,ω-diaminooligoethers[9] and diazapolyoxyethylene ethers[10]. Compounds II having decyl and methyl groups are more polar than those having one dodecyl group. However, the difference may, in part, arise from the lower total number of carbon atoms in the two alkyl chains.

An important factor influencing polarity is the type of heteroatom linked to the alkyl group(s) in compounds I–III (Fig. 5). The highest polarities are exhibited by derivatives of alkylamines and the lowest by derivatives of thio alcohols. The polarities of oxyethylene derivatives of alcohols and thio alcohols are similar, *e.g.*, the values of the polarity index (ethanol as solute) of $C_8H_{17}X(CH_2CH_2O)_2H$ are higher by 1.9 index units for derivatives of alcohols in comparison with derivatives of thio alcohols.

The influence of the group terminating the oxyethylene chain on retention was reported earlier[12]. Derivatives of alcohols having an oxyethylene chain terminated by a methoxy group exhibit higher retention indices on non-polar phases (Apiezon K, SE-30) than their analogues having a hydroxy group. An increase in the stationary

TABLE VII

SLOPES AND CORRELATION COEFFICIENTS FOR THE RELATIONSHIPS $P_i = a + bn$

n denotes the number of oxyethylene units. P_i is any polarity parameter.

Parameter	*Solute*	*Type of compounds*							
		I, $R=C_8H_{17}$		*II*, $R=C_8H_{17}$		*III*, $R=C_8H_{17}$		*IV*, $R=C_{10}H_{21}$	
		b	*r*	*b*	*r*	*b*	*r*	*b*	*r*
I_R	CH_3OH	28.7	0.9918	48.5	0.9979	37.7	0.9987	39.5	0.9232
	C_2H_5OH	23.2	0.9991	51.5	0.9999	28.1	0.9877	50.5	0.9865
PI	CH_3OH	6.47	0.9909	7.71	0.9999	5.31	0.9992	9.00	0.9831
	C_2H_5OH	3.87	0,9998	6.72	0.9991	5.62	0.9994	8.85	0.9903
ρ	CH_3OH	0.402	0.9913	1.55	0.9862	0.385	0.9964	0.27	0.9122
	C_2H_5OH	0.491	0.9867	2.31	0.9985	0.499	0.9965	0.455	0.9632
$\Delta G_s^m(OH)$	CH_3OH	−0.47	0.9910	−0.78	0.9999	−0.54	0.9883	−0.85	0.9979
	C_2H_5OH	−0.50	0.9978	−0.77	0.9999	−0.32	0.9825	−0.89	0.9926
$\Delta G_s^m(C=O)$	MEK*	−0.45	0.9946	−0.45	0.9996	−0.53	0.9848	−0.97	0.9843
	MPK*	−0.51	0.9990	−0.49	0.9997	−0.52	0.9908	−0.88	0.9971
$\sum_{i=1}^{5} \Delta I_i$	–	110.8	0.9984	230.4	0.9999	110.4	0.9901	82	0.9994
Criterion *A*	–	−0.02	0.9989	−0.06	0.9997	−0.08	0.9946	−0.04	0.9938
$\underline{\Delta G_s^m}(CH_2)$	–	0.06	0.9996	0.04	0.9995	0.04	0.9982	0.04	0.9968
$\underline{\Delta G^E}(CH_2)$	–	21.8	0.9998	21.5	0.9999	21.8	0.9996	17.5	0.9988
$\Delta G^E(CH_2)$	A**	16.2	0.9997	16.0	0.9993	16.0	0.9996	12.5	0.9997
	B**	14.4	0.9999	15.0	0.9999	15.0	0.9995	13.5	0.9890
	C**	35.1	0.9995	32.5	0.9992	34.4	0.9994	27.0	0.9998

* MEK = 2-Butanone; MPK = 2-pentanone.

** A = alkanes; B = ketones; C = alcohols.

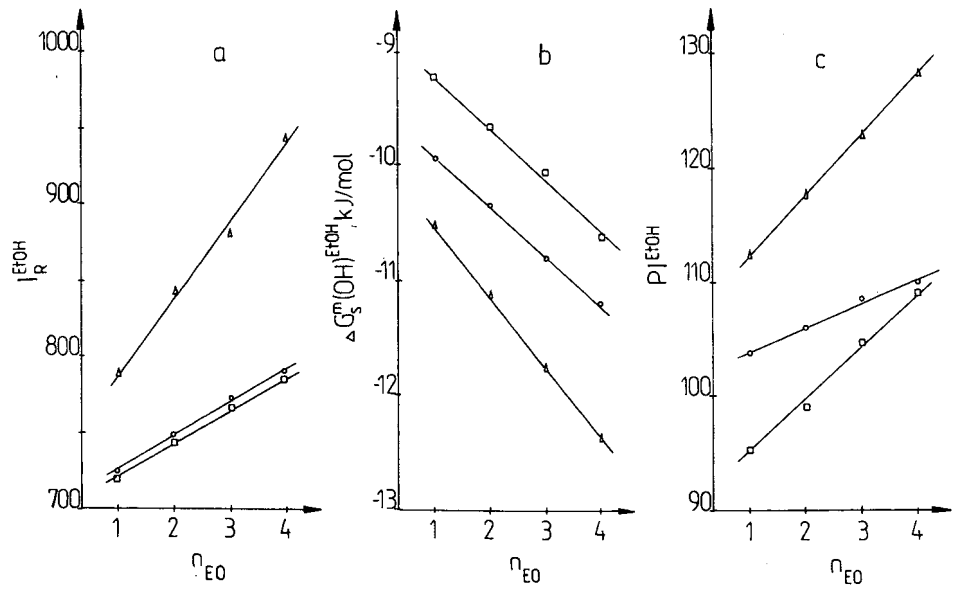


Fig. 5. Influence of the heteroatom linked to the alkyl group(s) in $C_6H_{13}X(CH_2CH_2O)_nH$ on (a) the retention index, (b) $\Delta G_s^m(OH)$ and (c) the polarity index (ethanol as solute) (○, X = –O–; △, X = –NH–; □, X = –S–).

TABLE VIII

INCREMENTS OF POLARITY PARAMETERS

Parameter	*Solute*	*–CH₂–*	*–O–*	*–NH–*	*–N=*	*–S–*	*–OH*	*–Cl*	*Constant*	*Error*	
										Abs.	*Rel.*
I_R	CH_3OH	−17.5	72.9	164.6	166.7	55.8	197.0	135.0	530.9	19	2.7
	C_2H_5OH	−17.4	70.2	152.7	156.8	48.2	183.9	105.1	610.9	21	2.8
PI	CH_3OH	−3.11	12.3	28.81	30.01	8.82	42.82	19.55	58.73	3.8	4.7
	C_2H_5OH	−2.67	10.4	21.85	22.85	5.03	30.54	14.26	81.26	3.5	3.7
ρ	CH_3OH	−0.39	1.77	4.14	4.32	1.26	4.18	3.37	−1.03	0.28	13.2
	C_2H_5OH	−0.56	2.30	5.13	5.56	1.96	5.44	3.60	0.07	0.29	13.4
$\Delta G_s^m(OH)$	CH_3OH	0.28	−1.1	−2.34	−2.48	−0.4	−2.12	−1.54	−8.86	0.4	3.5
	C_2H_5OH	0.23	−1.0	−1.64	−1.80	−0.1	−2.87	−1.49	−7.80	0.4	4.0
$\Delta G_s^m(C=O)$	MEK*	0.23	−1.0	−2.05	−2.22	−0.2	−2.66	−1.46	−7.45	0.4	4.2
	MPK*	0.26	−0.9	−1.84	−2.38	−0.3	−2.89	−1.83	−6.53	0.2	3.5
$\sum_{i=1}^{5} I_i$	–	−78	289	523	549	212	632	453	853	105	8.6
Criterion *A*	–	0.09	−0.1	−0.11	−0.11	−0.1	−0.14	−0.11	2.39	0.03	1.3
$\Delta G_s^m(CH_2)$	–	−0.03	0.11	0.26	0.29	0.05	0.28	0.26	−2.61	0.1	1.9
$\Delta G^E(CH_2)$	–	−8.21	37.9	40.2	47.2	31.5	56.7	36.1	254.0	5.0	1.8
$\Delta G^E(CH_2)$	A**	−6.26	28.2	28.2	35.3	23.7	46.1	32.1	149.2	5.0	2.9
	B**	−4.98	24.4	25.7	30.1	20.5	37.2	20.1	74.5	4.0	3.5
	C**	−13.3	61.4	66.0	76.0	50.4	87.1	56.1	537.1	8.0	1.3

* MEK = 2-Butanone; MPK = 2-pentanone.

** A = Alkanes; B = ketones; C = alcohols.

phase polarity reverses this relationship. Intermolecular interactions other than dispersive interactions increase the retention of derivatives having an oligooxyethylene chain terminated by a hydroxy group.

The polarities of compounds I and IV having different end-groups decrease in the order $RO(CH_2CH_2)_2CH_2CH_2OH > RO(CH_2CH_2)_2CH_2CH_2Cl > RO(CH_2CH_2)_2CH_2CH_2OCH_3$. This means that the change in polarity caused by the introduction of the chlorine atom, as measured by GC, is smaller than that of a hydroxy group but higher than that of a methoxy group.

The same conclusions result from the examination of all the parameters considered. They describe the polarities in a similar way, although their sensitivities to changes in the structures of the compounds are different.

All parts of the molecule influence the polarity and the effect of a particular group depends on the presence of other characteristic fragments. One should take into account that the structural increments of the polarity presented previously[8,13] and here are average values which can be attributed to the considered structural fragments. However, such increments allow polarities to be predicted from the molecular formulae of compounds and were also calculated for the tested group of oxyethylates.

The values of the structural increments for all the polarity parameters considered are given in Table VIII. Errors in the calculation of polarity parameters from structural increments are also presented. As expected, –OH and –N= groups have the most significant influence on the polarity of the compounds examined. The relative polarities of –OH, –N=, –NH–, –Cl, –O– and –S– are 2.19:1.90:1.81:1.57:1:0.74, respectively ($\sum_{i=1}^{5} \Delta I_i$ as a parameter). The relative polarities change for different parameters but generally they can be arranged in the above order (Table IX). These relative polarities are different from those presented earlier[6,13] for 1,3-bis(ω-alkoxy-

TABLE IX

RELATIVE POLARITIES OF STRUCTURAL FRAGMENTS

Parameter	*Solute*	*–OH–*	*–N–*	*–NH–*	*–Cl*	*–O–*	*–S–*
I_R	CH_3OH	2.69	2.28	2.26	1.85	1	0.76
	C_2H_5OH	2.62	2.24	2.17	1.50	1	0.69
PI	CH_3OH	3.47	2.43	2.33	1.58	1	0.71
	C_2H_5OH	2.95	2.21	2.11	1.38	1	0.48
ρ	CH_3OH	2.35	2.43	2.23	1.90	1	0.71
	C_2H_5OH	2.36	2.41	2.28	1.56	1	0.85
$\Delta G_s^m(OH)$	CH_3OH	2.65	2.24	2.19	1.39	1	0.35
	C_2H_5OH	2.85	1.79	1.62	1.48	1	0.13
$\Delta G_s^m(C=O)$	MEK*	2.68	2.24	2.07	1.47	1	0.22
	MPK*	3.15	2.60	2.01	2.00	1	0.35
$\sum_{i=1}^{5} I_i$	–	2.19	1.90	1.81	1.57	1	0.74
Criterion *A*	–	1.64	1.34	1.29	1.27	1	0.88
$\Delta G_s^m(CH_2)$	–	2.43	2.61	2.29	2.28	1	0.45
$\Delta G^E(CH_2)$	–	1.49	1.24	1.06	0.95	1	0.83
$\Delta G^E(CH_2)$	A**	1.63	1.25	1.01	1.14	1	0.84
	B**	1.52	1.23	1.05	0.83	1	0.84
	C**	1.42	1.24	1.07	0.92	1	0.82

* MEK = 2-Butanone; MPK = 2-pentanone.

** A = Alkanes; B = ketones; C = alcohols.

oligooxyethylene)-2-propanols and aminoether alcohols and their ethers. Much higher polarities of –N= and –NH– groups in relation to the –O– group are observed, but lower in comparison with the –OH group. Recently[6,13] the opposite relationship between the relative polarities of –N= and –OH groups has been observed. However, the hydroxy group in 1,3-bis(ω-alkoxyoligooxyethylene)-2-propanols is a secondary group screened by the long oligooxyethylene and alkyl chains. The relative polarity of the secondary hydroxy group is then lower than for the primary hydroxy group in compounds I–V. The –N= group in aminoether alcohols and their ethers is also screened by long chains and hence its relative polarity in comparison with the –O– group was reported to be lower than in compounds I–V.

CONCLUSIONS

The polarity of oligooxyethylene derivatives of alcohols, thio alcohols and alkylamines can be described satisfactorily by the polarity parameters considered. Each of these polarity parameters arrange the compounds examined in the same order according to their polarity. However, significant differences are observed when the sensitivity of the parameters to the changes in the structures of the compounds is examined. The most polar are derivatives of alkylamines, medium polarity is shown by derivatives of alcohols and the lowest polarity by derivatives of thio alcohols. The polarity increases with increasing length of the oligooxyethylene chain and decreasing number of carbon atoms in the alkyl chain(s). The distribution of carbon atoms in alkyl chains linked to the nitrogen atom (in II) significantly influences their polarity. Compounds having two short alkyl chains exhibit higher polarities than their analogues with one long alkyl chain. The average structural increments of the polarity parameters considered were calculated for characteristic structural fragments. The relative polarities of –OH, –N=, –NH–, –Cl, –O– and –S– are 2.19:1.90:1.81:1.57:1:0.74 for $\sum_{i=1}^{5} \Delta I_i$ used as a polarity parameter. These are different to those reported for aminoether alcohols and their ethers and 1,3-bis(ω-alkoxyoligooxyethylene)-2-propanols. The higher relative polarities, arranged in a different order, may be discussed in terms of the different structures of the compounds examined here and recently[4–6,13].

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SEPARATION OF FRIEND ERYTHROLEUKAEMIC CELL HISTONES AND HIGH-MOBILITY-GROUP PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HERBERT LINDNER*, WILFRIED HELLIGER and BERND PUSCHENDORF
Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck (Austria)
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SUMMARY

A procedure for the rapid separation of histones and high-mobility-group (HMG) proteins from Friend erythroleukaemic cells (line F4N) by reversed-phase high-performance liquid chromatography is reported. By using a Nucleosil 300-5 C_4 column and a multistep water–acetonitrile gradient containing 0.1% trifluoroacetic acid, the HMG-1 and HMG-2 proteins, several H1 subfractions including $H1^0$, H4, H2B, two H2A variants and two H3 subfractions were separated. Under changed conditions, by applying a varied acetonitrile gradient system, even two H2B variants were fractionated. The methods described seem to be a real alternative to the time-consuming polyacrylamide gel electrophoresis.

INTRODUCTION

In eukaryotic nuclei, DNA is assembled with histones into regular repeating units called nucleosomes[1]. The nucleosomal core region contains the histone octamer $(H2A, H2B, H3, H4)_2$, while the nucleosomal linker region is associated with H1[2]. Four of these five histone classes are composed of variants, differing in their primary structure[3–7]. Besides the histones, there is another class of so-called high-mobility-group (HMG) proteins present in chromatin. Both the histones and HMG proteins undergo a large number of postsynthetic modifications such as acetylation, phosphorylation, (ADP)ribosylation and methylation[8–12], which are believed to be important to regulatory functions[13–16].

Friend erythroleukaemic cells are used as a suitable model for studies of gene expression and differentiation since they can be induced to differentiate and synthesize haemoglobin[17]. In order to analyse the histones and HMG proteins from erythroleukaemic cells, sodium dodecyl sulphate[18,19], acetic acid–urea[20,21] and acetic acid–urea–Triton[22,23] polyacrylamide gel electrophoresis (PAGE) have been applied. The last mentioned gel system permits the separation of histones and some non-allelic variants as well as modified forms. The most effective separation of several H2A subspecies requires, however, a gel of a different composition to the one re-

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quired for the fractionation of the other core histones. Furthermore, this gel system is hardly suitable for separating HMG proteins. All three methods of PAGE, it is suggested, have their shortcomings; they do not properly fractionate H1 into several subspecies or subfractions, moreover they are laborious and time-consuming.

We have previously reported rapid and simple methods for separating calf thymus H1 and core histones by reversed-phase high-performance liquid chromatography (HPLC)[24,25]. Furthermore, we have demonstrated that reversed-phase HPLC, using modified conditions, is capable of fractionating chicken erythrocyte histones in a short time[26,27].

In this paper we describe a rapid and efficient method for separating HMG-1 and HMG-2 proteins, several H1 subfractions, $H1^0$ and all core histones including some H2A variants and H3 subfractions derived from Friend erythroleukaemic cells (line F4N) by reversed-phase HPLC. Furthermore, we have separated, under changed chromatographic conditions, two H2B variants by means of reversed-phase HPLC, a method which to our knowledge had not previously been demonstrated.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile (Type S) was obtained from Rathburn Chemicals (Walkerburn, U.K.), water was from Promochem (Wesel, F.R.G.) and trifluoroacetic acid (TFA) from Sigma Chemie (Munich, F.R.G.)

Cell line and culture conditions

Friend erythroleukaemic cells (line F4N) were maintained in Basal Medium Eagle (Serva Feinbiochemica, Heidelberg, F.R.G.) supplemented with 10% fetal calf serum (Boehringer, Mannheim, F.R.G.) and amino acids as described elsewhere[28]. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added routinely. Cell cultures were incubated at 37°C in 5% carbon dioxide–95% air.

Preparation of histones and HMG proteins

F4N cells [$(2–3) \cdot 10^9$] were collected by centrifugation (800 *g* for 10 min), washed two times in 0.01 *M* Tris–HCl, pH 7.0, 0.14 *M* sodium chloride, 0.01 *M* sodium butyrate, 0.01 *M* 2-mercaptoethanol, 0.1 m*M* phenylmethanesulphonyl fluoride at 4°C, pelleted and frozen at −70°C. The frozen cell pellet was washed once in the same buffer; the crude nuclei were prepared according to Kostraba *et al.*[29]. Cells were lysed in 10 volumes of distilled water containing 0.01 *M* sodium butyrate, 0.01 *M* 2-mercaptoethanol and 0.1 m*M* phenylmethanesulphonyl fluoride with 30 strokes of the L-pestle of a Dounce homogenizer and then centrifuged at 1000 *g* for 10 min at 4°C. The pellet was extracted with 0.4 *N* sulphuric acid and the proteins obtained according to Multhaup *et al.*[30].

High-performance liquid chromatography

All reversed-phase HPLC experiments were performed on a Beckman HPLC-gradient system using two 114M pumps and a 421A system controller. The eluent was monitored by the absorbance at 210 nm with a Model 165 variable-wavelength UV–

VIS detector. The detector signal was documented on a Shimadzu C-R3A integrator utilizing an automatic baseline correction.

The protein separations were performed on a Nucleosil 300-5 C_4 column (100 mm × 4 mm I.D.) and a Beckman Ultrapore™ C_8 column (75 mm × 4.6 mm I.D.), respectively. Both columns were packed with 5-μm spherical silica (pore size 30 nm). The lyophilized proteins were dissolved in water containing 0.1% TFA, and samples of 40–100 μg histones were injected onto the columns. The proteins were chromatographed at room temperature and at a constant flow-rate of 0.5 ml/min. Utilizing the Nucleosil C_4 column, the proteins were eluted with a 45-min multistep water/TFA–acetonitrile/TFA gradient. Applying the Beckman Ultrapore C_8 column, the proteins were chromatographed using a 52-min multistep water/TFA–acetonitrile/TFA gradient starting at 60% A–40% B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in 70% acetonitrile). The concentration of solvent B was increased linearly in the following order: from 40 to 54% (during 17 min), 54 to 56% (10 min), 56 to 60% (10 min) and 60 to 80% (15 min). In order to separate the histones including two H2B variants, the Nucleosil C_4 column was used applying a 75-min multistep water/TFA–acetonitrile/TFA gradient.

Gel electrophoresis

Protein fractions from the HPLC were collected, lyophilized and stored at −20°C. The histones were identified by using three different polyacrylamide slab gel systems: (i) SDS (sodium dodecyl sulphate) gels according to the method of Laemmli[31] (15% polyacrylamide–0.1% SDS); (ii) AU (acid–urea) gels according to the procedure of Panyim and Chalkley[32] (15% polyacrylamide–0.9 *M* acetic acid–6 *M* urea) and (iii) AUT (acid–urea–Triton) gels as described by Zweidler[33] (12% polyacrylamide–0.9 *M* acetic acid–8 *M* urea–0.37% Triton X-100). The gels were stained for 1 h with 0.1% Serva Blue R in 40% aqueous ethanol–5% aqueous acetic acid and destained overnight in 20% aqueous ethanol–5% aqueous acetic acid.

RESULTS AND DISCUSSION

Procedures have recently been developed for the fractionation of HMG proteins from different cells by reversed-phase HPLC[34,35]. However, these methods required long gradient times (80–300 min). Only the HMG-1 and HMG-2 proteins isolated from steer thymus were resolved by Kohlstaedt *et al.*[36] within 30 min. Furthermore, Elton and Reeves[37] separated HMG-1 and HMG-2 proteins prepared from Friend erythroleukaemic cell nuclei using a C_4 reversed-phase column during the course of 50 min, and HMG-14, HMG-17 and HMG-I, respectively, within 25–30 min[38]. Moreover, the same authors reported the simultaneous separation of HMG-1, HMG-2, H1, HMG-I, HMG-14 and HMG-17 proteins from Friend erythroleukaemic cells by using a different gradient system (45 min)[39]. This method, however, permits no fractionation of different H1 subspecies.

Utilizing our preceding studies[24–27] on a rapid separation of histones by means of reversed-phase HPLC, we have attempted to develop a simple and fast method for a simultaneous fractionation of HMG proteins, H1 variants and core histones and their variants, respectively, isolated from Friend erythroleukaemic cell nuclei. Using a Nucleosil 300-5 C_4 column and a 45-min multistep water/TFA–acetonitrile/TFA gra-

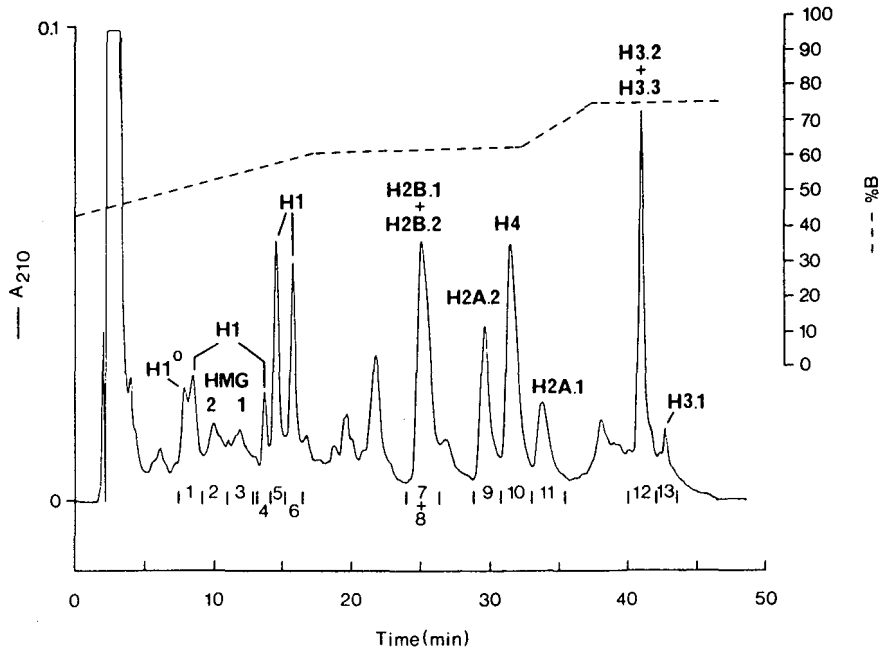

Fig. 1. Separation of total histones and HMG proteins (40 μg) from Friend erythroleukaemic cells (line F4N) by reversed-phase HPLC. Column: Nucleosil 300-5 C_4 (100 mm × 4 mm I.D.). Flow-rate: 0.5 ml/min. Monitoring wavelength: 210 nm. Eluent: 45-min multistep gradient, where solvent A is water and solvent B is 70% acetonitrile, both solvents containing 0.1% TFA; starting at 56% A–44% B, the concentration of solvent B was increased linearly in the following from 44 to 61% (17 min), 61 to 63% (15 min), 63 to 76% (5 min). The isocratic conditions (76% B) were continued for 8 min. In order to obtain the fractions 1–13, 100 μg of proteins were injected onto the column. Numbered fractions were lyophilized and used for the electrophoretic analyses in Fig. 2.

dient we have separated in the course of one single experiment the HMG-1 and HMG-2 proteins and all major histone classes, including numerous variants, within 45 min (Fig. 1). The fractions of the eluate indicated on the abscissa were collected, lyophilized and analysed by means of SDS-PAGE (Fig. 2a) with the following result: fraction 1, $H1^0$ and an histone H1 variant; 2, HMG-2, 3, HMG-1; 4, H1; 5, H1; 6, H1; 7+8, H2B; 9, H2A; 10, H4; 11, H2A; 12, H3; 13, H3. As shown in Fig. 1, histone $H1^0$ and an H1 variant were separated from each other, however, the two proteins were jointly collected in fraction 1. To verify the assignment of fractions 2 and 3 to HMG-2 and HMG-1, these fractions were additionally subjected to an AU-PAGE (Fig. 2b). The proteins exhibited the electrophoretic mobility characteristic of HMG-2 and HMG-1, respectively. Due to their negligible concentration in the standard, the HMG proteins may, however, hardly be detected in Fig. 2b. Noteworthy is that, despite an heavy enrichment by the chromatographic separation, the proteins are highly pure. To characterize the core histones with regard to variants the fractions 7+8 and 9–13 were analysed by AUT-PAGE (Fig. 2c) and identified as follows: fractions 7+8, H2B.1 + H2B.2; 9, H2A.2; 10, H4; 11, H2A.1; 12, H3.2 + H3.3; 13, H3.1.

We also tested a Beckman Ultrapore C_8 column using a 52-min multistep

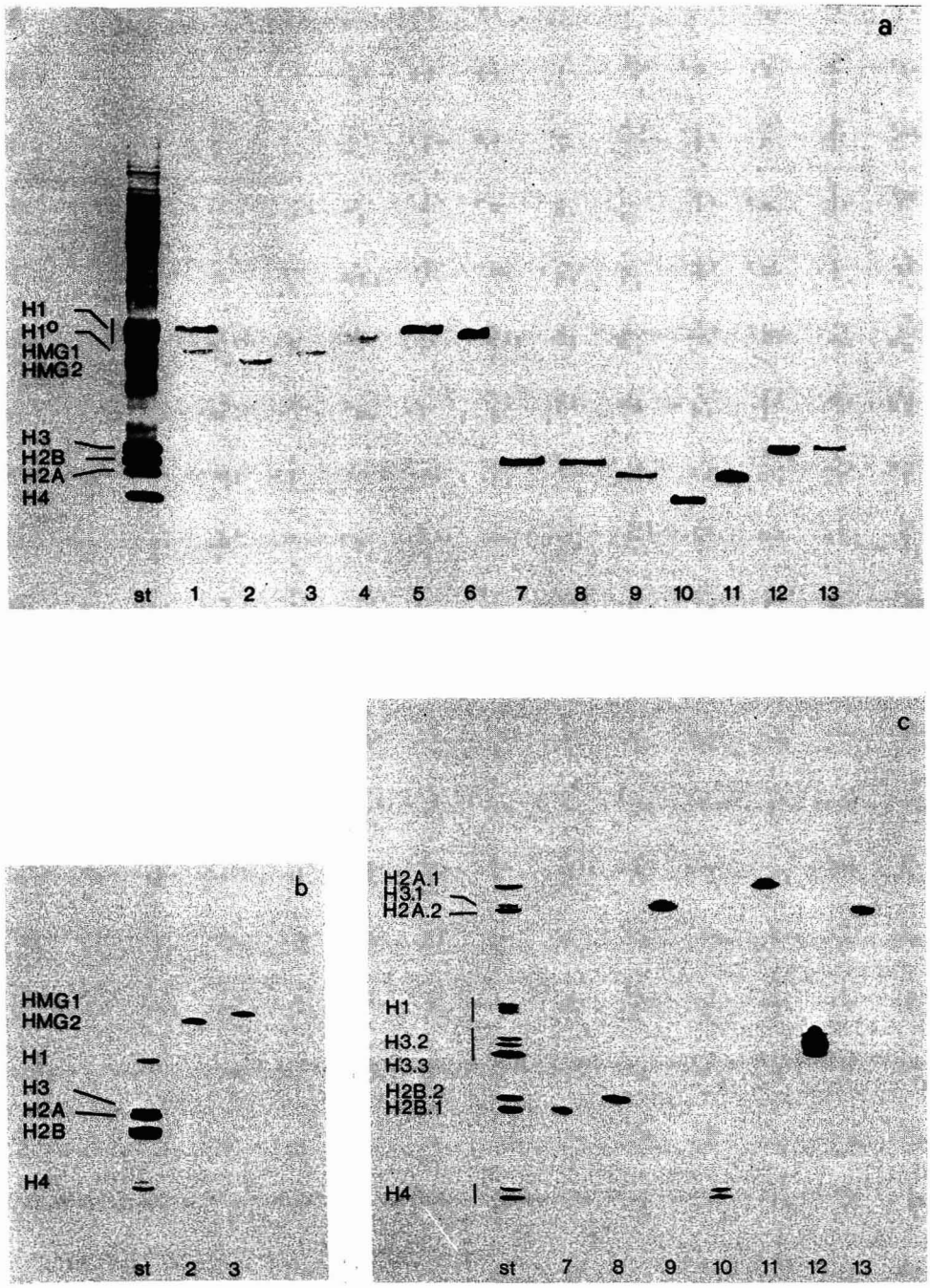

Fig. 2. Gel electrophoresis of histones and HMG fractions separated with the HPLC system used in Figs. 1 and 3, respectively. (a) SDS-PAGE of fractions 1–13; (b) AU-PAGE of fractions 2 and 3; (c) AUT-PAGE of fractions 7–13. The sample containing histones and HMG proteins used for the HPLC separation in Fig. 1 was also used as a mobility standard (st).

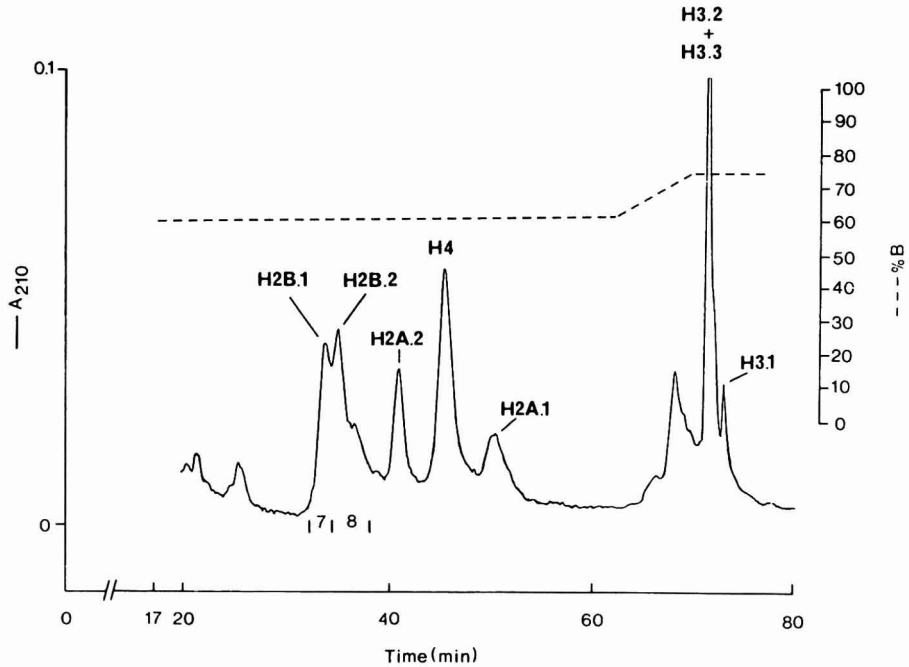

Fig. 3. Separation of core histones (40 μg) from Friend erythroleukaemic cells (line F4N) by reversed-phase HPLC. Column: Nucleosil 300-5 C_4 (100 mm × 4 mm I.D.). Flow-rate: 0.5 ml/min. Monitoring wavelength: 210 nm. Eluent: 75-min multistep gradient, where solvent A is water and solvent B is 70% acetonitrile, both solvents containing 0.1% TFA; starting at 57% A—43% B, the concentration of solvent B was increased linearly from 43 to 58% (17 min), 58 to 61% (45 min), 61 to 75% (5 min). The isocratic conditions (75% B) were continued for 8 min. In order to obtain the fractions 7 and 8, 100 μg of histones were injected onto the column. These fractions were lyophilized and subjected to the electrophoretic analyses in Fig. 2.

water/TFA–acetonitrile/TFA gradient, as described in Experimental. Essentially identical elution profiles compared to Fig. 1 were obtained (data not shown).

Moreover, we also attempted to separate the variants H2B.1 and H2B.2 from each other. A 75-min multistep water/TFA–acetonitrile/TFA gradient allowing optimum resolution using a Nucleosil 300-5 C_4 column is shown in Fig. 3. The fractions 7 and 8 were identified by SDS- (Fig. 2a) and AUT-PAGE (Fig. 2c), respectively. Consequently, fraction 7 comprises practically pure H2B.1 and fraction 8, H2B.2 slightly contaminated by H2B.1.

Considering the results of the studies of Elton and Reeves[38,39], with the system described by us it should also be possible to separate HMG-14, HMG-17 and HMG-I. This fractionation would require an extension of the gradient from 44 to 20% B (solvent B, 0.1% TFA in 70% acetonitrile), maintaining the gradient slope. However, these proteins were not present in our preparation.

We may summarize the results of our examination as follows:

(1) The HMG-1 and HMG-2 proteins may be separated from H1 and $H1^0$ histones, respectively, and all other major histone classes isolated from Friend erythroleukaemic cell nuclei using reversed-phase HPLC within 45 min

(2) Two H2A variants, two H3 subfractions and four H1 subspecies may be

fractionated in one experiment as may be H4 from H2A.1. This H4/H2A.1 separation raises a difficult problem of histone fractionation which has frequently been examined[25,40,41].

(3) By varying the gradient, both H2B variants may be resolved by means of reversed-phase HPLC, a method which, so far as we know, has not previously been demonstrated.

ACKNOWLEDGEMENTS

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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THIAMINE AND ITS PHOSPHATE ESTERS IN ANIMAL TISSUES

HEITAROH IWATA*, TOSHIO MATSUDA and HIDEO TONOMURA
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565 (Japan)
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SUMMARY

An improved method for the determination of thiamine and its phosphate esters in animal tissues using reversed-phase high-performance liquid chromatography with precolumn derivatization is described. Thiamine and its phosphate esters were converted into fluorophores by alkaline cyanogen bromide, and the derivatives were applied to an ODS packed column. Then the effluent obtained by an acidic mobile phase was mixed with an alkaline methanol solution to increase the fluorescence intensity of the derivatives which was determined spectrofluorometrically. A complete, rapid and quantitative separation of thiamin and its phosphate esters was achieved and the use of the acidic buffer as a mobile phase improved the column stability. The fluorophores of thiochrome ester peaks on the chromatogram were sensitive to pretreatment with thiamine triphosphatase or acid phosphatase. The applicability of the mehod to the determination of the form of thiamin in various tissues of rat is demonstrated.

INTRODUCTION

Thiamine and its phosphate esters in animal tissues have been determined mainly by column chromatography[1–3] and paper electrophoresis[4–6]. Most recent studies, however, used high-performance liquid chromatography (HPLC) because of its rapidity and sensitivity[7–14]. The HPLC method requires the conversion of thiamine compounds into fluorescent derivatives for sensitive determination. The conversion was done by alkaline oxidation with potassium ferricyanide[9–14] or cyanogen bromide[7,8] either prior to[7,8,12–14] or after[9–11] the chromatography. Among these methods, only a precolumn derivatization with oxidation by cyanogen bromide allows an adequate blank experiment which excludes the contamination with non-thiochrome compounds. The precolumn method[7,8] seems to be most suitable for determination of thiamine compounds in biological preparations which contain unidentified fluorescent materials. This method, however, has a shortcoming as pointed out by Bontemps *et al.*[12,13]: the column packed with silica-based material is

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not stable in the alkaline solution as a mobile phase which is necessary for maintaining the fluorescence intensity. It is known that the fluorescence intensity of thiochrome is decreased by lowering the pH of the buffer. Bontemps *et al.*[13,14] have recently reported that a column packed with poly(styrene–divinylbenzene) beads which is stable in the alkaline solution is well suited for the analysis of thiamine derivatives after the precolumn chemical oxidation. On the other hand, we have found that the decrease in fluorescence intensity under acidic conditions can be reversed by bringing the pH back to an alkaline value. This finding has allowed us to develop an improved method using an economic ODS column for the determination of thiamine and its phosphate esters in animal tissues.

EXPERIMENTAL

Chemicals

Thiamine monophosphate (TMP) and acid phosphatase (from sweet potato) were obtained from Sigma. Thiamine triphosphate (TTP) and thiamine diphosphate (TDP) were synthesized by the method of Matsukawa *et al.*[15]. O-Carbamoyl-thiochrome (Carb-Thc) was synthesized by the procedure of Ogawa *et al.*[16]. TTPase (128 μmol per mg protein per min) was purified from bovine brain supernatant as previously reported[17]. All other reagents were of the best grade commercially available.

Apparatus

The HPLC system comprised an LC-3A pump, an SIL-1A injector, a Shim pack CLC-ODS column (150 mm × 6 mm I.D.), a CTO-2A column oven (50°C), a CRB-1B incubator box (50°C), a PRR-1A proportioning pump, an FLD-1 fluorescence detector (flow cell, 14 μl), a strip-chart recorder and a Chromatopac C-E1B. All the equipment was obtained from Simadzu (Kyoto, Japan).

Preparation of tissue extracts

Male Sprague–Dawley rats, weighing 200–300 g, were killed by decapitation. Their tissues were quickly removed and homogenized with cold 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 16000 for 15 min, the extract was lyophilized after removing TCA from the deproteinized supernatant with water-saturated diethyl ether and stored at −20°C until used. Under these conditions, the decomposition of thiamine phosphate esters during the preparation seemed to be negligible; killing of the animals in a microwave oven gave similar results to those previously reported[17].

Procedure

The procedure is shown in Fig. 1. For the conversion of thiamine compounds into fluorophores[18], the extract was divided. To one aliquot (200 μl), 25 μl of 0.3 *M* cyanogen bromide were added, and the resulting solution was mixed for about 1 min using a Vortex mixer. Then, 25 μl of 1 *M* sodium hydroxide were added, again followed by Vortex mixing for *ca.* 1 min. As the sample may contain not only thiochrome derivatives but also non-thiochrome fluorescent materials[3], a blank experiment should also be carried out. Therefore, to a second aliquot (200 μl) of the extract, first 25 μl of

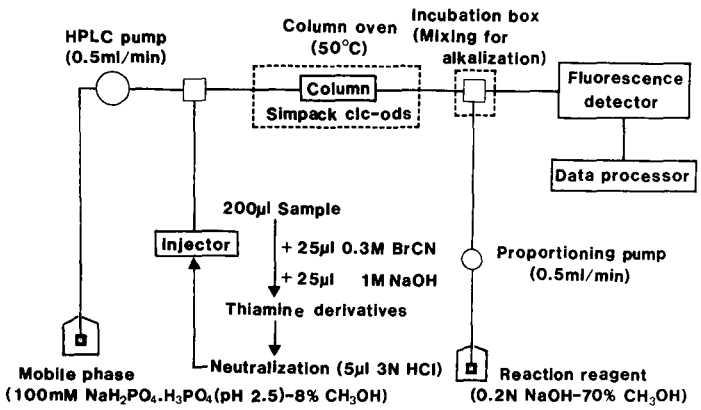

Fig. 1. Procedure for determination of thiamine and its phosphate esters.

1 *M* sodium hydroxide were added (Vortex mixing for *ca.* 1 min), to destroy thiamine compounds; then 25 µl of 0.3 *M* cyanogen bromide were added (again Vortex mixing for *ca.* 1 min). After neutralization of both aliquots with 3 *M* hydrochloric acid, 20–40 µl volumes were injected onto the column. The mobile phase, 100 m*M* sodium dihydrogenphosphate–phosphoric acid buffer (pH 2.5)–8% methanol, was pumped at a flow-rate of 0.5 ml/min, and the reagent (0.2 *M* sodium hydroxide–70% methanol) was applied at the same flow-rate by a proportioning pump to increase the pH of the effluent: the alkaline methanol solution was mixed with the mobile phase in the incubator box before the fluorometric determination. The peak area on the chromatogram was analyzed by the integrator. The contents of thiamine compounds were calculated from the difference between the sample and the blank.

RESULTS AND DISCUSSION

Thiamin compounds were first oxidized by cyanogen bromide under alkaline conditions and the resulting derivatives were separated under acidic conditions by HPLC. The mobile phase and the reagent were optimized with respect to the separation of thiochrome derivatives and the fluorescence intensity. Higher concentrations of phosphate buffer in the mobile phase resulted in an incomplete separation of thiochrome triphosphate (ThcTP) and thiochrome diphosphate (ThcDP), and conversely lower concentrations prolonged the analysis (data not shown). The fluorescence intensity of the effluent after the chromatography was increased not only by alkalization of the effluent, but also by including methanol in the reagent (data not shown), in agreement with previous reports[19,20]. Based on these observations, 0.2 *M* sodium hydroxide–70% methanol was used as the reagent: the pH value of the effluent was 8.6.

Fig. 2 shows a typical chromatogram obtained with a solution containing thiamine, TMP, TDP and TTP. Thiamine phosphate esters were converted into the corresponding thiochrome (Thc) derivatives such as ThcTP (peak 1), ThcDP (peak 2) and thiochrome monophosphate (ThcMP, peak 3), while the oxidation of thiamin

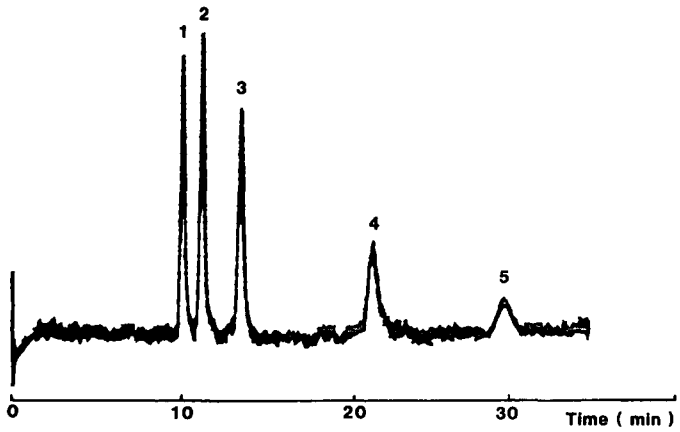

Fig. 2. Chromatogram of a standard solution containing thiamine TMP, TDP and TTP. Thiamine compounds (each 1 pmol) were oxidized and applied on a column as described in Experimental. Peaks 1, 2, 3, 4 and 5 correspond to ThcTP, ThcDP, ThcMP, Thc and Carb-Thc, respectively.

produced two derivatives, ThcI (peak 4) and ThcII (peak 5). Ogawa *et al.*[16] have reported that the oxidation of thiamine by cyanogen bromide, but not $K_3Fe(CN)_6$, produces a new fluorescent compound, Carb-Thc, besides Thc. Nosaka *et al.*[21] also showed that thiamine oxidized with cyanogen bromide gave two peaks in HPLC, though they did not comment on these peaks. To identify peaks 4 and 5 on the chromatogram, we synthesized Thc and Carb-Thc and injected them onto the column. From their HPLC retention times, these peaks were considered to be Thc and Carb-Thc, respectively (data not shown). Since the fluorescence characteristics of these compounds were essentially identical[16], the thiamin content can be estimated from the sum of these peaks.

When samples extracted from rat brain, liver, kidney and muscle were subjected to HPLC, the first peak always appeared at 5.6 min. If t_0 is 5.6 min, the capacity factors for peaks 1, 2, 3, 4 and 5 were calculated to be 0.79, 1.01, 1.41, 2.86 and 4.34, respectively. This result indicates that this method is satisfactory for the separation of each thiamin and its phosphate esters.

Fig. 3 shows calibration graphs for standard solutions of TTP, TDP, TMP and thiamin. Linearity was observed up to 100 pmol of each derivative. The minimum amounts reproducible determined were 0.1 pmol for thiamin phosphate esters and 0.5 pmol for thiamin. The sensitivity of this method is comparable to that of the previous HPLC methods[7,8], but less than that of the methods[9–14] recently reported. In the latter methods, however, there may be a limitation on the application to biological preparations, since it is difficult to carry out a proper blank experiment as shown in the former methods. Bontemps *et al.*[12] showed that TTP in rat heart could not be quantified by their method. In general, the sensitivity may be improved by using a smaller cell and a more sensitive fluorometer.

The analytical pattern of thiamine and its phosphate esters in rat cerebral cortex by the HPLC method described here is shown in Fig. 4. Though several peaks were detected, peaks 1, 2, 3, 4 and 5 were found to correspond to ThcTP, ThcDP, ThcMP,

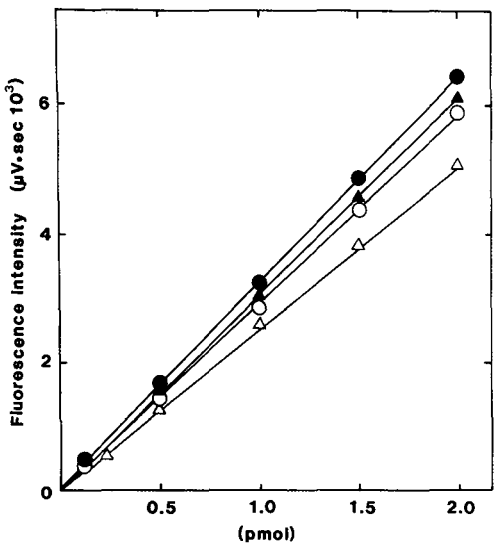

Fig. 3. Calibration graphs for the determination of thiamine and its phosphate esters. Each point represents the mean of three determinations and the deviation is within the symbol. ○, TTP; ●, TDP; △, TMP; ▲, thiamine.

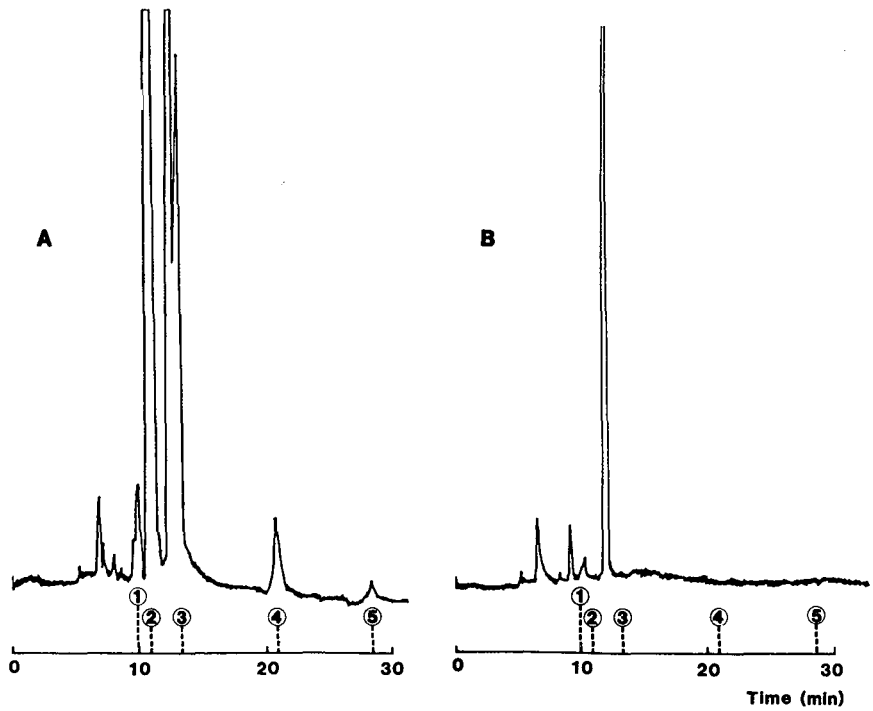

Fig. 4. Elution profile of thiamine and its phosphate esters in rat cerebral cortex. The extract from rat cerebral cortex was divided as described in Experimental: one was used as the sample (A) and the other as the blank (B). The retention times of ThcTP (1), ThcDP (2), ThcMP (3), Thc (4) and Carb-Thc (5) are indicated in both experiments.

TABLE I

EFFECT OF TREATMENT OF THE TISSUE EXTRACT WITH TTPase OR ACID PHOSPHATASE ON ITS THIAMINE PHOSPHATE ESTERS

The tissue extract was treated with 0.38 mg TTPase in a medium of 0.4 ml containing 50 m*M* Tris–HCl (pH 9.0) and 6 m*M* magnesium chloride at 37°C for 30 min (experiment A) or with 5 U acid phosphatase in a medium of 0.4 ml containing 25 m*M* imidazole buffer (pH 5.8) at 37°C for 60 min (experiment B). The control experiment was simultaneously carried out without the enzyme. In this experiment, an extract from rat soleus muscle was used since the TTP content was higher in this issue than in the other tissue[23], but a similar result was obtained for an extract from the cerebral cortex. The data from a typical experiment are given which was repeated three times with similar results.

	Distribution (%)			
	TTP	*TDP*	*TMP*	*Thiamine*
Experiment A				
Control	6.0	76.0	17.9	0.1
TTPase	0	82.8	16.8	0.4
Experiment B				
Control	6.5	86.7	6.2	0.6
Acid phosphatase	0	0	94.0	6.0

Thc and Carb-Thc, respectively, from their retention times. The addition of each authentic thiamin compound in the tissue extract enlarged the corresponding peaks in HPLC (data not shown), and the recovery of the thiamine compounds added was 95–100%. Since the ThcTP and ThcDP, but not ThcMP and Thc, peaks were contamined a little by the blank peaks (Fig. 4B), the measurement of Thc derivatives should be based on the difference between the sample and blank fluorescence as described in Experimental.

In the previous HPLC methods, the peaks were identified by comparing the retention times with those of authentic compounds or by co-chromatography with

TABLE II

CONTENTS OF THIAMINE AND ITS PHOSPHATE ESTERS IN VARIOUS RAT TISSUES

The results (nmol/g tissues) are the means ± S.D. from four or five experiments and the values in parentheses are percentages of the total thiamine.

	Total	*TTP*	*TDP*	*TMP*	*Thiamin*
Cerebral cortex	7.25 ± 1.17	0.02 ± 0.01 (0.3)	5.85 ± 1.04 (80.7)	1.07 ± 0.05 (14.8)	0.30 ± 0.08 (4.1)
Crebellum	8.65 ± 0.63	0.03 ± 0.01 (0.3)	6.83 ± 0.40 (79.3)	1.49 ± 0.21 (17.2)	0.31 ± 0.14 (3.6)
Heart	17.9 ± 2.1	0.05 ± 0.02 (0.3)	16.1 ± 1.7 (89.4)	1.76 ± 0.36 (9.8)	0.16 ± 0.03 (0.9)
Kidney	18.6 ± 1.6	0.03 ± 0.01 (0.2)	14.6 ± 1.6 (78.5)	1.51 ± 0.37 (8.1)	2.45 ± 0.13 (13.2)
Liver	34.3 ± 1.5	0.18 ± 0.12 (0.5)	28.7 ± 1.4 (83.7)	4.20 ± 0.17 (12.2)	1.33 ± 0.15 (3.9)

added authentic compounds. These criteria, however, may not confirm that the fluorophore is indeed due to a thiamine compound as previously pointed out[3,5,17]. In this study, the tissue extract was treated with TTPase or acid phosphatase, in order to ensure the specificity of the fluorophores (Table I). The TTPase treatment resulted in disappearance of the ThcTP peak and a corresponding increase in the ThcDP peak. Further, in the sample pretreated with acid phosphatase the ThcTP and ThcDP peaks were not observed. When the extract was treated with adequate amounts of acid phosphatase, all the Thc esters disappeared and a large peak of Thc was observed (data not shown). In these experiments, the blank peaks as shown in Fig. 4B were not influenced by the treatments (data not shown). This is the first demonstration that thiamine ester peaks from a biological preparation subjected to HPLC are indeed derived from thiamin compounds.

The applicability of the method is demonstrated by determination of thiamine and its phosphate esters in rat tissues (Table II). In these tissue samples, the contamination with the blank fluorescence was as follows; in TTP, 2.5 (brain), 2.8 (heart), 10.4 (kidney) and 0.8% (liver); in TDP, 1.0 (brain), 3.1 (heart), 3.6 (kidney) and 1.0% (liver); in TMP and thiamine, no contamination was detectable. TDP was the most abundant thiamine compound and TTP was the least (less than 1% of the total thiamine) in all the tissues. The low level of TTP is in agreement with a recent report[22].

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CHROMATOGRAPHIC AND SPECTRAL INVESTIGATIONS ON THE *IN VIVO* METABOLITES OF 6-NITROBENZO[*a*]PYRENE

CHITTA RAHA*
Eppley Institute for Research in Cancer and Allied Diseases and Department of Biochemistry, University of Nebraska Medical Center, Omaha, NE 68105-1065 (U.S.A.)
DANIEL WILLIAMSON
Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE (U.S.A.)
PURNIMA THAKRAN
Department of Pharmacology, University of Nebraska Medical Center, Omaha, NE (U.S.A.)
DAVID CASSADA
Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE (U.S.A.)
and
PATRICK IVERSEN
Eppley Institute for Research in Cancer and Allied Diseases and Department of Pharmacology, University of Nebraska Medical Center, Omaha, NE (U.S.A.)
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SUMMARY

6-Nitrobenzo[*a*]pyrene (6-NBaP) occurs in the environment, is mutagenic in the Ames assay in the presene of added S9 and is carcinogenic to male but not female mouse liver when injected intraperitoneally (i.p.) into mice. In order to understand what kinds of active metabolites could have been produced *in vivo*, both male and female mice were injected i.p. with 6-NBaP in dimethyl sulfoxide. Twenty-four hours after injection, urine, feces, blood, liver and spleen (non-target tissue) were examined for metabolites by chromatographic and high-resolution mass spectral means. On the basis of the mass spectral fragmentation patterns of synthetic and metabolic standards, it was observed that both male and female animals excreted ring-hydroxylated metabolites of 6-NBaP in the urine to differing extents. Male animals additionally excreted 6-aminobenzo[*a*]pyrene and the significance of this observation is discussed.

INTRODUCTION

Nitro-substituted polycyclic aromatic hydrocarbons (nitro-PAHs) occur in the environment and many of them are direct-acting bacterial mutagens[1]. Benzo[*a*]pyrene (BaP), a carcinogenic PAH, occurs widely in the atmosphere[2] and can undergo nitration in a simulated environment[3] to produce 6-nitrobenzo[*a*]pyrene (6-NBaP) in addition to 1- and 3-NBaP. 6-NBaP has been detected in air pollution samples[4], and its

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bacterial mutagenicity requires exogenous activation with Arochlor-induced rat liver S9[5]. To investigate the nature of the metabolites of 6-NBaP that could have been produced during the exogenous activation process, Fu *et al.*,[6] examined the rat liver microsomal metabolism of 6-NBaP and characterized 3-hydroxy-6-NBaP (3-OH-6-NBaP) as the major metabolic product, together with smaller amounts of 1-OH-6-NBaP, 1,9-dihydroxy-6-NBaP (1,9-di-OH-6-NBaP) and 3,9-di-OH-6-NBaP (hydroquinones). A mixture of 3-OH-6-NBaP and 1-OH-6-NBaP requires activation with liver S9 to be mutagenic, suggesting the posibility that the monohydroxy compounds were undergoing further metabolism. Later it was suggested, without experimental details, that the hydroquinones are direct-acting mutagens[7].

As 6-NBaP was found to be mutagenically active[1,5], the question naturally arose as to whether it can pose a risk to human health. Therefore, the carcinogenicity of 6-NBaP and other nitro-PAHs was examined in a highly responsive newborn mouse assay system by Wislocki *et al.*[8]. During this bioassay it was found that when 6-NBaP was injected intraperitoneally (i.p.) into newborn CD_1 male and female mice, it was the male and not the female animal that developed hepatocellular adenoma and carcinoma. Like Fu *et al.*[6], we carried out a number of *in vitro* aerobic metabolism experiments with 6-NBaP[5,9,10] and observed that both liver and lung tissue produced ring-hydroylated metabolites of 6-NBaP. The lung tissue is not a target of the carcinogen 6-NBaP in either sex. Many nitro-PAHs may be activated via nitro-reduction[7]. As 6-NBaP induced hepatocellular tumors in male and not female mice after i.p. injections, showing a sex difference, it was our objective to determine what kinds of metabolites were produced *in vivo*.

EXPERIMENTAL

Chemicals

The preparation and chromatographic purification of 6-NBaP and its spectral properties have been described[5,11].

Synthesis of 6-aminobenzo[a]pyrene (6-ABP). Palladium–carbon reduction of 6-NBaP to 6-ABP has been described[12]. However, we prepared 6-ABP according to a new method, as follows.

6-NBaP (6 mg) was transferred into a 100-ml round-bottomed flask with 2 ml of toluene. The flask was fitted with a reflux condenser and an adapter for a nitrogen gas inlet. A 25-ml volume of 85% hydrazine hydrate (Matheson, Coleman and Bell, East Rutherford, NJ, U.S.A.) and 1.4 mg of zinc dust were added. The apparatus was evacuated, filled with nitrogen gas and then refluxed for 1 h. The reaction mixture was cooled under nitrogen gas, the product was extracted into 500 ml of ethyl acetate, the extract was washed with 4 × 50 ml of water and then dried over 100 g of anhydrous sodium sulfate. The solvent was removed, the greenish fluorescing solid residue was dissolved in 1 ml of benzene and resolved into its components by silica gel thin-layer chromatography (TLC) (see later for details). The greenish fluorescent (360 nm light) band with R_F values 0.08 and 0.49 in solvents 2 and 3 (Table I) was scraped off, extracted with acetonitrile, benzene and dichloromethane, the solvents removed and the residue (1–1.5 mg) was again chromatographed to remove minor amounts of impurities. The material, R_F 0.49 in solvent 3, was extracted as above, the solvent removed and the residue was characterized by UV, mass and proton NMR spectra.

Synthesis of 6-(N-acetylamino)benzo[a]pyrene (6-AABaP). Many of the metabolites of 6-NBaP are unstable; therefore, as we described earlier[5,9] and also describe later, the crude mixture of metabolites isolated was subjected to acetylation before subjecting them to extensive analytical processing. As 6-ABP was an *in vivo* metabolite present in the mixture of metabolites (see later), we wished to prepare 6-AABaP for reference purposes.

6-ABP (0.5 mg) in 0.5 ml of dimethyl sulfoxide (DMSO) was diluted with ethyl acetate to 21 ml, then a mixture of 0.1 ml of acetic anhydride and 0.1 ml of pyridine was added and the solution was stirred overnight (this is the method used for acetylation of the metabolic mixture). In the morning 11 ml of water were added, the mixture was stirred for 2 h to decompose the acetic anhydride, a further 90 ml of ethyl acetate were added, the organic layer was washed with 5 × 10 ml of water and the ethyl acetate layer was dried over 10 g anhydrous calcium chloride and then 25 g of anhydrous sodium sulfate. The solvent was removed and the residue was chromatographed (Table I). After a benzene run the mono-N-acetylamino derivative, R_F 0.92, was characterized by taking its high-resolution mas spectrum. It showed a molecular ion ($M^{+\cdot}$) at m/z 309 and a de-acetylation fragment (due to loss of ketene) at m/z 267.

Although the data were satisfactory for reference purposes, we wished to examine whether the 6-AABaP can be prepared by reducing 6-NBaP in the presence of acetic anhydride. To achieve this, a mixture of 6.5 mg of 6-NBaP, 14 ml of acetic anhydride and 14 g of zinc dust (Mallinckrodt, St. Louis, MO, U.S.A.) was refluxed under a nitrogen atmosphere for 30 min. The reaction mixture was cooled, 35 g of ice were added to decompose the acetic anhydride, the mixture was shaken with 250 ml of ethyl acetate and the ethyl acetate extract was washed with 2 × 25 ml of water, 2 × 25 ml of 5% sodium hydrogencarbonate solution and finally 1 × 25 ml water. The organic extract was dried over 50 g of anhydrous sodium sulfate, the solvent removed and the residue chromatographed (Table I). The major product (*ca.* 5 mg) had an R_F value of 0.08 in benzene. Mass and NMR spectral data (not shown) showed the substance to be predominatly 6-(N,N-diacetylamino)-BaP. We attempted to release the free amine by removing the acetyl groups with ammonia [2 ml of methanol + 2 ml of ammonia solution (58%)] but failed and, therefore, this approach for preparing 6-AABaP was not pursued any further.

Instrumentation

For the characterization of synthetic substances, we used UV, NMR and mass spectrometry. UV spectra were obtained with a Cary 15 UV–VIS spectrophotometer[9]. NMR spectra were obtained on a Varian XL-300 (300 MHz) spectrometer with C^2HCl_3 (99.96 atom% ^2H) as the solvent.

In vivo metabolites were obtained in small amounts and, therefore, high-resolution mass spectral determination was the method of choice for their identification.

Mass spectral data were acquired with an AEI MS-9 mass spectrometer operating in the electron ionization mode (*ca.* 70 eV) at an acceleration voltage of 8 kV. Calibration of each spectrum (Table II) was obtained by using perfluorokerosene 755 (SCM Specialty Chemicals, Gainesville, FL, U.S.A.). Data were obtained and processed by the DS-2000 data system. The resolution of the instrument was $M/\Delta M$ = 10 000 and the temperature of the source was *ca.* 220°C.

TLC

Synthetic and metabolic samples were resolved on silica gel soft layer plates containing 13% calcium sulfate binder and zinc–cadmium silicate as the fluorescence indicator (Anasil) (Foxboro, North Haven, CT, U.S.A.). The separated products were extracted with organic solvents in hich the inorganic binder and the indicator were insoluble. The synthetic materials could be extracted from the gel and then analysed spectrally without much interference.

However, 1 or 0.25 mm thick plates were used according to the size of the samples. The solvent systems used in this work are shown in Table I. Other TLC procedures used in this kind of work have been described[9,11,13].

Animal experiments

The *in vitro* microsomal aerobic metabolism of 6-NBaP has been described[5,6,9–11,14] and was shown to produce ring-hydroxylated metabolites of 6-NBaP.

Many nitro-PAHs are activated via reduction of the nitro group and *in vitro* such reduction occurs under reduced oxygen tension or anaerobic incubation[7]. With the objective of determining whether under anaerobic incubation conditions the nitro group of 6-NBaP underwent reduction, the following experiment was carried out.

All biochemicals were obtained from Sigma (St. Louis, MO, U.S.A.). The isolation of rat liver microsomal protein has been described[5,11]. The anaerobic incubation was carried out in an argon atmosphere. After the addition of each reactant, the 125-ml erlenmeyer reaction flask was evacuated and filled with argon gas. A total incubation volume of 34 ml of 50 m*M* Tris (pH 7.5) contained 69 mg of protein, 0.72 mg (70 μM) of 6-NBaP in 0.61 ml of acetone, 1 m*M* FMN, 3 m*M* $MgCl_2 \cdot 6H_2O$, 20 m*M* glucose-6-phosphate, 10 m*M* NADP and 157 units of glucose-6-phosphate dehydrogenase. Incubation was carried out in white light for 30 min. The reaction was arrested by placing the flask on ice and adding 0.1 g of zinc sulfate and 20 ml of methanol to precipitate protein. After centrifugation the supernatant was extracted with 2 × 60 ml of dichloromethane and then 2 × 60 ml of ethyl acetate. The combined organic extract was dried over 80 g of anhydrous sodium sulfate, the solvent was removed and the residue chromatographed (Table I). The metabolic 6-ABP was characterized by comparing its chromatographic and mass spectral properties with those of our synthetic standard.

In vivo metabolites

In carrying out the carcinogenesis experiment, Wislocki *et al.*[8] used male and female CD_1 mice and injected 6-NBaP i.p. We used male and female Swiss mice, available at the Eppley Institute.

Age of the mice

In order to isolate, chromatographically resolve and spectrally characterize the *in vivo* metabolites, one needs to administer a large dose of the test substance, as a number of metabolites, each occurring in small amounts, are excreted in the urine. We acetylated the metabolites, resolved them by TLC and then characterized them by mass spectrometry.

In many preliminary experiments using 17-days old mice (10–14 g), as were used by Wislocki*et al.*[8], we observed that deaths occurred when we tried to inject more than

32 nmol (9.5 μg) of 6-NBaP in 52 μl of DMSO. We therefore decided to use 5-week-old (27–30 g) male and female mice. The tolerance of i.p. DMSO was found to be 0.2 ml per mouse, above which deaths occurred in female mice.

Dose of 6-NBaP administered

In each experiment twelve mice were used. With male mice, to each mouse 960 nmol of 6-NBaP in 0.15 ml of DMSO were given i.p. in the first experiment. In a second experiment, 800 nmol of 6-NBaP in 0.1 ml of DMSO were used for each male mouse. Thus, two doses of 6-NBaP were examined in the male mouse. With female mice, each received i.p. 800 nmol of 6-NBaP in 0.1 ml of DMSO. After injections the mice were transferred to cages.

Metabolism cages

"Metabowl" from Jencons (Hemel Hempstead, Hertfordsire, U.K.) was used. Each cage accommodated four mice. A gentle stream of air was sucked through the cages during the 24-h experiment. The mice had free access to water but no food.

Collection of samples

The duration of each experiment was 24 h, during wich both urine and feces were collected and kept frozen over dry-ice. During collection of liver and spleen for preparing the respective cytosols, the twelve male mice were decapitated and the blood (8.8 g) was collected in 75 ml of ice-cold 1% sodium citrate (pH 8.4). Both liver (ca. 15 g) and spleen (*ca.* 1 g) were collected (1 g per 10 ml) in ice-cold 0.15 *M* sodium chloride–50 m*M* Tris (pH 7.5)–200 μ*M* Desferal (STD buffer).

Isolation of metabolites

As described by Alexander *et al.*[15], blood proteins were precipitated with tungstic acid, sedimented, the pH of the supernatant was adjusted to 5.5 and free metabolites were extracted with an equal volume of ethyl acetate. The ethyl acetate extract was processed as described below.

With liver and spleen, 20% homogenate was prepared in STD buffer, cytosols were prepared by differential centrifugaton, cytosolic proteins were precipitated by adding equal volumes of acetone and then free metabolites, if any, were extracted with an equal volume of ethyl acetate. As described below, free ring-hydroxylated metabolites of 6-NBaP and 6-ABP present in the ethyl acetate extract, if any, were acetylated, separated by TLC and then analysed by mass spectrometry. By this procedure, blood, liver and spleen, at the sample size we have used, did not show any metabolites (Tables II and III). These are not further discussed.

Urinary and fecal metabolites

Both urine and feces were collected for 24 h and frozen over dry-ice. As soon as the dry-ice was removed care was taken that both urine and feces were treated with organic solvents as soon as possible so that bacteria could not consume the organic metabolite(s).

In a typical experiment involving twelve male mice in 24 h, 26 ml of yellow urine (pH 6.0) mixed with feces was obtained. Urine and feces were separated by filtering through a coarse (ASTM 40–60) funnel. Urine was immediately extracted with 2 × 52

ml of ethyl acetate. Feces (4.7 g) were homogenized with a Polytron (Brinkmann, Westbury, NY, U.S.A.) in 30 ml of STD buffer (which also contained both chloroform and toluene as bacteriostats). The fecal homogenate was centrifuged for 5 min on a bench-top centrifuge at speed 3 (International Equipment, Needham Heights, MA, U.S.A.). The supernatant was collected and extracted with 2 × 40 ml of ethyl acetate. To the aqueous portions obtained from both urine and feces after organic extractions, both chloroform and toluene were added and left in the cold room while the free metabolites were being processed.

Free urinary metabolites: treatment of ethyl acetate extracts

The 104 ml of ethyl acetate extract was washed with 2 × 1 ml water, the organic layer was shaken with 10 g of anhydrous calcium chloride until turbid, the upper layer was decanted into another separating funnel, the lower aqueous layer was drained out and the organic layer was further dried with 20 g of anhydrous sodium sulfate. The organic extract was transferred into a 500-ml erlenmeyer flask which contained 10 g of 3 Å molecular sieve (Mallinckrodt) to act as a drying aent. To the extract 0.4 ml (4 μl/ml of the ethyl acetate) of acetic anhydride and 0.4 ml of pyridine were added, mixed and allowed to stand for 5 h. A batch of 0.1 ml each of acetic anhydride and pyridine was again added, mixed, the acetylation allowed to proceed overnight, and finally a last batch of 0.1 ml each of acetic anhydride and pyridine was added, the reaction allowed to proceed for further 1 h and then terminated by adding a further 400 ml of ethyl acetate and washing with 5 × 30 ml of water to remove the acetylating reagents. The organic extract was dried with anhydrous calcium chloride and sodium sulfate as described above, the solvents were removed and the residue was chromatographed (Table I).

Free fecal metabolites

As with urinary free metabolites, the fecal free metabolites in the 80 ml of ethyl acetate extract (above) were treated with acetylating reagents, the products chromatographed (Table I) and materials in chromatographic bands selected with reference to standards (Table I) were analysed by mass spectrometry.

Conjugated metabolites

Tong and Selkirk's work[16] with *in vitro* cell culture systems suggested that the ring-hydroxylated metabolites of 6-NBaP may be conjugated with glucuronides. According to Hande *et al.*[17], sometimes glucuronides are found to be resistant to β-glucuronidase, and according to their recommendation we used large excesses of β-glucuronidase and prolonged incubation.

Conjugated metabolites in urine. After the free urinary metabolites had been removed in ethyl acetate (above), an aqueous layer, 54 ml (including washings of ethyl acetate extract), was obtained. The aqueous layer was taken in a stoppered 250-ml erlenmeyer flask, 50 m*M* acetate buffer (pH 5.5) was added, followed by 5.4 mg (0.1 mg/ml aqueous layer) of β-glucuronidase (containing sulfatase activity from *Helix pomatia* type H-1; Sigma), 0.5 ml each of chloroform and toluene and the mixture was incubated at 37°C for 20 h. After the first 10 h another batch of chloroform and toluene was added, the reaction mixture cooled, the pH adjusted to 6.5 and the released metabolites were extracted into 108 ml of ethyl aceate. The ethyl acetate extract was

subjected to the acetylation process (see above, free urinary metabolites), the acetylated products were resolved by chromatography (Table I) and then bands were selected for mass spectrometry with reference to R_F values of reference substances (Table I).

Conjugated metabolites in feces. For this work the method used for releasing conjugated urinary metabolites (above) was followed. After removing the free fecal metabolites with ethyl acetate (see above), 25 ml of aqueous layer containing the conjugated metabolites, if any, were obtained. To the aqueous layer, acetate buffer (pH 5.5), β-glucuronidase, chloroform and toluene were added, the mixture was incubated and the released materials. if any, were processed as for conjugated urinary metabolites.

RESULTS

Synthetic material

The 6-ABP synthesized by the zinc–hydrazine method had the chromatographic properties shown in Table I. The UV, NMR and mass spectra of the product shown in Fig. 1 were consistent with the structure of 6-ABP. Two protons in the NH_2 group were seen at 6.92 ppm (not shown in Fig. 1B).

In vitro anerobic metabolite

In order to determine whether, under reduced oxygen tension, 6-NBaP was converted into 6-ABP, an *in vitro* experiment was carried out. It was found that an amount of 6-ABP was formed (Table I). The metabolic amine had a mass spectrum identical with that of the synthetic substance (Fig. 1C). During the *in vitro* incubation the raction flask was repeatedly evacuated and filled with argon gas, but the residual oxygen content, if any, was not determined. As such, in addition to the formation of the amine, some ring-hydroxylated metabolites were also found to be present. The

TABLE I

R_F VALUES OF BaP, ITS DERIVATIVES AND *IN VITRO* METABOLITES OF 6-NBaP

Compound	*Solvent 1: hexane–benzene (3:1)*	*Solvent 2: hexane–benzene (1:1)*	*Solvent 3: benzene*
Synthetic:			
BaP	0.43	–	–
6-Acetoxy-BaP	0.13	0.28	0.57
6-ABP	–	0.08	0.49
6-(N-Acetylamino)-BaP	–	–	0.92, 0.96
6-(N,N-Diacetylamino)-BaP	–	0.01	0.08
6-NBaP	0.34	–	–
BaP-1,6- and -3,6-quinone (mixture)	0.19	0.27	0.2, 0.3
In vitro metabolites[5,6,9,10]*:*			
Monoacetoxy-6-NBaP	0.19	0.27	0.44
Diacetoxy-6-NBaP	0.19	0.20	0.15
6-ABP	0.0	0.08	0.52

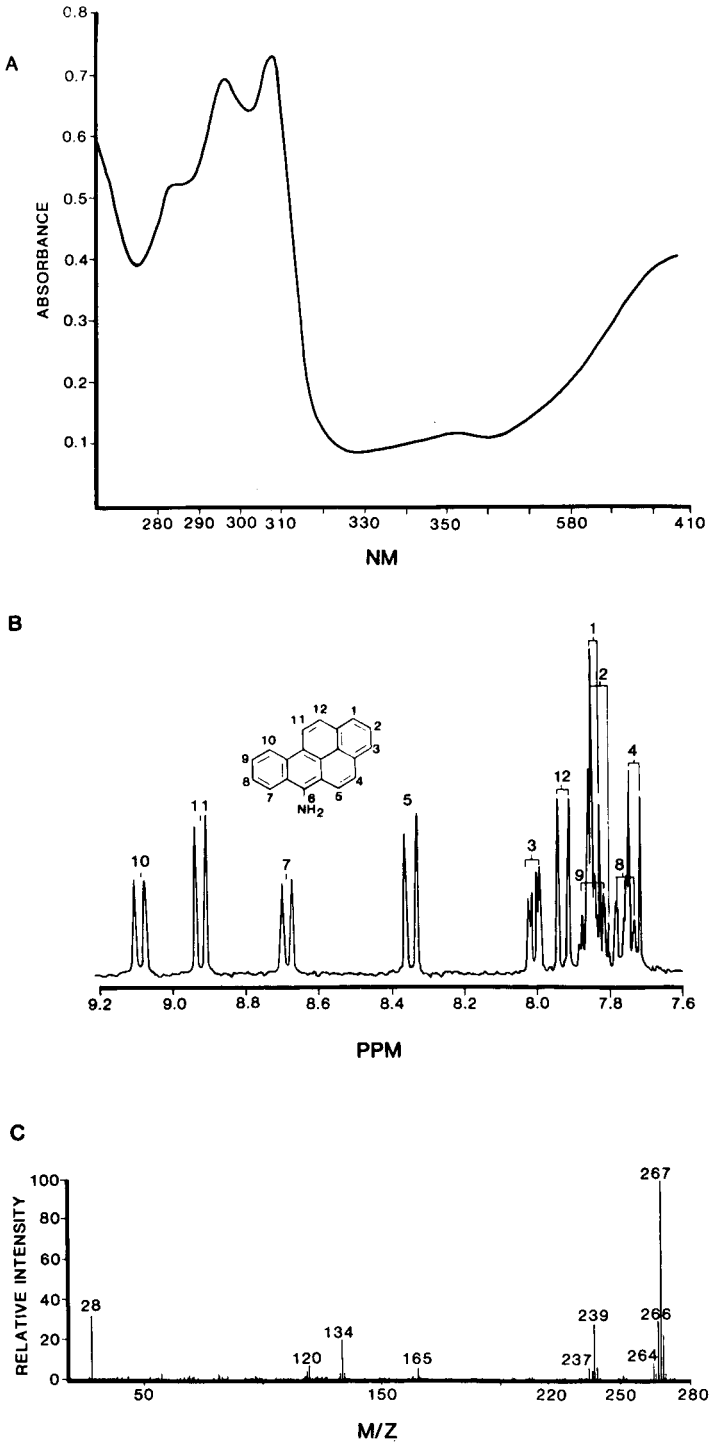

Fig. 1. Spectral properties of 6-ABP synthesized by the zinc–hydrazine method. (A) Ultraviolet absorption spectrum in acetonitrile. (B) Proton (aromatic) NMR spectrum of the substance in [$^2H_6$]DMSO. Tetramethylsilane was used as internal reference substance. (C) Mass spectrum.

chromatographic, UV and mass spectral properties of monohydroxy-6-NBaP have been reported[10].

Urinary metabolites

Accurate mass measurements are given in Table II. Most of the metabolites, both free and conjugated, were seen in urine. As we have often mentioned, many of the 6-NBaP metabolites are unstable but are stabilized after acetylation. One such substance is 6-hydroxy-BaP, which we characterized earlier from *in vitro* experiments, via UV, NMR and mass spectra of both the free and the acetylated products[5]. In one *in vivo* male mouse experiment we found 6-acetoxy-BaP (after acetylation) (*m/z* 310, Table II), suggesting the occurrence of 6-hydroxy-BaP (*m/z* 268) as a urinary metabolite. We also see the excretion of both BaP (*m/z* 252) and 6-NBaP (*m/z* 297) by both male and female (designated F, Table II) mice. In one male mouse experiment UV-detectable amounts of 6-NBaP were excreted.

It as been suggested that activation of 6-NBaP occurred via ring oxidation[6] and it has been reported that the nitro-PAH was carcinogenic to male and not to female mouse liver. In Table II we find that both male and female mice produce

TABLE II

MASS SPECTRAL CHARACTERISTICS OBSERVED IN MOUSE URINARY METABOLITES

(F) means samples from female urine.

m/z	*Molecular formula*	*Observed mass*	*Calculated mass*	*Probable compound*
252	$C_{20}H_{12}$	252.0924 252.0927 252.0933(F)	252.0939	BaP
267	$C_{20}H_{13}N$	267.1028 267.1052	267.1048	6-ABP
	$C_{19}H_9NO$	267.0671	267.0684	Fragment of mono-OH-6-NBaP
268	$C_{20}H_{12}O$	268.0873 268.0884 268.0886	268.0888	Hydroxy-BaP
	$C_{19}H_{10}NO$	268.0754 268.0759(F)	268.0762	Fragment of mono-OH-6-NBaP
282	$C_{20}H_{10}O_2$	282.0658 282.0664 282.0676	282.0681	Fragment of mono-OH-6-NBaP
283	$C_{20}H_{11}O_2$	283.0740 283.0764	283.0759	Fragment of mono-OH-6-NBaP
284	$C_{20}H_{12}O_2$	284.0833 284.0850(F) 284.0858	284.0837	Product of nucleophilic displacement of NO_2 group of mono-OH-6-NBaP
297	$C_{20}H_{11}NO_2$	297.0764 297.0781 297.0808 297.0795(F)	297.0790	6-NBaP
310	$C_{22}H_{14}O_2$	310.0979	310.0993	6-Acetoxy-BaP
313	$C_{20}H_{11}NO_3$	313.0725(F)	313.0739	Mono-OH-6-NBaP
329	$C_{20}H_{11}NO_4$	329.0704(F)	329.0688	Di-OH-6-NBaP
371	$C_{22}H_{13}NO_5$	371.0826(F)	371.0794	Monoacetoxy derivative of di-OH-6-NBaP

TABLE III
MASS SPECTRAL FRAGMENTATION OF 3-OH-6-NBaP AS REPORTED EARLIER[6,14]

Charged ion	*Mol. formula*	*Mol.wt.*	*Calculated mass*
$M^{+}\cdot$	$C_{20}H_{11}NO_3$	313	313.0739
$[M-NO]^{+}\cdot$	$C_{20}H_{11}O_2$	283	283.0759
$[M-NO-H]^{+}\cdot$	$C_{20}H_{10}O_2$	282	283.0681
$[M-CHO-O]^{+}\cdot$	$C_{19}H_{10}NO$	268	268.0762
$[M-CHO-O-H]^{+}\cdot$	$C_{19}H_9NO$	267	267.0684

ring-hydroxylated metabolites. However, in male experiments there were more chromatographic bands that contained the ring-hydroxylated metabolites. In characterizing the mass spectral fragmentation patterns of the ring-hydroxylated metabolites isolated in these experiments and shown in Table II, we utilized, for purposes of comparison, the fragmentation pattern suggested by Fu *et al.*[6] and shown in Table III for 3-OH-6-NBaP. The parent peak ($M^{+}\cdot$) at *m/z* 313 for a monohydroxy-6-NBaP was not seen in any of the male samples, although the appropriate fragments at *m/z* 283, 282, 268 and 267 were present (Table II). In the female sample there was a peak at *m/z* 313 for a monohydroxy-6-NBaP. Also, there was a peak at *m/z* 329 for a hydroquinone. The peak at *m/z* 371 is due to a monoacetoxyhydroquinone.

When we looked for the amine (*m/z* 267) we found that both male experiments produced the amine. We carried out one experiment with females and could not detect the formation of the amine. It is not known whether more experiments with females will show excretion of the amine. In both male and female experiments dihydroxy-BaP (*m/z* 284) was detected[9].

DISCUSSION

In Fig. 2, Scheme A, the mechanism of formation of ring-hydroxylated metabolites of 6-NBaP is shown[14]. A monohydroy-6-NBaP may be the source of a dihydroxy-BaP shown in Scheme C. In Scheme C we suggest that in the formaton of both 6-hydroxy-BaP and the dihydroxy-BaP (Table II)[5,9] water acts as a nucleophile which displaces the NO_2 group to produce the hydroxylated compounds[18]. In Scheme B the formation of BaP is suggested to involve the formation of a nitroanion radical involving electron transfer from NADPH via NADPH cytochrome *P*-450(*c*) reductase to the nitro group. A carbon-centered radical may be produced from the nitroanion radical[19]. The carbon-centered radical may be reduced to BaP. In Scheme D the reduction of the NO_2 group to the amine is shown to involve the intermediacy of a hydroxylamine, which is sometimes regarded as an active intermediate in the reduction of a nitro-PAH[7].

Whether it is the formaton of the epoxide (Fig. 2, Scheme A) or the hydroxylamine (Fig. 2, Scheme D) in the activation pathway of 6-NBaP, the metabolites are electrophiles capable of binding to DNA. The extent of DNA binding sometimes correlated with the carcinogenic potency of the substance[20]. In Table II, we find that both male and female mice can produce ring-hydroxylated (*i.e.*, epoxide intermediary) metabolites of 6-NBaP. Therefore, epoxide-mediated DNA binding of 6-NBaP will have lesser importance as 6-NBaP is carcinogenic to male and not female

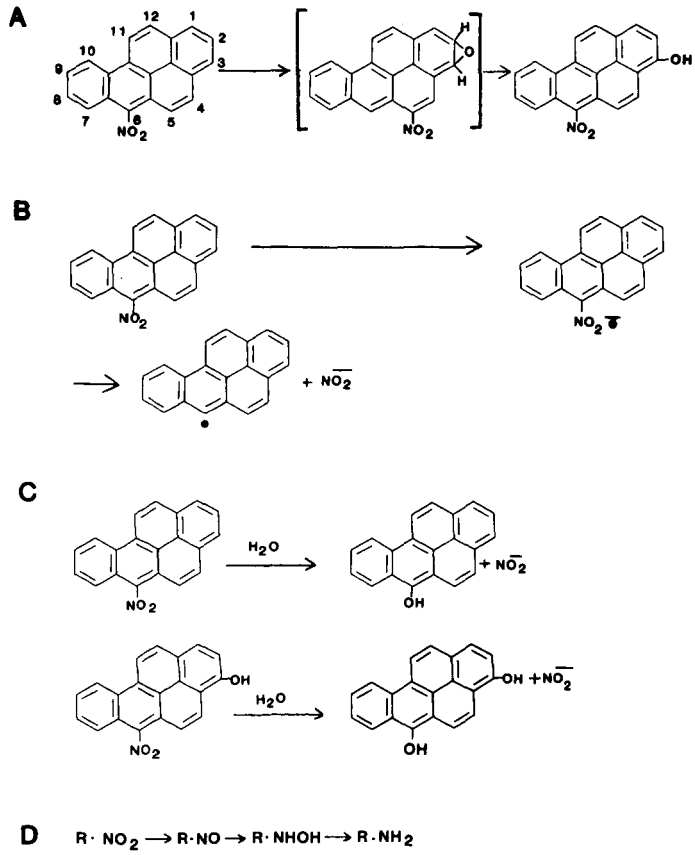

Fig. 2. Suggested schemes of product formation during the metabolism of 6-NBaP. (A) Microsomal ring oxidation of 6-NBaP. (B) Probable route of BaP formation via a nitroanion radical. (C) Route for the probable nucleophilic displacement of the NO_2 group by water. (D) Route for the reduction of the NO_2 group to an amino group.

mice. We examined *in vivo* metabolites produced in one female and two male experiments. 6-ABP was found to be produced by the males. The activation of the intermediate hydroxylamine (Fig. 2, Scheme D) may be different in male and female mice, as was found by Miller and Miller[21] with 2-acetylaminofluorene. Such activation involved sulfation of a hydroxy group.

Sulfotransferase activity may be different in males and females[21] and also may be different among the species[22]. In explaining the fact that 6-NBaP is carcinogenic to male and not to female mouse liver, it is possible that in the male liver a hydroxylamine is produced via nitroreduction and the hydroxylamine is activated via sulfotransferase activity. This possibility is just speculation and the verification of its correctness should form the subject matter of future research.

ACKNOWLEDGEMENTS

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CHROM. 20 733

DETERMINATION OF OXYBUTININ CHLORIDE IN PHARMACEUTICALS BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY WITH TWO COUNTER-IONS IN THE ELUENT

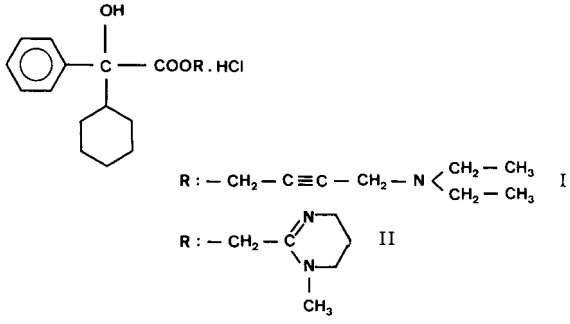
J. A. DE SCHUTTER* and P. DE MOERLOOSE
Department of Pharmaceutical Chemistry and Drug Quality Control, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)
(Received June 8th, 1988)

SUMMARY

A reliable stability-indicating method using reversed-phase ion-pair high-performance liquid chromatography for the determination of the anticholinergic drug oxybutinin chloride in pharmaceuticals is reported. Sample extraction is easy and fairly rapid and recovery and precision of the method are excellent. Due to the simultaneous use of an organic amine and of an alkanesulphonate in the mobile phase, good selectivity towards related (quaternary ammonium) anticholinergic drugs was obtained.

INTRODUCTION

Oxybutinin chloride (OBU), α-cyclohexyl-α-hydroxyphenylacetic acid 4-(diethylamino)-2-butynyl ester hydrochloride (I), is an anticholinergic drug used to relieve the symptoms of neurogenic bladder[1]. A method was developed for the quantitation of OBU in pharmaceutical formulations for routine quality control purposes since we were not aware of any published chromatographic method for OBU.



The analytical procedure was based on an octadecylsilane column with an acidic methanol–water mixture containing two counter-ions of opposite charge, *i.e.*, sodium octanesulphonate (SOS) and N,N-dimethyloctylamine (DMOA). Due to the ex-

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treme simplicity and quantitative recoveries of the extraction procedure and to the use of oxyphencyclimine hydrochloride (OXY) (II) as an internal standard a very good precision could be obtained (coefficient of variation $< 1\%$). The method was suitable for quality control, content uniformity and stability indication.

The selectivity of the chromatographic separation towards related anticholinergics was excellent. Moreover, the method can be applied as such or with minor modifications to the analysis of several tertiary or quaternary ammonium anticholinergic drugs such as glycopyrrolate, penthienate bromide, oxyphenonium bromide, hexocyclium, isopropamide iodide, fenpiverinium bromide and mepenzolate bromide.

EXPERIMENTAL

Chemicals

Oxybutinin hydrochloride (kindly supplied by Therabel, Brussels, Belgium) and oxyphencyclimine hydrochloride (kindly supplied by Pfizer, Brussels, Belgium) were used without further purification. α-Cyclohexyl-α-hydroxyphenylacetic acid was a kind gift from Ciba-Geigy (Brussels, Belgium).

Methanol (UCB, Leuven, Belgium), 1-octanesulphonic acid sodium salt (Janssen, Beerse, Belgium), N,N-dimethyloctylamine (Aldrich, Milwaukee, WI, U.S.A.), 85% orthophosphoric acid and 30% hydrogen peroxide (Merck, Darmstadt, F.R.G.), all of analytical reagent grade, were used. Water was purified by ion-exchange chromatography and subsequent distillation.

Apparatus

The liquid chromatographic equipment consisted of a Spectra-Physics SP 8000 liquid chromatograph, a Model 770 variable-wavelength detector (Spectra-Physics) and a BD 8 single channel recorder (Kipp and Zonen, Delft, The Netherlands).

Chromatography

A 5-μm particle size RSil C_{18} column (150 mm × 4.1 mm I.D.) (Alltech-Europe, Eke, Belgium) was used. All separations were conducted at 25°C (flow-rate 1 ml/min) and the column effluent was monitored at 220 nm. Injections of 10 μl were made with a Valco six-port injection valve.

The mobile phase was prepared by dissolving 6.49 g of SOS (30 mmol/l) and 1.50 ml of DMOA (7.5 mmol/l) in *ca.* 990 ml of methanol–water (65:35), adjusting the pH to 5.0 with orthophosphoric acid and diluting to 1000 ml in the same methanol–water mixture. Before chromatography, the mobile phase was filtered through a 5-μm filter and degassed with helium.

Analytical procedure

OBU was analysed in Ditropan® tablets and syrup (Therabel) stated to contain 5 mg per tablet and 5 mg per 5 ml syrup respectively.

Calibration standards

Stock solutions containing *ca.* 145 mg of OBU and *ca.* 60 mg of OXY (internal standard) were prepared in methanol–water (65:35). Working standards were pre-

pared by pipetting 7.0, 8.0, 9.0 and 10.0 ml of the OBU stock solution and 10.0 ml of the internal standard stock solution into a 50-ml volumetric flask and diluting to volume in methanol–water (65:35).

Calibration graphs were constructed by plotting the peak area ratios of OBU to OXY *versus* the weights of OBU in the standard solutions and analysed by least-squares regression methods.

Determination of the oxybutinin chloride content of tablets

Twenty tablets were weighed and powdered in a mortar. An amount of powder, equivalent to 2.5 average tablets, was transferred to a 50-ml volumetric flask. About 25 ml of methanol–water (65:35) and 10.0 ml of the internal standard stock solution were added. The contents were mixed, sonicated for 2 min in an ultrasonic bath and diluted to volume in the same methanol–water mixture. A portion of this suspension was centrifuged at 3000 *g* for 5 min. Aliquots of the supernatant were injected into the liquid chromatograph.

Determination of the oxybutinin chloride content of syrup

An amount of syrup, equivalent to 12.5 ml of syrup, was weighed into a 50-ml flask. About 25 ml of methanol–water (65:35) and 10.0 ml of the internal standard stock solution were added. The contents were mixed, sonicated for 2 min in an ultrasonic bath and diluted to volume in the same methanol–water mixture. Aliquots of this solution were injected into the liquid chromatograph.

Accuracy analysis

Tablets. Known amounts between *ca.* 95 and 125% of the tablet claim of OBU were added to a placebo tablet mixture. This sample was treated as described for the determination of the OBU content of the tablets.

Syrup. A known amount of OBU at the 100% level of the syrup claim was added to a placebo syrup mixture. This sample was treated as described for the determination of the OBU content of the syrup.

Precision analysis

The precision of the method was tested by subjecting portions of the appropriate sample preparation to the entire assay procedure and calculating the coefficient of variation (C.V.) of the results.

RESULTS AND DISCUSSION

Control of separation

The reversed-phase liquid chromatographic (RP-HPLC) separation of basic drugs such as OBU and OXY often involves the interaction with residual silanol groups[2–5] resulting in poor chromatographic performance[6–8]. The addition of an organic amine such as DMOA to acidic aqueous–organic eluents greatly improves the efficiency and peak shape of basic drugs but also reduces the capacity factors (k') to such an extent that the resolution quickly decreases[9,10]. An advantage of the simultaneous incorporation in the eluent of a counter-ion such as SOS is the possibility to increase retention of the basic drugs through ion-pair formation while the selectivity,

peak shape and the efficiency are kept high or even improved. Hence, the resolution of basic drugs will be enhanced[10]. Since the retention of neutral and acidic compounds remains almost unaffected by the presence of SOS in the eluent, important selectivity changes are easily achieved[11].

A test mixture consisting of OBU, OXY, methyl *p*-hydroxybenzoate and α-cyclohexyl-α-hydroxyphenylacetic acid (CPA) was used in the present chromatographic optimization study. CPA is expected to be the main degradation (hydrolysis) product of OBU as was confirmed by the stability experiments; the *p*-hydroxybenzoate ester is present as preservative in the syrup formulation. Application of the optimization strategy reported earlier for the quantitative analysis of quaternary ammonium drugs in pharmaceuticals[12] resulted in a mobile phase consisting of methanol–water (65:35), 7.5 mmol/l DMOA and 30 mmol/l SOS at pH 5.0. The pH of the eluent controls the retention of the acid CPA; below pH 5.0, CPA might interfere with the elution of OBU. The combined effects of DMOA and SOS allow a baseline separation of OBU and OXY (resolution = 1.8) in about 10 min with good peak shape (asymmetry factor = 1.40 for OBU) and sufficient efficiency (plate count = 2600 for OBU). Since no excipient or decomposition peaks interfere with the elution of OBU (k' = 5.0), this eluent composition was used for the quantitation (Fig. 1).

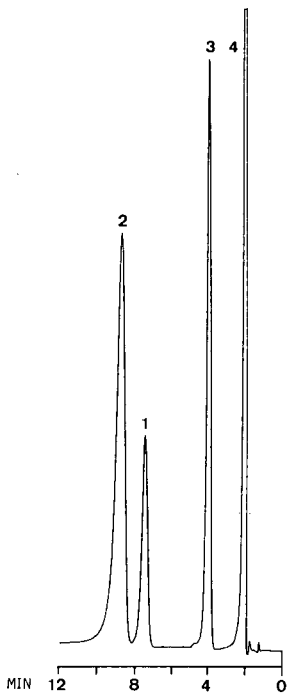

Fig. 1. Ion-pair liquid chromatographic separation of a standard mixture of oxybutinin chloride and related compounds. Stationary phase: RSil C_{18}, 5 μm, 150 mm × 4.1 mm I.D. Mobile phase: methanol–water (65:35), containing 30 mmol/l SOS and 7.5 mmol/l DMOA, pH 5.0. Chromatographic conditions: column temperature 25°C; flow-rate 1 ml/min; detection wavelength 220 nm. Peaks: 1 = OBU; 2 = OXY; 3 = CPA; 4 = methyl *p*-hydroxybenzoate.

Stability

Accelerated and extreme degradation of OBU in aqueous solution was achieved by several methods: acid and base hydrolysis, oxidative decomposition in the presence of hydrogen peroxide, photolytic decomposition and decomposition by boiling. No peaks interfering with the elution of OBU were observed in all cases. Several minor peaks were detected in the OBU solution after oxidative decomposition, mostly eluting with k' values less than 2.5. Analysis of the mixture formed after photolytic decomposition for 1 week in daylight or after boiling an aqueous OBU solution revealed no significant additional peaks. Decomposition occurred by acid and especially by base-catalysed hydrolysis was most significant and rapid, CPA being the only degradation product observed by UV detection at 220 nm, as expected.

From these experiments and from Fig. 1, it was concluded that the present determination method is stability-indicating.

Specificity

The specificity of the analysis for related anticholinergic drugs is illustrated in Fig. 2. A specific identification of OBU in such mixtures is possible since most related drugs are well separated from OBU. Only oxyphenonium bromide showed some interference but by careful evaluation of the retention times, OBU could unambig-

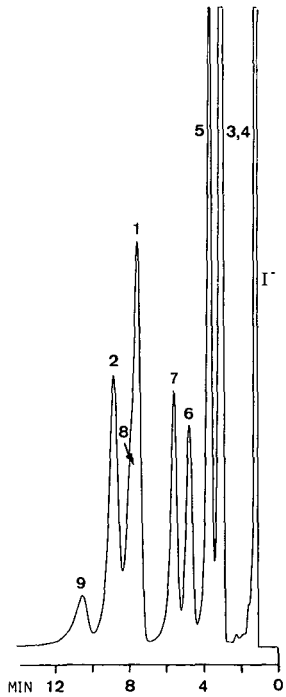

Fig. 2. Ion-pair liquid chromatographic separation of a standard mixture of anticholinergic drugs. Mobile phase, stationary phase and chromatographic conditions as in Fig. 1. Peaks: 1 = OBU; 2 = OXY; 3 = isopropamide iodide; 4 = fenpiverinium bromide; 5 = mepenzolate bromide; 6 = penthienate bromide; 7 = glycopyrrolate; 8 = oxyphenonium bromide; 9 = hexocyclium.

uously be identified in the mixture also in that case. From Fig. 2 it appeared that the chromatographic separation and probably the analytical method presented for OBU would also be suitable for the determination of these related drugs since they are all eluted efficiently as symmetrical bands in a reasonable analysis time.

Quantitative analysis

Typical chromatograms obtained for the quantitative analysis of the syrup and the tablets were very similar to that presented in Fig. 1, except that CPA could not be detected in either case.

The coefficient of variation (C.V.) of eleven replicate standard injections was 0.65%. Calibration standard solutions were prepared such that a range of 81–116% of the concentration claimed on the label was covered. A plot of the peak area ratios of OBU to OXY *versus* the amount of OBU in the standard solutions was linear as indicated by the correlation coefficient, $r = 0.9998$.

Analysis of the spiked placebo mixtures showed a mean recovery of 100.8% ($n = 6$, C.V. = 0.83%) for the tablets and of 100.9% ($n = 6$, C.V. = 0.96%) for the syrup. The results of the quantitation, calculated for four independently prepared commercial samples of both the tablets and the syrup were 99.2% (C.V. = 0.62%) and 104.8% (C.V. = 0.67%) respectively. In both cases, the amount claimed on the label agreed well with the results of the analysis.

CONCLUSION

The ion-pair liquid chromatographic method presented for the quantitation of OBU in pharmaceuticals is simple, selective, precise and accurate, owing to a reproducible chromatographic elution system, a proper choise of internal standard and a simple extraction procedure. The analytical methodology described for OBU might be of use for the determination of several related anticholinergic drugs.

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CHROM. 20 737

BORON ISOTOPE SEPARATION BY ION-EXCHANGE CHROMATOGRAPHY USING AN ANION-EXCHANGE RESIN IN HALIDE FORMS

SEPARATION FACTORS AT 25°C

TAKAO OI*, TETSURO TSUKAMOTO, HIDEKI AKAI and HIDETAKE KAKIHANA
Department of Chemistry, Sophia University, 7-1 Kioicho, Chiyodaku, Tokyo 102 (Japan)
and
MORIKAZU HOSOE
Department of Chemistry, The National Defense Academy, 1-10-20 Hashirimizu, Yokosuka, Kanagawa 239 (Japan)
(Received June 15th, 1988)

SUMMARY

A series of chromatographic experiments were carried out to determine the values of the separation factors for the $^{10}B/^{11}B$ isotopic pair in the boron isotope separation systems of an anion-exchange resin in halide forms at 25°C. In every experiment, the lighter isotope, ^{10}B, was enriched in the rear part of the boron zone formed in the chromatographic column. The separation factors obtained were 1.0099 for the fluoride-form resin, 1.0018 for the chloride-form resin and 1.0014 for the bromide-form resin, independent of the boron concentration. A theoretical consideration of them is given.

INTRODUCTION

Boron-10 is one of the most important nuclides in nuclear-related fields including neutron capture therapy for melanotic cancer and brain tumours because of its large neutron absorption cross-section. Methods for separating ^{10}B from its heavier isotope ^{11}B have been developed, among which ion-exchange chromatography is promising. Many studies on the ion-exchange chromatographic separation of boron isotopes have been carried out, not only to find practical separation systems but also to obtain knowledge on the fundamental boron isotope effect; recently, they were briefly reviewed[1].

In 1973, Hirao *et al.*[2] reported that a boric acid band formed in a chromatographic column packed with a strongly basic anion-exchange resin in the fluoride form can be eluted by pure water and ^{10}B was markedly enriched in the rear part of the band and ^{11}B in the front part after migration for about 45 cm. Their experiments were all carried out in band elution chromatography and, unfortunately, values of separation factor, S, were not given.

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As an extension of the work by Hirao *et al.*[2], we performed a series of chromatographic experiments in the breakthrough or reverse breakthrough manner in order to determine S values in systems of a strongly basic anion-exchange resin in halide forms at 25°C. In this paper, the results of such experiments are reported and a comparison with theory is given.

EXPERIMENTAL

The ion-exchange resin used was a strongly basic anion-exchange resin, Diaion SA-20A (20–50 mesh). All reagents were of analytical reagent grade and were used without further purification.

Eight experiments were carried out, four in the breakthrough manner with the resin in the chloride or bromide form and four in the reverse breakthrough manner with the resin in the fluoride form. These operational modes were chosen from the adsorption isotherms depicted in Fig. 1, in order to obtain sharper edges of the boron adsorption zones. The experimental conditions are summarized in Table I.

In breakthrough experiments, the resin was packed in a chromatographic column of Pyrex glass (150 cm × 1 cm) and was converted into the chloride or bromide form by the usual method. After washing out the conditioning agent by pure water, an aqueous boric acid solution was fed to the column at a constant flow-rate. The effluent from the column was collected and divided into fractions (each 15 cm^3). In reverse breakthrough experiments, first the resin packed in a chromatographic column (Pyrex glass, 100 cm × 1 cm) was converted into the fluoride form by using 0.5 M aqueous sodium fluoride solution and excess of sodium fluoride was washed out by pure water. Boric acid solution was fed to the column until the boron concentration in the effluent became equal to that of the feed. Boron adsorbed on the resin was then eluted by pure water at a constant flow-rate and the effluent was collected and divided into fractions (each 15 cm^3).

The column temperature was kept constant at 25°C throughout an experiment by circulation of thermostatted water through a jacket surrounding the column.

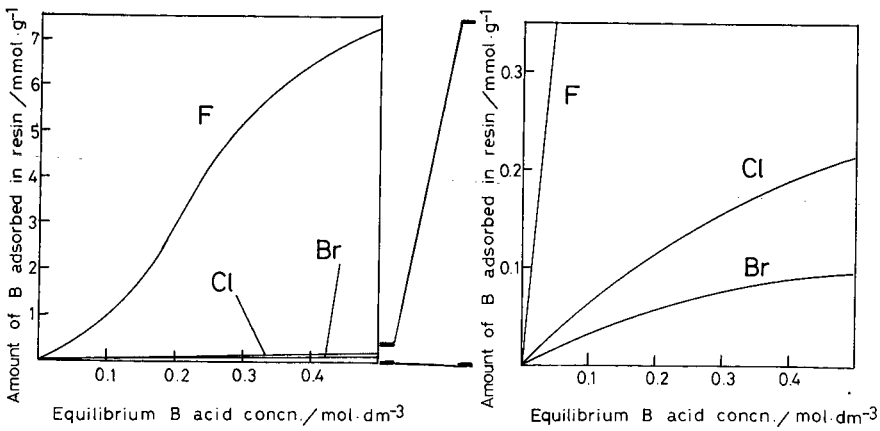

Fig. 1. Adsorption isotherms for the halide-form resin at 25°C. The vertical axis is the amount of boron adsorbed in 1 g of resin in the chloride form.

TABLE I

EXPERIMENTAL CONDITIONS* AND SEPARATION FACTORS

Experiment	*Resin form*	*Operating mode*	*Boron solution (M)*	*Bed height (cm)*	*Flow-rate ($cm^3\ cm^{-2}\ h^{-1}$)*	*S*
01	F^-	Reverse breakthrough	0.010	95.0	12.5	1.0098
02	F^-	Reverse breakthrough	0.053	93.5	12.9	1.0097
03	F^-	Reverse breakthrough	0.101	94.0	13.3	1.0103
04	F^-	Reverse breakthrough	0.500	94.0	12.2	1.0098
05	Cl^-	Breakthrough	0.106	150	17.6	1.00184
06	Cl^-	Breakthrough	0.661	146	17.1	1.00184
07	Br^-	Breakthrough	0.100	150	17.0	1.00137
08	Br^-	Breakthrough	0.677	140	18.4	1.00133

* Temperature = 25.0 ± 1.0°C; eluent = pure water.

The boron concentration of each fraction from each experiment was determined by neutralization titration with 0.1 *M* sodium hydroxide solution after adding a sufficient amount of mannitol and drops of Bromthymol Blue (BTB) indicator. The boron isotopic ratios, $^{10}B/^{11}B$, of selected fractions were measured by the surface ionization method[3] with a MAT 261 mass spectrometer. The fact that halide ions were not eluted into the effluents was verified by using a complex of zirconium ion with *p*-dimethylaminoazobenzenearsenic acid[4] in the case of fluoride ion and silver nitrate solution in the cases of chloride and bromide ions.

RESULTS

Chromatograms and boron-10 atomic fractions for the eight experiments are shown in Figs. 2–4. In each figure, the step-like line shows the boron concentration profile, the open circles the atomic fractions and the "original" line that in the feed solution. Note that the scaling of the axes is different from figure to figure.

Except for two cases, 01 and 04, the boron zones have satisfactorily sharp boundaries. In all the experiments with the resin in the fluoride form, small fractions of the boron loaded on the column appear to remain in the resin phase without being eluted by pure water, which is consistent with the description by Hirao *et al.*[2] that the boric acid loaded was eluted "almost completely" by pure water.

In every experiment the lighter isotope, ^{10}B, was enriched at the rear part of the boron zone, *i.e.*, it was preferentially fractionated into the resin phase. This trend is consistent with the one reported by Hirao *et al.*[2] and is independent of the resin form (F^-, Cl^-, Br^-).

For each of the experiments, the single-stage separation factor, $S(= 1 + \varepsilon)$, for the $^{10}B/^{11}B$ isotopic pair, defined as

$$S = (\overline{[^{10}B]}/\overline{[^{11}B]})/([^{10}B]/[^{11}B]) \quad (1)$$

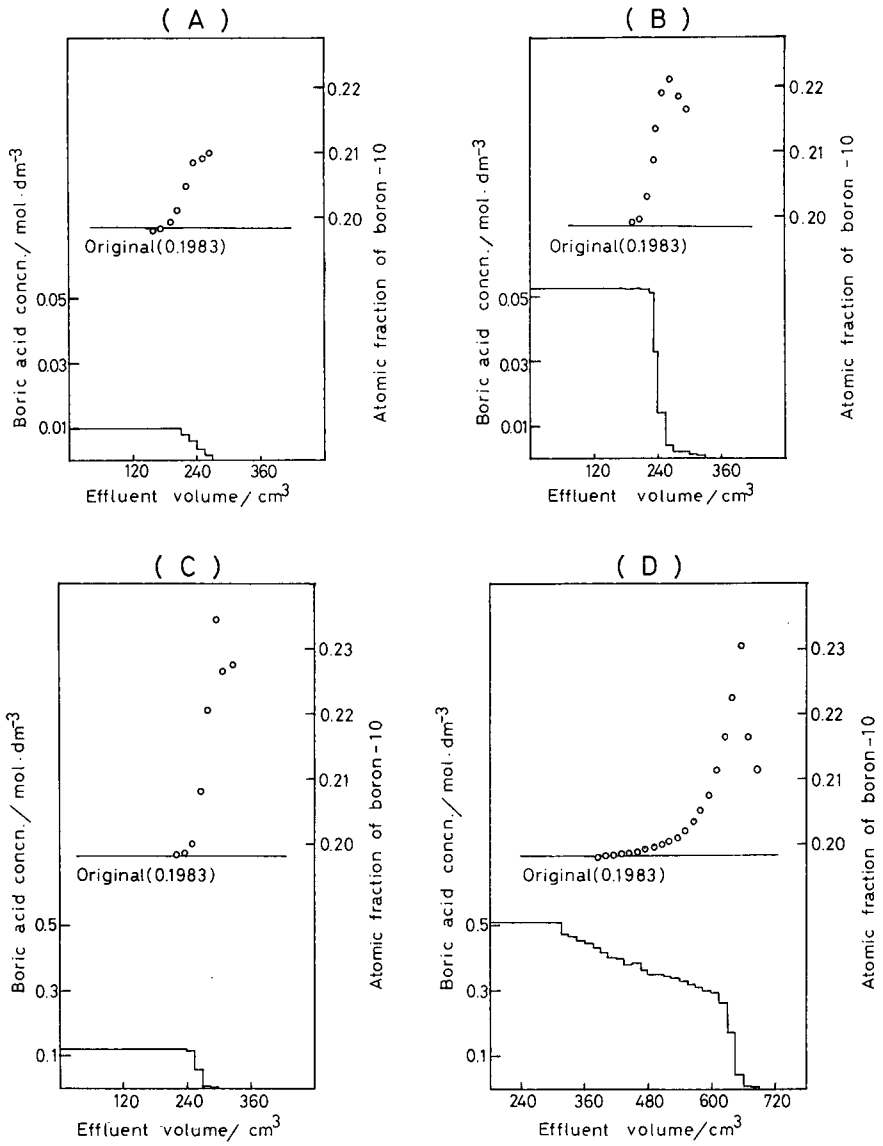

Fig. 2. Chromatograms and ^{10}B atomic fractions of the separation systems of the fluoride-form resin. Experiments: (A) 01; (B) 02; (C) 03 and (D) 04. Conditions as summarized in Table I.

where [A] denotes the isotopic concentration of A and the bar the resin phase, was calculated using the equation[5]:

$$\varepsilon = \Sigma|R_i - R_0|f_i/[QR_0(1 - R_0)] \quad (2)$$

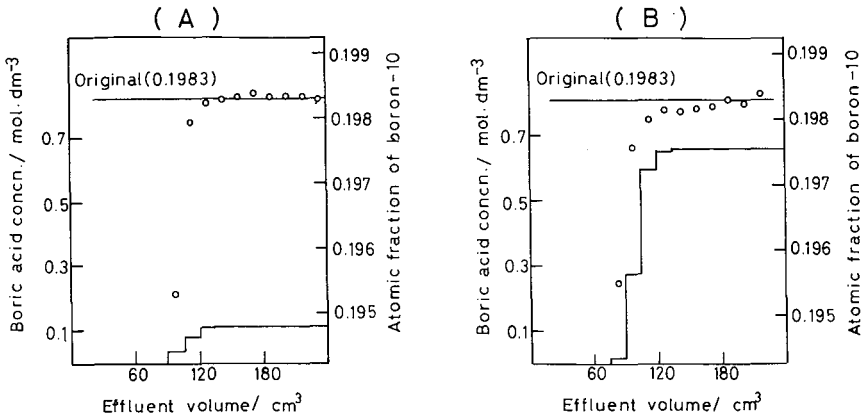

Fig. 3. Chromatograms and ^{10}B atomic fractions of the separation systems of the chloride-form resin. Experiments: (A) 05 and (B) 06. Conditions as summarized in Table I.

In eqn. 2, R_0 is the atomic fraction of ^{10}B in the feed solution (the "original"), R_i that in the ith fraction, f_i the amount of boron in the ith fraction, Q the total exchange capacity of the column and the summation is taken over all fractions that are enriched or depleted in ^{10}B. The ε values obtained are listed in the last column of Table I. From these values, S is independent of the boron concentration in the feed for a given form of the resin within experimental errors, and decreases in the sequence $F^- > Cl^- > Br^-$, which is the same order as that of the exchange capacity of the resin (*cf.*, Fig. 1).

DISCUSSION

Based on a theory of isotope distribution between two phases[6], the separation factor is expressible in terms of the ^{10}B-to-^{11}B isotopic reduced partition function ratio (RPFR) of the boron species involved and their mole fractions in and outside the resin phase. From the solution chemistry of boron[7] it is known that $B(OH)_3$ is the predominant species existing in the solution phase in all the experiments. The boron chemistry in the resin phase, on the other hand, is more complicated.

In general, the resin phase of the form $R\text{–}X^-$ (X = F, Cl or Br) can be regarded as a concentrated solution of X^-. For the experiments with X = F, three series of reactions involving boron species are conceivable in the resin phase. The first one is the direct reaction of $B(OH)_3$ with F^-. Studies on the complex formation between $B(OH)_3$ and fluoride in aqueous solution[8–10] indicated that the following successive reactions occur:

$$B(OH)_3 + F^- \rightarrow B(OH)_3F^- \quad (3a)$$

$$B(OH)_3F^- + F^- \rightarrow B(OH)_2F_2^- + OH^- \quad (3b)$$

$$B(OH)_2F_2^- + F^- \rightarrow B(OH)F_3^- + OH^- \quad (3c)$$

$$B(OH)F_3^- + F^- \rightarrow BF_4^- + OH^- \quad (3d)$$

The equilibrium constants were reported[10] as $\log K_1 = 1.71$ (eqn. 3a) and $\log K_2 = -7.15$ (eqn. 3b), which means that reactions 3b, 3c, 3d are negligible in the present experiments. Hence

$$\overline{B(OH)_3} + \overline{F^-} \rightarrow \overline{B(OH)_3F^-} \tag{3a'}$$

is the only viable reaction in the fluoride-form resin phase among 3a to 3d. The second series that may occur in this resin are

$$\overline{F^-} + \overline{H_2O} \rightarrow \overline{HF} + \overline{OH^-} \tag{4a}$$

and

$$\overline{B(OH)_3} + \overline{OH^-} \rightarrow \overline{B(OH)_4^-} \tag{4b}$$

i.e., the hydrolysis of fluoride and the successive formation of $B(OH)_4^-$. If reaction 4a does occur in the resin phase, part of the HF formed is brought into the solution phase and, in due course, will be found in the effluent. The very fact that fluoride was not detected experimentally in the effluents, although the sensitivity was not very good, indicates that reactions 4a and 4b are not the main reactions occurring in the resin phase. The third series of reactions involve the formation of polynuclear species. It was reported[7] that in aqueous boric acid solutions of concentrations greater than 0.025 *M* there are polynuclear species such as $B_3O_3(OH)_4^-$ and $B_3O_3(OH)_5^{2-}$ in addition to the monomeric species $B(OH)_3$ and $B(OH)_4^-$. The kinds of the species and their distribution depend on the boron concentration and the pH of the solution. The adsorption isotherm in Fig. 1 suggests that at higher boron concentrations in the solution phase, there are polynuclear species in the resin phase. However, the nature of the species, their structures and their distribution pattern are unknown at present. In the absence of such knowledge the best approximation is to regard a polynuclear

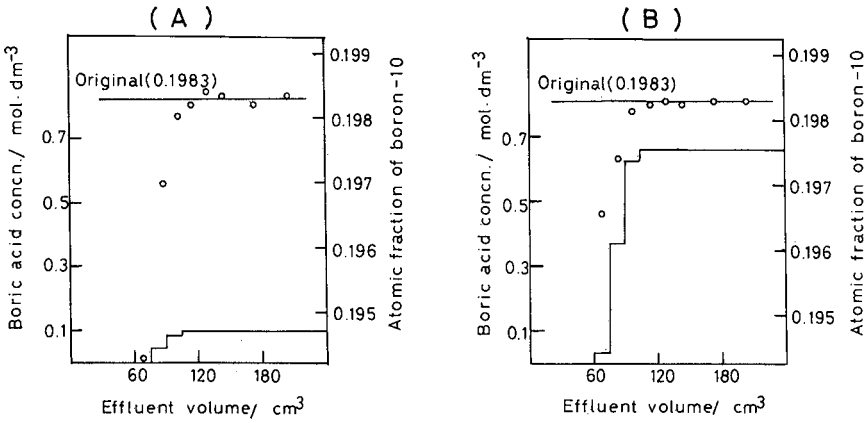

Fig. 4. Chromatograms and ^{10}B atomic fractions of the separation systems of the bromide-form resin. Experiments: (A) 07 and (B) 08. Conditions as summarized in Table I.

species as consisting of monomeric parts such as $B(OH)_3$, $B(OH)_4^-$ and $B(OH)_3F^-$ and to substitute its ln(RPFR) value by a weighted sum of the ln(RPFR) values of the monomeric species. This approximation method was successfully applied to the boron isotope separation systems of hydroxide-form resins[11]. For example, the polyborate species $B_3O_3(OH)_4^-$ was regarded as comprised of two triangular groups $B(OH)_3$ and a tetrahedral group $B(OH)_4^-$ and hence its RPFR value was approximated as $\ln f_{B_3O_3(OH)_4^-} = (2/3)\ln f_{B(OH)_3} + (1/3)\ln f_{B(OH)_4^-}$, where f_A is the RPFR of the species A. To summarize, in the experiments with the fluoride-form resin, the boron species in the solution phase is $B(OH)_3$ alone and those in the resin phase can be considered to comprise $\overline{B(OH)_3}$, $\overline{B(OH)_3F^-}$ and $\overline{B(OH)_4^-}$.

In the case of the resin in the chloride or bromide form, the possibility of the occurrence of the second series of reactions is much smaller than in the case of the fluoride-form resin because the degree of hydrolysis of Cl^- and Br^- is expected to be much less than that of F^-. In addition, the existence of polynuclear species is negligible, judging from the adsorption isotherms in Fig. 1. Hence, for the chloride or bromide form the only reaction of importance in the resin phase will be

$$\overline{B(OH)_3} + \overline{Cl^-} \rightarrow \overline{B(OH)_3Cl^-} \tag{5}$$

or

$$\overline{B(OH)_3} + \overline{Br^-} \rightarrow \overline{B(OH)_3Br^-} \tag{6}$$

Thus, in the experiments with these resins the boron species in the solution phase is $B(OH)_3$ and those in the resin phase are $\overline{B(OH)_3}$ and $\overline{B(OH)_3X^-}$.

Knowing the kinds of boron species in and outside the resin phase, the separation factor in eqn. 1 can be rewritten as:

$$S = ([\overline{^{10}B}]/[\overline{^{11}B}])/([^{10}B]/[^{11}B])$$

$$= \frac{[^{11}B(OH)_3]}{[^{10}B(OH)_3]} \cdot \frac{[\overline{^{10}B(OH)_3}] + [\overline{^{10}B(OH)_3X^-}] + [\overline{^{10}B(OH)_4^-}]}{[\overline{^{11}B(OH)_3}] + [\overline{^{11}B(OH)_3X^-}] + [\overline{^{11}B(OH)_4^-}]}$$

$$= \frac{[^{11}B(OH)_3]}{[^{10}B(OH)_3]} \cdot \frac{[\overline{^{10}B(OH)_3}]}{[\overline{^{11}B(OH)_3}]} \cdot \frac{[\overline{^{11}B(OH)_3}] + \bar{K}_X[\overline{^{11}B(OH)_3X^-}] + \bar{K}_{OH}[\overline{^{11}B(OH)_4^-}]}{[\overline{^{11}B(OH)_3}] + [\overline{^{11}B(OH)_3X^-}] + [\overline{^{11}B(OH)_4^-}]}$$

$$= K_{phase}(\bar{x}_{B(OH)_3} + \bar{x}_{B(OH)_3X^-}\bar{K}_X + \bar{x}_{B(OH)_4^-}\bar{K}_{OH}) \tag{7}$$

In eqn. 7, $\bar{x}_A$ is the mole fraction of the boron species A in the resin phase ($\bar{x}_{B(OH)_3} + \bar{x}_{B(OH)_3X^-} + \bar{x}_{B(OH)_4^-} = 1$ and, in the case of X = Cl or Br, $\bar{x}_{B(OH)_4^-} = 0$) and K_{phase}, $\bar{K}_X$ *and* $\bar{K}_{OH}$ are the equilibrium constants of the following boron isotope exchange reactions:

$$^{10}B(OH)_3 + \overline{^{11}B(OH)_3} \xrightarrow{K_{phase}} {}^{11}B(OH)_3 + \overline{^{10}B(OH)_3} \tag{8}$$

$$\overline{^{10}B(OH)_3} + \overline{^{11}B(OH)_3X^-} \xrightarrow{\bar{K}_X} \overline{^{11}B(OH)_3} + \overline{^{10}B(OH)_3X^-} \quad (9)$$

and

$$\overline{^{10}B(OH)_3} + \overline{^{11}B(OH)_4^-} \xrightarrow{\bar{K}_{OH}} \overline{^{11}B(OH)_3} + \overline{^{10}B(OH)_4^-} \quad (10)$$

Since K_{phase}, $\bar{K}_X$ and $\bar{K}_{OH}$ are expressed as the ratios of the RPFRs of the two species involved in the reactions, eqn. 7 is also expressible in terms of the RPFRs

$$S = (f_{B(OH)_3}/\bar{f}_{B(OH)_3})\,(\bar{x}_{B(OH)_3} + \bar{x}_{B(OH)_3X^-}\,\bar{f}_{B(OH)_3}/\bar{f}_{B(OH)_3X^-} + \\ + \bar{x}_{B(OH)_4^-}\,\bar{f}_{B(OH)_3}/\bar{f}_{B(OH)_4^-}) \quad (11)$$

where f_A and $\bar{f}_A$ are the RPFRs of the species A in the solution phase and in the resin phase, respectively:

$$K_{phase} = f_{B(OH)_3}/\bar{f}_{B(OH)_3} \quad (12)$$

$$\bar{K}_X = \bar{f}_{B(OH)_3}/\bar{f}_{B(OH)_3X^-} \quad (13)$$

and

$$\bar{K}_{OH} = \bar{f}_{B(OH)_3}/\bar{f}_{B(OH)_4^-} \quad (14)$$

The separation factor accompanying the phase change is usually much smaller than those accompanying chemical reactions, and consequently, to a good approximation, $K_{phase} = 1 (f_{B(OH)_3} = \bar{f}_{B(OH)_3})$.

Eqns. 7 and 11 are then simplified as:

$$S = \bar{x}_{B(OH)_3} + \bar{x}_{B(OH)_3X^-}\,\bar{K}_X + \bar{x}_{B(OH)_4^-}\bar{K}_{OH} \quad (7')$$

$$= \bar{x}_{B(OH)_3} + \bar{x}_{B(OH)_3X^-}\,\bar{f}_{B(OH)_3}/\bar{f}_{B(OH)_3X^-} + \bar{x}_{B(OH)_4^-}\,\bar{f}_{B(OH)_3}/\bar{f}_{B(OH)_4^-} \quad (11')$$

Kahihana and Kotaka[12] had calculated the PRFR values of $B(OH)_3$ and $B(OH)_4^-$. The RPFR values of the species $B(OH)_3X^-$ are calculable from those of $B(OH)_4^-$ and BX_4^-, the RPFR values of which had also been given by Kakihana and Kotaka, by using the rule of the geometric mean:

$$\ln \bar{f}_{B(OH)_3X^-} = (3/4) \ln \bar{f}_{B(OH)_4^-} + (1/4) \ln \bar{f}_{BX_4^-} \quad (15)$$

The values thus obtained at 25°C are listed in Table II, together with those for $B(OH)_3$ and $B(OH)_4^-$ and those of $\bar{K}_X$ and $\bar{K}_{OH}$.

TABLE II

VALUES OF THE REDUCED PARTITION FUNCTION RATIOS OF BORON SPECIES, AND EQUILIBRIUM CONSTANTS AT 25°C

Boron species	$B(OH)_3$	$B(OH)_4^-$	$B(OH)_3F^-$	$B(OH)_3Cl^-$	$B(OH)_3Br^-$
	1.2008	1.1780	1.1884	1.1602	1.1556
Equilibrium constant		$\bar{K}_{OH}$	$\bar{K}_F$	$\bar{K}_{Cl}$	$\bar{K}_{Br}$
		1.0194	1.0104	1.0350	1.0391

For the systems of the chloride and bromide forms, we can estimate the mole fractions of the species $B(OH)_3X^-$ in the resin phase from the data on the experimental separation factors and $\bar{K}_X$ in Table II and eqn. 7′. For the chloride form resin systems, $S = 1.00184$, $\bar{K}_{Cl} = 1.0350$ and $\bar{x}_{B(OH)_4^-} = 0$, then $\bar{x}_{B(OH)_3Cl^-} = 0.053$. That is, only 5.3% of total boron in the resin phase is in the form of $B(OH)_3Cl^-$ and the rest is in the form of $B(OH)_3$ which gives rise to no boron isotope effect. Similarly, for the bromide-form resin systems, $S = 1.00135$, $\bar{K}_{Br} = 1.0391$ and $\bar{x}_{B(OH)_4^-} = 0$, then $\bar{x}_{B(OH)_3Br^-} = 0.035$. For the fluoride-form resin systems, it is not possible to estimate the values of the mole fractions. However, it is interesting that the experimentally obtained S values are nearly equal to the equilibrium constant of the isotope exchange reaction observed when the boron species in the solution phase is $B(OH)_3$ alone and that in the resin phase is $B(OH)_3F^-$ alone. Quantitatively, assuming $\bar{x}_{B(OH)_4^-} = 0$, $\bar{x}_{B(OH)_3F^-}$ is calculated to be 0.952. The decreasing order of $\bar{x}_{B(OH)_3F^-} > \bar{x}_{B(OH)_3Cl^-} > \bar{x}_{B(OH)_3Br^-}$ explains why the sequence of the separation factors is S (fluoride-form resin) > S (chloride-form resin) > S(bromide-form resin) while that of the theoretical $\bar{K}_X$ is $\bar{K}_{Br} > \bar{K}_{Cl} > \bar{K}_F$.

CONCLUSION

The present work is summarized as follows.

(1) The lighter isotope ^{10}B was enriched in the rear parts of the boron zones formed in chromatographic columns packed with an anion-exchange resin in halide forms.

(2) The values of the separation factor were 1.0099 for the fluoride-form resin, 1.0018 for the chloride-form resin and 1.0014 for the bromide-form resin at 25°C and were independent of the boron concentration.

(3) A theoretical consideration suggested that the boron isotope fractionations observed resulted mostly from the boron isotope effects between $B(OH)_3$ in the solution phase and $B(OH)_3X^-$ (X = F, Cl or Br) in the resin phase.

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DETERMINATION OF INORGANIC ARSENIC SPECIES IN AQUEOUS SAMPLES BY ION-EXCLUSION CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

E. C. V. BUTLER
CSIRO Division of Oceanography, Marine Laboratories, GPO Box 1538, Hobart, Tasmania 7001 (Australia)
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SUMMARY

Arsenic(III) and -(V) were separated by ion-exclusion chromatography, using 0.01 *M* orthophosphoric acid eluent. Both forms of arsenic can be monitored by UV detection at 200 nm, but sensitivity is poor. Amperometric detection with a platinum-wire electrode at an applied potential of + 1.00 V allows arsenic(III) to be determined down to 0.012 μM. Detector response was shown to be linear to 1.00 μM, at which concentration, ten replicate injections of arsenic(III) gave a relative standard deviation of 1.3%.

In an application of the chromatographic procedure with amperometric detection to analysis of bottled mineral waters, arsenic(III) was measured by direct injection, and total inorganic arsenic was determined as arsenic(III) after reduction of arsenic(V) by sulphur dioxide.

INTRODUCTION

The measurement of arsenic in natural waters, particularly analysis for specific chemical forms (chemical speciation), is important not only in identifying toxic levels of the element, but also for gaining insight into its biogeochemical cycling. Arsenic species also have potential to act as hydrologic tracers[1], an application that can only be fully exploited with suitable analytical methods. Previous reports of As determination in natural waters have used a wide variety of methods, with hydride generation–atomic absorption spectrophotometry being the most popular[2].

High-performance liquid chromatography (HPLC) is a powerful technique for determining trace elements[3,4], as it separates the analyte from the sample matrix and possible interferents. Ion-exchange has been the usual model for the chromatographic separation of inorganic anions[4]. This approach has been successful in measuring As species, inorganic and organic, with detection by graphite furnace atomic absorption spectrophotometry[5], hydride generation–atomic absorption spectrophotometry[6], and inductively-coupled plasma atomic emission spectrophotometry[7]. However, weak acid anions such as arsenite are often poorly resolved by conventional ion-

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exchange techniques. Ion-exclusion chromatography very effectively separates As (III) from As(V) with no interference from most other inorganic anions[8]. In this chromatographic procedure neutral species such as undissociated acid molecules are strongly retained within the pores of the polymeric cation-exchange resin, whereas Donnan exclusion causes anionic species to elute early[9,10].

As(III) is well suited to anodic electrochemical detection in flowing streams[11,12]. Tan and Dutrizac [13] determined As(III) and As(V) simultaneously in metallurgical solutions by ion chromatography. Careful optimisation of the operational parameters of an electrochemical detector was required to measure the former species, while the latter was monitored by a conductivity detector.

In the present study, As(III) is separated by ion-exclusion chromatography and detected by its oxidation at a platinum-wire electrode. This procedure, with simple chemical pretreatment, is used to analyse the As(III) and As(V) content of bottled mineral waters.

EXPERIMENTAL

Instrumentation

The chromatography system comprised commercially available equipment apart from the electrochemical detector cell. Separation was achieved on a Bio-Rad organic acid analysis column (Aminex HPX-87H ion-exclusion packing, 300 × 7.8 mm) using a Waters M45C pump, 0.2-μm in-line filter, guard cartridge (Aminex HPX-85H resin, 40 × 4.6 mm) and a Rheodyne 7125 injector with a 200-μl sample loop. The amperometric detector consistes of a BAS LC-3A controller and a flow-cell (described below) incorporating a platinum-wire electrode. An LDC Spectromonitor II spectrophotometric detector was also used in preliminary work. Chromatograms were displayed on a YEW Type 3056 dual-pen recorder. Pulsation in the column effluent was attenuated hydropneumatically by placing an empty glass column, stoppered, in a tee (dead-end) configuration before the amperometric detector.

The flow cell for the amperometric detector (Fig. 1) is similar to that described by Lown *et al.*[14]. It was fabricated from a 50-mm cube Perspex block. The working electrode is 0.34 mm I.D. platinum wire, with the sensing portion being 21 mm in length. The platinum wire used for the auxiliary electrode has a diameter of 0.71 mm. A Metrohm mini Ag/AgCl reference electrode (6.0727.000) is screwed into an appropriately threaded chamber opposite the auxiliary electrode. All channels are 0.80 mm I.D. except for the 1.15 mm I.D. channel that exits through the reference electrode chamber. Seals of silicone rubber (Silastic J RTV; Dow Corning) are compressed by LDC polypropylene tube-end fittings, while leakage about the reference electrode is prevented by an O-ring. For satisfactory performance of the amperometric detector, the eluent is grounded via an HPLC tube fitting.

Reagents

All solutions were prepared from analytical reagent-grade chemicals and distilled, deionised water from a Millipore Milli-Q water purification system. A stock standard As(III) solution (5.00 m*M*) was prepared by dissolving 0.1237 g As_2O_3 in about 10 ml of a 10% potassium hydroxide solution. After addition of 1.0 ml concentrated sulphuric acid it was diluted to 250 ml. Working standards were prepared by

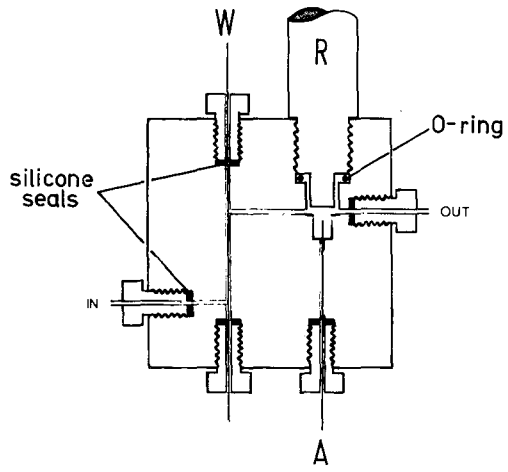


Fig. 1. Cross-sectional diagram of the flow cell for the platinum-wire amperometric detector. Platinum-wire working electrode (W) and auxillary electrode (A) with Metrohm mini Ag/AgCl reference electrode (R).

serial dilution with Milli-Q water or eluent. A primary As(V) standard solution was obtained by treatment of As(III) solution with aqua regia[13]. As(V) solutions prepared from $Na_2HAsO_4 \cdot 7H_2O$ were calibrated by reference to the primary standard solution. The eluent used was 0.01 *M* orthophosphoric acid, which was filtered through a Whatman GF/F filter and degassed under vacuum in an ultrasonic bath.

Procedures

The eluent was pumped through the system at 0.6 ml/min. The working electrode was operated at a potential of +1.00V *vs.* Ag/AgCl as recommended by Lown and Johnson[11]. Preliminary surface treatment of the electrode involved polishing with diamond (6 μm) and alumina (0.05 μm) pastes, immersion in hot concentrated perchloric acid for 1 min, and a thorough rinsing with distilled/deionised water. The filter control on the BAS LC-3A controller was set at 1.0 s. All detector cables were shielded and connected to a common ground. When in use the spectrophotometric detector was placed before the amperometric detector and operated at a wavelength of 200 nm. Chromatography was performed at 19 ± 1°C.

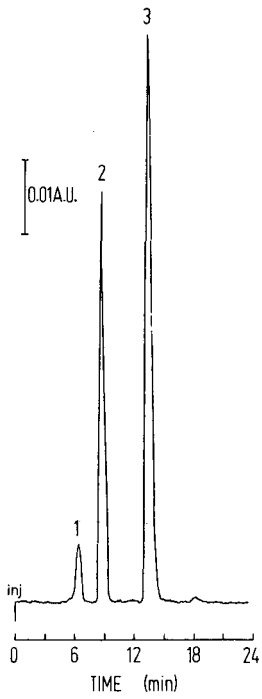
To analyse mineral waters, the undiluted sample was ultrasonicated for 10 min. The decarbonated sample was then injected directly onto the column to determine As(III). To determine total inorganic As, and hence As(V), the reduction procedure of Bodewig *et al.*[15] was used with minor modification. Instead of acidifying the sulphur dioxide-treated sample (25 ml) with 1 ml of concentrated sulphuric acid, 50 μl of concentrated orthophosphoric acid (low in As) was added and the solution was bubbled with nitrogen for 45 min at 90°C. No interference from residual sulphur dioxide was observed after this step. Arsenic concentrations were calculated by comparing sample peak heights with those from injection of standard As(III) solutions.

RESULTS AND DISCUSSION

Performance characteristics

A standard mixture of As(III) and As(V) (0.50 m*M* of each dissolved in the eluent) was used to evaluate the chromatographic separation of the As species (Fig. 2). Output from the spectrophotometric detector shows the peak for the mildly acidic arsenate [As(V)] species, $AsO(OH)_3$ appearing well before As(III), which is present in solution as the very weak acid, $As(OH)_3$. Strong acid anions such as Cl^-, which are excluded from the resin, elute with the void volume, and are adequately resolved from the As(V) peak. Anodic detection at the platinum-wire electrode is specific for As (III), since As(V) cannot be oxidised. This permits trace amounts of As(III) to be easily discerned from a 1000-fold excess of As(V) (Fig. 3).

Previous research has shown that adsorbed hydroxyl groups on platinum metal (*i.e.* PtOH species) are important in the oxidation of As(III) to As(V)[16]. PtOH forms rapidly on the metal surface at potentials, $E > 0.7$ V, but its longer-term conversion to PtO at these potentials results in some deterioration of electrode response for As(III). The decline in sensitivity was evaluated by repeated injections of 1.0 μM As(III) solution at intervals over several days (Fig. 4). The background current was



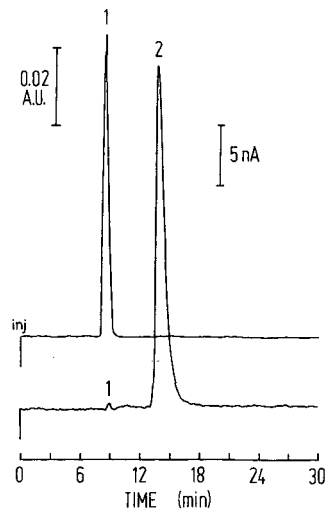


Fig. 2. Separation of As species by ion-exclusion chromatography. Chromatographic conditions as in text. UV detection at 200 nm. Peaks: 1 = anions; 2 = As(V), 0.5 m*M*; 3 = As(III), 0.5 m*M*.

Fig. 3. Comparison of response of UV (upper trace) and amperometric (lower trace) detectors to As species. UV detector: 200 nm. Amperometric detector: +1.00 V *vs.* Ag/AgCl. Peaks: 1 = As(V), 1.00 m*M*; 2 = As(III), 1.00 μM.

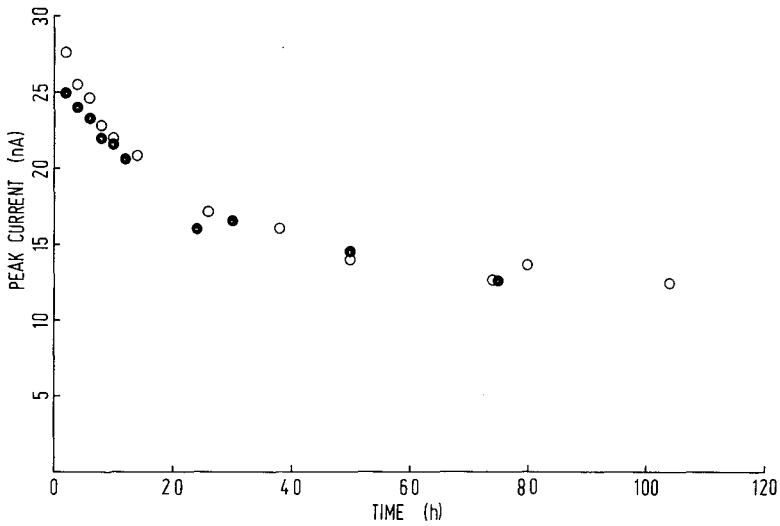


Fig. 4. Response decay for As(III), 1.0 μM, detection at platinum-wire electrode (0.34 mm I.D.). (●) +0.80 V *vs.* Ag/AgCl; (○) +1.00 V *vs.* Ag/AgCl.

allowed 2 h to stabilise after applying potential to the electrode. Trials were performed at +0.80 V and +1.00 V. In both cases it was evident that peak current, after steady decline, levels off after 50 h to about 50% of the 2-h value. This gradual loss of sensitivity is not, however, a problem when monitoring HPLC chromatograms, because samples would be routinely interspersed with injections of standard solutions. In fact reproducibility in the short term is very good. About 27 h after initial application of +1.00 V, ten replicate injections of 1.0 μM As(III) standard solution were made over a 1-h interval. They yielded a mean peak current of 15.9 nA, range 15.8–16.1 nA, and a relative standard deviation of 1.3%. The response index, r, defined in the power function:

$$y = Ac^r$$

(where y is detector output, c is solute concentration and A is a constant) is a measure of detector linearity[17]. Linear regression analysis of peak height data in the concentration range 0–1.00 μM gave an r value of 0.98. For a truly linear detector $r = 1$, but the practical bounds for response linearity ascribed by Scott[17] are 0.98–1.02. Above 10.0 μM the relationship between concentration and peak height became non-linear. For a similar platinum-wire amperometric detector, Lown and Johnson[11] recorded a linear calibration curve, based on peak areas, from 0.017 to 50 μM. Dispersion in the chromatographic system, as well as the reasons given by the above authors contribute to non-linearity in detector response at higher concentrations. The minimum detectable As(III) concentration (signal-to-noise ratio = 2) with this system of ion-exclusion chromatography and amperometric detection is 0.012 μM.

Anodic (oxidative) detection at platinum has been described for many inorganic and organic compounds[12]. Whereas a complex mixture in the flow-stream passing

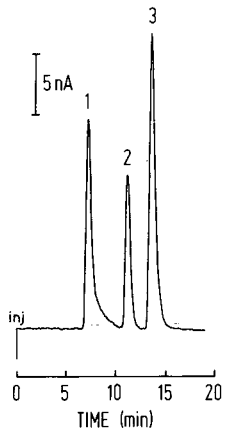
over the electrode may produce chemical interference in As(III) determination, preliminary chromatographic separation often provides additional information on a sample. Electroactive species such as sulphite and oxalic acid are well resolved from As(III) after ion-exclusion chromatography (Fig. 5), while oxidisable ionic compounds, such as iodide, are not retained on the column and so do not interfere.

The sample may not be the only source of interference. Impurities derived from the eluent or components of the HPLC system can also hamper accurate analysis. In preliminary studies here it was observed that 0.01 *M* sulphuric acid eluent leached interfering agents from the stainless-steel fittings of the chromatograph. Hydrochloric acid, another acid used for ion-exclusion chromatography, was not considered because of its known corrosive action and its unsuitability as an electrolyte for As(III) determination with the platinum-wire amperiometric detector[11]. Orthophosphoric acid proved to be suitable for both good chromatography and sensitive electrochemical detection of As(III).

Application

Several bottled mineral waters on sale in the U.K. have been reported to contain As in excess of what might be considered a safe limit of 10 μg/l (0.13 μ*M*)[18].

Three representative bottled mineral waters available in Tasmania (Australia) were analysed. Two were national brands (samples 1 and 2) and the third was imported from France (sample 3). As(III), if present, could be determined by direct injection of the decarbonated mineral water (Fig. 6). Total inorganic As was measured after reducing any As(V) to As(III) by treating the sample with sulphur dioxide. The original As(V) concentration can be evaluated by difference. A standard addition of 1.0 μ*M* arsenate to a mineral water proved the reduction procedure effective, with



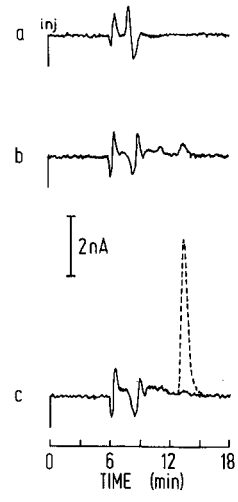


Fig. 5. Separation of electroactive compounds by ion-exclusion chromatography. Amperometric detector: +1.00 V *vs.* Ag/AgCl. Peaks: 1 = oxalic acid, 50 μ*M*; 2 = sulphite, 2.0 μ*M*; 3 = As(III), 1.0 μ*M*.

Fig. 6. Analysis of bottled mineral waters, (a) direct injection of mineral water 2, decarbonated; (b) mineral water 2, after treatment with sulphur dioxide; (c) mineral water 3, after treatment with sulphur dioxide, and standard addition of As(III) (0.25 μ*M*, dashed line).

95% recovered as As(III). In each of the selected mineral waters concentrations of As were below the minimum detectable concentration($< 0.012\ \mu M$) for either As(III) or As(V), except for mineral water 2 where As(V) was just measurable at 0.013 μM. Farmer and Johnson[18] have observed that As in mineral waters exists mostly in the higher oxidation state.

The analytical procedure as described here would be suited to screening of groundwaters for elevated inorganic As levels (arsenite and arsenate), monitoring of mining and industrial effluents, and conceivably, analysis of biological material after appropriate acid digestion. In many natural waters, however, inorganic As concentration is close to or less than the minimum detectable concentration. For accurate As speciation studies in such waters a prior preconcentration step will be needed. The organoarsenic species, monomethylarsonate and dimethylarsinate are frequently present in natural waters. Although these compounds may be ineffectively separated from inorganic As species by some strong cation-exchange resins[5], they will not interfere in procedures reported here. Neither monomethylarsonate nor dimethylarsinate elicited a response from the amperometric detector at 5 μM levels.

Arsenic is not the only metalloid detectable at positive potentials at the platinum-wire electrode. Antimony(III) produced a similar response when the detector was used in the "flow-injection analysis" mode. At pH 2.2 of the 0.01 M orthophosphoric acid eluent antimony(III) is cationic to a small degree, so simultaneous determination of the two metalloids, As and Sb, by ion-exclusion chromatography is not possible under the conditions reported here.

In on-going work, a simple preconcentration technique for As(III) is being sought, and chromatographic conditions are being modified to enable determination of arsenic(III) and antimony(III) with a single injection.

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I am grateful to D. J. Mackey and D. C. Johnson for helpful advice during the development of the procedure. The skilled work of N. McQueen is acknowledged in the construction of the flow cell for the amperometric detector. This research was made possible by the award of a CSIRO Postdoctoral Fellowship.

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ION CHROMATOGRAPHIC DETERMINATION OF LOW LEVEL CADMIUM(II), COBALT(II) and MANGANESE(II) IN WATER

V. CHEAM*

Analytical Chemistry Research Project, Research and Applications Branch, National Water Research Institute, Canada Centre for Inland Waters, 867 Lakeshore Road, P.O. Box 5050, Burlington, Ontario L7R 4A6 (Canada)

and

E XUE LI

Institute of Environmental Health Monitoring, Chinese Academy of Preventive Medicine, 29 Nan Wei Road, Beijing (China)

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SUMMARY

An automated ion chromatographic method for analysis of low- and sub-ppb (μg/l) levels of cadmium in water preserved with 0.2% nitric acid is described. The detection limit of 0.1 ppb satisfies the various guidelines and objectives of 0.2 ppb cadmium. The method is also applicable to determination of cobalt and manganese down to 0.1 ppb. The automation, on-line concentration and simultaneous determination of the three elements is an advantage over the flame and furnace atomic absorption spectrometry.

INTRODUCTION

Cadmium is a toxic priority-element which has created a great deal of attention and concern[1–6]. In trend and long-term monitoring studies, reliable low-level Cd data are not available due to lack of sensitive methods. Consequently, it has been recommended that sensitive and reliable methods be developed[2].

Routine analyses of trace metals including cadmium are commonly carried out using atomic absorption spectrometry (AAS) and inductively coupled plasma (ICP) atomic emission spectrometry[7,8]. Lum and Callaghan[9] claimed to have validated a direct injection graphite furnace-AAS method giving a <2 ppt* detection limit. However, Sturgeon[10] recently pointed out some difficulties with direct trace analysis by graphite furnace and stated that direct quantitative determination of low-ppt Cd levels is not feasible without sample preconcentration.

Separate sample preconcentration is required for AAS and ICP analysis of low levels[7,8]. Since ion chromatography (IC) techniques are potentially cost-effective as

* Throughout this article, the American billion (10^9) and trillion (10^{12}) are meant.

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TABLE I

VARIOUS WATER QUALITY GUIDELINES AND OBJECTIVES

Water type	*Permissible concentration Cd (μg/l)*			
	Great Lakes Water Quality agreement (1987)[23]	*U.S. Environmental Protection Agency Water Quality criteria (1973 and 1980) (see ref. 5)*	*International Joint Commission–Great Lakes Water Quality objectives (see ref. 5)*	*Canadian water quality guidelines (1987)*[6]
Drinking water, supply/ambient water	–	10	–	5
Freshwater, aquatic life	0.2	1.5 (in soft water) 6.3 (in very hard water)	0.2	0.2 (in soft water) 1.8 (in very hard water)
Irrigation water	–	10	–	10

recently shown for anions and cations[11], and are suitable for studying metals and their speciation[12,13], we decided first to test IC in investigating low-level Cd samples. It has been stated that a detection limit as low as 0.1 ppb Cd^{2+} is attainable by IC[12,13] but Mn^{2+} would coelute, which is unacceptable in water analysis. Recently, Rubin and Herberling[14] reported an IC method that separates Cd^{2+} and Mn^{2+}, but it is not very sensitive for sub-ppb (μg/l) Cd^{2+}.

In this study, we concentrated on developing an IC method which not only can separate Mn^{2+} but also can detect Cadmium down to a 0.2-ppb limit as required by various guidelines and objectives (Table I). As will be shown low- and sub-ppb Cd^{2+}, Co^{2+} and Mn^{2+} levels can be simultaneously determined with recoveries ranging from 95 to 107% in 20 out of 21 attempted recoveries.

EXPERIMENTAL

Chemicals

Very-high-purity chemicals were used: acetic acid (Seastar Chemicals and Merck 739055 Suprapur), oxalic acid · $2H_2O$ (Fluka 75700), lithium hydroxide · H_2O (Fluka 62530), pyridine-2,6-dicarboxulic acid (PDCA) (Fluka 82790), nitric acid (Seastar Chemicals and Baker Instra-analyzed), 4-(2-pyridylazo)resorcinol (PAR) (Fluka 83970), ammonium hydroxide (Seastar Chemicals and Merck 010983 Suprapur), sodium chloride, sodium bicarbonate, sodium carbonate, sodium sulphate, and sodium acetate (Merck Suprapur), 2-dimethylaminoethanol (DMAE) (Fluka 38990, Aldrich D15,740-6), metal standard stocks (Fisher Scientific). Milli-Q water (18 MΩ) was used. Labware was cleaned with 30% nitric acid[15].

Equipment and operation conditions

The system comprises a Dionex chromatograph 2000 equipped with an analytical pump, a reagent delivery module and a membrane reactor, a Kratos visible spectrophotometric detector (Spectroflow 757), an HP 3390 recorder-integrator, a Technicon autosampler IV, two loading pumps (Dionex DQP-1) and an Auto Ion 100 for automation.

Fig. 1 shows the system schematic and valve configuration for sample concentration, rinsing and injection. A typical run starts with sample loading via valve I INJECT, sample flushing via port 5 of valve II to flush out any carryover, and sample concentrating via ports 7 of valves II and III into the concentrator column CG3 (the right one) and to waste. The run continues with sample rinsing by MQ water (18 MΩ, stripped of trace metals by CG5 column, via valve I LOAD), followed by sample injection via valve III INJECT. The analyte metals are separated by CS5 column, and in the reaction chamber react with PAR to form metal chelates, which are detected and measured at 520 nm. The operating conditions are summarized in Table II. Calibration curves and one-point calibrations were used.

RESULTS AND DISCUSSION

Chelate formation

The chelating reagent PAR is known to be a very sensitive reagent for spectrophotometric determination of metals. For example, it has been successfully

TABLE II

OPERATION CONDITIONS

Sample handling				*Eluent (ml/min)*	*Concentrator*	*Separator*	*Detection*	
Flushing	*Loading*	*Rinsing*	*Injection*				*Post-column chamber*	*Detector*
4 ml	10 ml	3 ml		3 m*M* PDCA 4.3 m*M* Lithium hydroxide	HPIC-CG3 (50 × 4 mm)	HPIC-CS5 (250 × 4 mm)	0.2 m*M* PAR 0.5 *M* Ammonium hydroxide	520 nm 0.1(0.05) a.u.f.s.
Valve I ON	Valve I ON	Valve I OFF	Valve I ON	2 m*M* Sodium sulphate			1.0 *M* DMAE	
Valve II OFF	Valve II ON	Valve II ON	Valve II OFF	25 m*M* Sodium chloride			(0.6 ml/min through	
Valve III OFF	Valve III OFF	Valve III OFF	Valve III ON	(1.0 ml/min)			membrane reactor)	

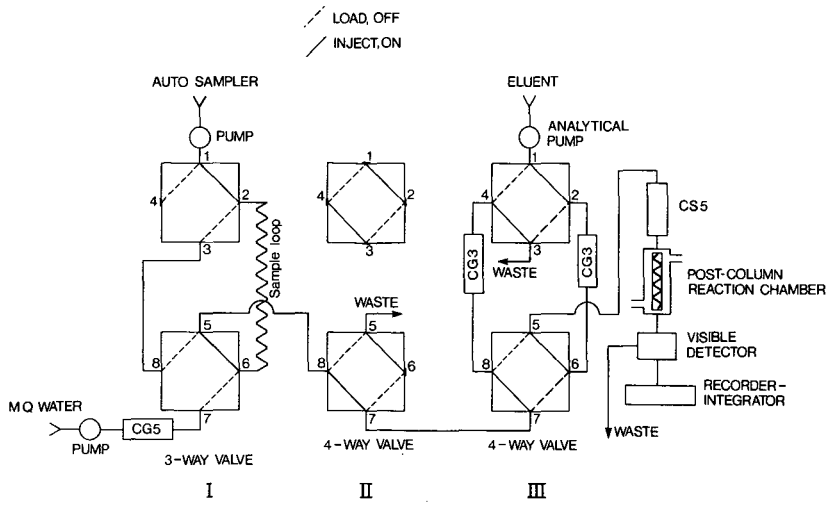

Fig. 1. Valve positioning for sample enrichment and injection, and system schematic. MQ = Milli-Q.

used in spectrophotometric studies of trace cadmium and vanadium[16–19]. PAR is used here for post-column chelating Cd^{2+} eluted from the cation-exchange column CS5 as labile Cd–$PDCA_n$ complexes. The stable chelate is most likely Cd · PAR_2 (ref. 20), perhaps formed by the following reaction

$$Cd\text{–}PDCA_2^{2-} + 2PAR\text{–}H = Cd \cdot PAR_2 + 2HPDCA^-$$

and is measured at 520 nm.

Choice of eluents and post-column reactants

Oxalic acid eluent was found to give very good sensitivity for Cd^{2+} determination but was incapable of separating Cd^{2+} from Mn^{2+}. It is therefore unsuitable in any water analysis of metals.

PDCA was found to be capable of separating Cd^{2+} and Mn^{2+} but several variations and combinations of eluent/post-column reactant had to be tested for desired sensitivity. The chemicals for various eluents were PDCA, lithium hydroxide, sodium chloride, sodium acetate, acetic acid and sodium sulphate. Those for various post-column reactants were PAR, ammonium hydroxide, acetic acid, sodium bicarbonate, sodium carbonate and dimethylaminoethanol (DMAE). The combination giving optimum sensitivity and reproducibility for Cd^{2+} was (PDCA + lithium hydroxide + sodium sulphate + sodium chloride)/(PAR + ammonium hydroxide + DMAE), as detailed in Table II.

Enrichment process

On-line enrichment was necessary to achieve sub-ppb Cd^{2+} levels. The sample is loaded and concentrated on CG3 in valve III OFF after the system has been flushed with same sample to avoid carryover (Fig. 1 and Table II). It was found that 10 ml of sample was enough to attain the desired sensitivity.

The enrichment process reveals that the background contamination can be

serious for several metals, most notably Zn^{2+}, giving variable baseline peaks. For ultra-trace works, a class 100 type environment is required to avoid this type of contamination. Our laboratory is not such an environment. Nevertheless, for sub-ppb Cd^{2+} levels and in this case also sub-ppb Co^{2+} and Mn^{2+} levels, the background contamination is not a problem as we get consistantly clean baselines.

Effect of sample acidity

Unacidified natural or standard samples are easy to analyze, giving well-defined chromatograms, but are in practice useless due to metal loss with storage time. Our common practice is to preserve water samples with 0.2% (v/v) nitric acid. But this acidity could elute Cd^{2+} from the CG3 concentration column and prevent concentration process. Thus an acidity effect was studied on Cd^{2+} along with Ni^{2+}, Co^{2+} and Mn^{2+}, and plotted in Fig. 2. It can be seen that the metals are well-retained at 0.2% and 0.5% nitric acid, but at 1.0% the acid starts acting as eluent, noticeably for Co^{2+} and Mn^{2+}.

The 0.2% acidity, however, upsets the behaviour of chromatograms, giving rise to negative dip at the early part of the elution pattern. Coupled with the background contamination mentioned above, the early peaks are a problem. Fortunately, this

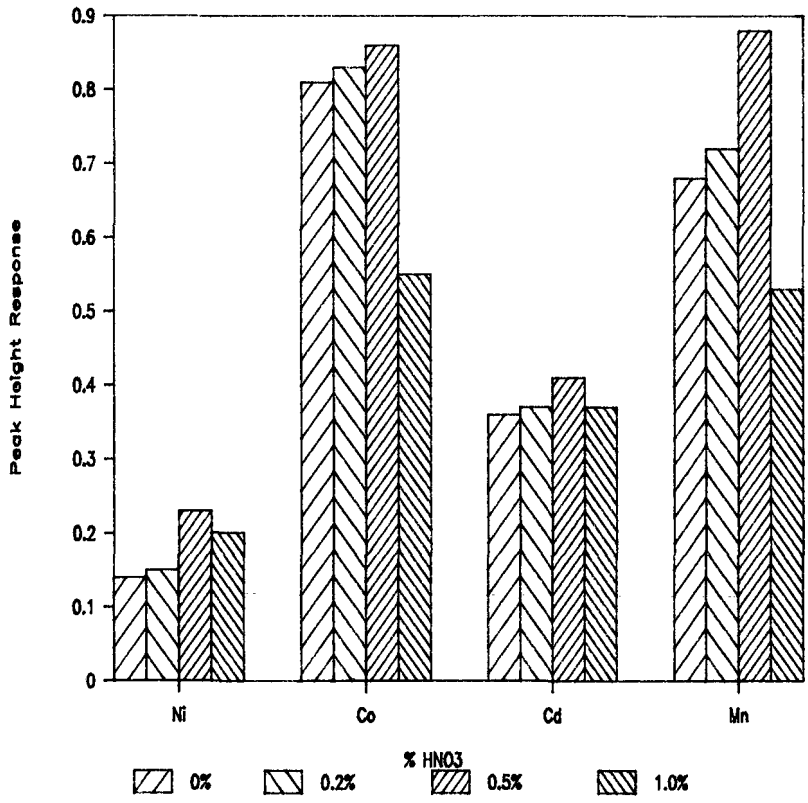

Fig. 2. Acid effect on response of Ni^{2+}, Co^{2+}, Cd^{2+} and Mn^{2+}.

effect does not significantly upset the Cd^{2+} peak; in fact, a rinse of CG3 with 3 ml of Milli-Q water ensures sensitive and reproducible Cd^{2+} peaks to meet our objective of 0.2 ppb cability (Table I). Furthermore, under these conditions Co^{2+} and Mn^{2+} peaks were also more reproducible than other metals, and thus were included in this study (Fig. 3). Rinsings with very dilute carbonate solutions, instead of water, were also tested but showed no advantages.

Fig. 3 is a chromatogram of a spiked precipitation sample from Quebec. Peaks 1–4 were identified by matching the retention times (in min) of the standard, the unspiked and the spiked sample as follows

Metal	*Standard*	*Unspiked*	*Spiked*
Zn^{2+}	7.94	8.03	8.03
Co^{2+}	8.78	Blank	8.84
Cd^{2+}	9.43	9.43	9.45
Mn^{2+}	10.52	10.53	10.51

Satisfactory recoveries of Co^{2+}, Cd^{2+} and Mn^{2+} (to be discussed below, Table VI) also confirm the peaks identification. Furthermore, the interference studies (discussed next) show no co-elution of other metals with the above peaks.

Interferences

As discussed above, the hydronium ion is an interferent at 1% concentration (pH 0.8). The concentration at which the ion starts interferring lies between 0.5–1.0%, which is well above our normal use of 0.2%.

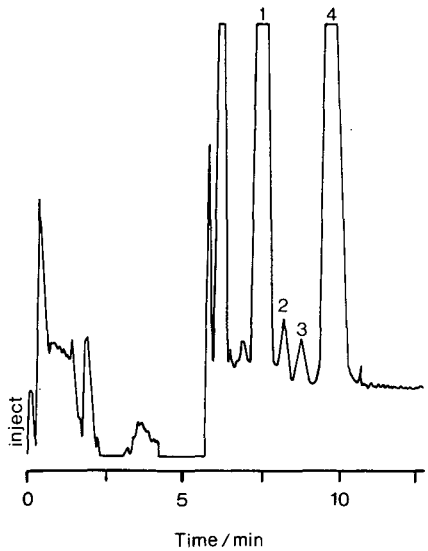

Fig. 3. Chromatogram of a spiked precipitation sample from Québec. Peaks: 1 = Zn^{2+}, *ca.* 60 ppb; 2 = Co^{2+}, 0.53 ppb; 3 = Cd^{2+}, 0.87 ppb; 4 = Mn^{2+}, 9.39 ppb. Conditions as in Table I.

A standard of each of the heavy metals routinely analyzed here by AAS (V^{5+}, Cr^{6+}, Mo^{6+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Al^{3+}, Pb^{2+}, As^{3+}, Se^{4+}) was first tested for identification of individual retention time. Ten metals were detected, and a mixture of them was analyzed in the same run, giving the following sequential retention times: Pb^{2+} 5.80 min, Fe^{3+} 6.10 min, Cu^{2+} 6.57 min, Ni^{2+} 7.27 min, Zn^{2+} 7.90 min, Co^{2+} 8.73 min, Cd^{2+} 9.30 min, Mn^{2+} 10.53 min, Fe^{2+} 11.45 min, and Hg^{2+} 14.16 min. Some other metals (Ag^{+}, Tl^{+}, Tl^{3+}, In^{3+}, Sn^{2+}, Sb^{3+}, Bi^{3+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, and La^{3+}) were then individually tested with the same mixture. No extra peak (except In^{3+} at 4.19 min) nor response increase in any of the existing peaks was observed, which indicates non-interference from any of these metals on the analysis of Co^{2+}, Cd^{2+}, and Mn^{2+}.

Zn^{2+} and Mn^{2+} concentrations are often higher than Co^{2+} and Cd^{2+} in waters and can become concentration interferents if present at much higher concentrations. However, 100 ppb Zn^{2+} and 100 ppb Mn^{2+} do not interfere with analysis of 0.5 ppb Co^{2+} and 0.5 ppb Cd^{2+}. In general, if the analytes are present in minute quantity relative to other metals eluting nearby, the concentration interferences can be a problem. This can, however, be solved by properly managing the waste solution from the detector. By identifying and discarding the interfering-fraction of the solution, one collects and re-analyzes the analyte-containing fraction.

Performance characteristics

Standard reference materials. A standard reference material, SRM 1643b (trace metals in water) of the National Bureau of Standards was used. The materials containing 0.5 *M* nitric acid had to be diluted to our normal working acid content of 0.2% nitric acid. The results found agree well with the certified values, taking dilution into account (Table III). Two spikes were also added to the diluted material and the recoveries were satisfactorily (Table IV). Calibration curves were used, one of which is shown in Fig. 4.

Quality control samples. Two samples, regularly used in our interlaboratory quality assurance (QA) programs, were tested. The first quality control sample, TM201, was prepared using Lake Ontario water for direct aspiration atomic absorption technique (high levels) and for solvent extraction technique after proper dilution (low levels).

The sample was diluted 200 times, reacidified to 0.2% nitric acid, and analyzed. The results found are in good agreement with the design values, which are the multi-laboratory and multi-study averages (Table V).

The second sample, synthetic rain A, which was specifically prepared for an acid

TABLE III

SRM 1643b RECOVERY RESULTS

Metal	*Certified concentration (ppb)*	*Recovery ± S.D. (n = 9) (ppb)*	*Recovery (%)*
Co^{2+}	1.69	1.64 ± 0.14	97
Cd^{2+}	1.32	1.30 ± 0.03	98
Mn^{2+}	1.82	1.83 ± 0.09	101

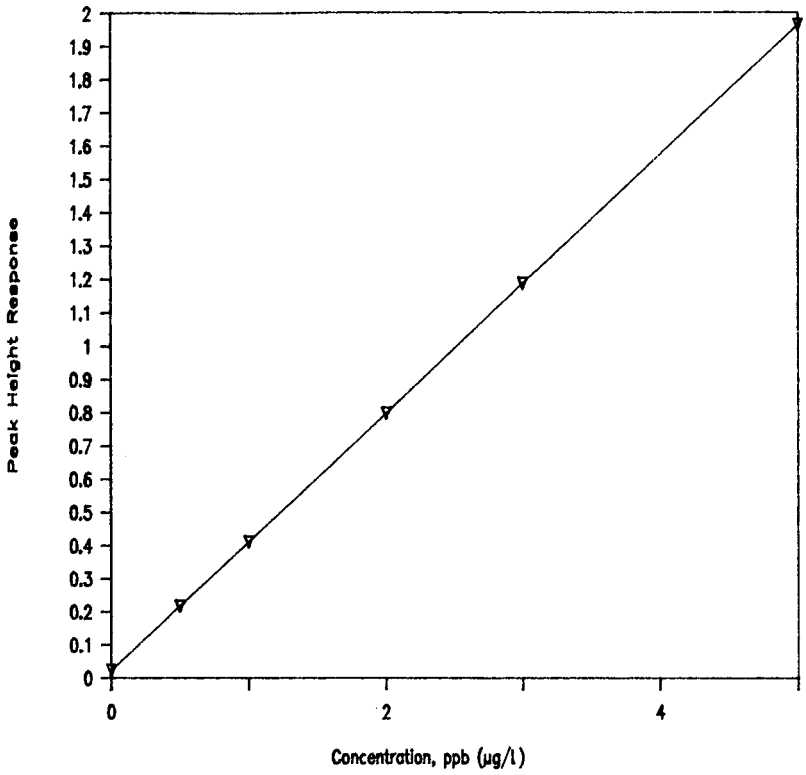

Fig. 4. Cd^{2+} calibration curve.

rain-related QA program (the Long Range Transport of Airborne Pollutants, LRTAP) was tested for the three metals. The results compared well with the median of 33 interlaboratory results (Table V)[21].

Precipitation samples. Two precipitation samples preserved with 0.2% nitric acid was analyzed for, and then spiked respectively with Co^{2+}, Cd^{2+} and Mn^{2+} as follows: 0.5, 0.5 and 2.0 ppb for Quebec sample; 0.5, 0.5 and 1.5 ppb for British Columbia sample. Table VI shows satisfactory recoveries and precision.

Analytical range. The detection limits under the given conditions are 0.1 ppb for each of the three elements and compare favourably with the revised detection limits of the AAS solvent extraction methods of 0.5, 0.1 and 1.0 ppb[7,8] and those of the

TABLE IV

RECOVERY OF TWO SPIKES ADDED TO SRM 1643b

Metal	*Total recovery ± S.D. (n = 5) from 1.5 ppb spike*	*Recovery (%)*	*Total recovery ± S.D. (n = 5) from 3.0 ppb spike*	*Recovery (%)*
Co^{2+}	3.13 ± 0.07	100	4.64 ± 0.23	99
Cd^{2+}	2.80 ± 0.11	99	4.34 ± 0.19	100
Mn^{2+}	3.23 ± 0.11	97	5.14 ± 0.20	107

TABLE V

RESULTS FOUND IN TWO QUALITY CONTROL SAMPLES

Metal	*TM 201*			*Synthetic rain A*		
	Design value (ppb)	*Found ± S.D. (n = 6)*	*Recovery (%)*	*Median value (ppb)*	*Found ± S.D. (n = 6)*	*Recovery (%)*
Co^{2+}	1.49	1.44 ± 0.11	97	1.10	1.16 ± 0.03	105
Cd^{2+}	0.49	0.50 ± 0.06	102	1.20	1.26 ± 0.05	105
Mn^{2+}	0.50	0.57 ± 0.07	114	5.55	5.63 ± 0.19	101

TABLE VI

RECOVERY DATA FOR TWO PRECIPITATION SAMPLES

$n = 5$.

Metal	*Quebec sample*			*British Columbia sample*		
	Original concentration found ± S.D. (ppb)	*Total recovery ± S.D. (ppb)*	*Recovery (%)*	*Original concentration found ± S.D. (ppb)*	*Total recovery ± S.D. (ppb)*	*Recovery (%)*
Co^{2+}	0.0	0.53 ± 0.01	106	0.55 ± 0.03	1.03 ± 0.02	98
Cs^{2+}	0.37 ± 0.03	0.87 ± 0.05	100	0.32 ± 0.05	0.82 ± 0.08	100
Mn^{2+}	7.26 ± 0.14	9.39 ± 0.26	101	1.51 ± 0.07	2.85 ± 0.06	95

interlaboratory "specification" studies of 2.0, 1.0, 2.0[22] for Co^{2+}, Cd^{2+}, and Mn^{2+}, respectively.

As this work centers around low- and sub-ppb analyses, the upper limit was not studied. This limit, however, should be quite high since concentrated samples can be easily dealt with by decreasing the concentrating volume and/or by sample dilution.

Advantages and disadvantages. The automation, on-line concentration and simultaneous determination is a definite advantage over the flame and graphite AAS. If a lower detection limit is required, one simply on-line concentrates more than 10 ml of sample. For AAS and ICP on the other hand, routine analysis of low-level metals first requires separate preconcentration of 100 ml sample by solvent extraction or evaporation technique. The IC is also relatively cheap to operate.

Although it is a good complementary method to AAS and ICP, the somewhat slow analysis time of IC would be a disadvantage in routine use when compared to ICP.

CONCLUSIONS

A sensitive IC method has been developed for simultaneous determination of low- and sub-ppb Co^{2+}, Cd^{2+} and Mn^{2+} in ditute water samples. It is fully automated and is a good complementary method to AAS and ICP.

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USE OF CYCLODEXTRINS IN ISOTACHOPHORESIS

VI. CYCLODEXTRINS AS LEADING ELECTROLYTE ADDITIVES FOR THE SEPARATION OF BILE ACIDS

JIŘÍ SNOPEK* and EVA SMOLKOVÁ-KEULEMANSOVÁ
Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
IVAN JELÍNEK and JIŘÍ DOHNAL
Research Institute for Pharmacy and Biochemistry, Kouřimská 17, 130 60 Prague 3 (Czechoslovakia)
and
JIŘÍ KLINOT and EVA KLINOTOVÁ
Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
(Received June 1st, 1988)

SUMMARY

Cyclodextrins (CDs) and some of their methyl derivatives have been used for the optimization of the isotachophoretic separation of bile acids in aqueous electrolyte systems. The addition of heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin to the leading electrolyte proved useful for both the solubilization and the structural differentiation of the solutes studied and led to the successful separation of their mixtures. Other CDs tested, even if they gave a satisfactory solubilization effect, did not support the resolution of bile acid mixtures.

INTRODUCTION

Bile acids are biologically active compounds synthesized from cholesterol in the liver and secreted into the bile. They have been widely studied from the clinical and pharmacological point of view. A close connection between serious hepatobiliary diseases and bile acid metabolism failure was found. Some of bile acids proved to be very active gallstone-dissolving substances which could be potentially utilized in human medicine. Especially this fact initiated great progress in the isolation and synthesis of bile acids, and a number of pure and stable semi-synthetic compounds with high biological and clinical activity have been successfully prepared.

The monitoring of the synthesis of bile acids and their determination in biological materials makes heavy demands on the choice of a suitable analytical technique, because of the small structural differences between individual bile acid derivatives. Capillary isotachophoresis (ITP) in non-aqueous solvents has been successfully introduced into bile acid analysis[1]. The use of a non-aqueous medium solves especially the problems concerning the low solubility of bile acids in aqueous

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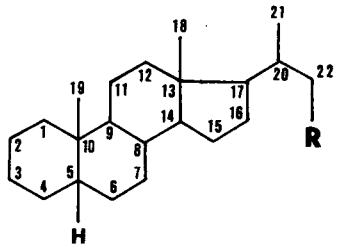
TABLE I

STRUCTURAL FORMULAE OF THE COMPOUNDS INVESTIGATED



Compound	*Substituents in position*			*R*	*Name*
	3	*7*	*12*		
I	HO(α), H	HO(α), H	HO(α), H	CH_2COOH	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (cholic acid)
II	HO(α), H	HO(α), H	H, H	CH_2COOH	3α,7α-Dihydroxy-5β-cholan-24-oic acid (chenodeoxycholic acid)
III	HO(α), H	HO(β), H	H, H	CH_2COOH	3α,7β-Dihydroxy-5β-cholan-24-oic acid (ursodeoxycholic acid)
IV	HO(α), H	H, H	HO(α), H	CH_2COOH	3α,12α-Dihydroxy-5β-cholan-24-oic acid (deoxycholic acid)
V	HO(α), H	H, H	H, H	CH_2COOH	3α-Hydroxy-5β-cholan-24-oic acid (lithocholic acid)
VI	HO(α), H	O=	H, H	CH_2COOH	3α-Hydroxy-7-oxo-5β-cholan-24-oic acid
VII	O=	O=	O=	CH_2COOH	3,7,12-Trioxo-5β-cholan-24-oic acid (dehydrocholic acid)
VIII	O=	O=	H, H	CH_2COOH	3,7-Dioxo-5β-cholan-24-oic acid
IX	O=	H, H	O=	CH_2COOH	3,12-Dioxo-5β-cholan-24-oic acid
X	O=	H, H	H, H	CH_2COOH	3-Oxo-5β-cholan-24-oic acid (oxolithocholic acid)
XI	O=	O=	H, H	COOH	3,7-Dioxo-24-nor-5β-cholan-23-oic acid
XII	HO(α), H	HO(α), H	H, H	COOH	3α,7α-Dihydroxy-24-nor-5β-cholan-23-oic acid
XIII	$OCOCH_3$(α), H	$OCOCH_3$(α), H	H, H	COOH	3α,7α-Diacetoxy-24-nor-5β-cholan-23-oic acid
XIV	$OCOCH_3$(α), H	O=	H, H	CH_2COOH	3α-Acetoxy-7-oxo-5β-cholan-24-oic acid
XV	HO(α), H	$OCOCH_3$(α), H	H, H	CH_2COOH	3α-Hydroxy-7α-acetoxy-5β-cholan-24-oic acid
XVI	HO(α), H	HO(α), H	H, H	C(OH)HCOOH	3α,7α,23R-Trihydroxy-5β-cholan-24-oic acid (β-phocaecholic acid)
XVII	$OCOCH_3$(α), H	$OCOCH_3$(α), H	H, H	$CH(OCOCH_3)COOH$	3α,7α,23R-Triacetoxy-5β-cholan-24-oic acid

solutions and specific interactions with an organic solvent ensure a satisfactory resolution.

The aim of this paper is to propose an aqueous electrolyte system that could be used in commercially available equipment made from Perspex. The search for an aqueous electrolyte system suitable for the resolution of 17 investigated bile acid standards is based on a study of cyclodextrin complex formation, which has proved many times to be the decisive factor in altering the resolution of structurally related compounds and isomers in ITP[2–6].

EXPERIMENTAL

Chemicals

Doubly distilled water from a quartz apparatus was used in the preparation of the solutions of the electrolytes and compounds investigated.

All chemicals were of the highest quality commercially available: 37% hydrochloric acid, tris(hydroxymethyl)aminomethane (Tris), histidine (His) (Merck, Darmstadt, F.R.G.); 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (Calbiochem, Lucerne, Switzerland); Natrosol 250 HR (hydroxyethylcellulose, HEC) (Hercules, Wilmington, DE, U.S.A.); Zerolit DM-F (indicator) (BDH, Poole, U.K.); α- and γ-cyclodextrins (α- and γ-CD) (Astec, Whippany, U.S.A.); β-cyclodextrin (β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (dimethyl-β-CD) and heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (trimethyl-β-CD) (Chinoin, Budapest, Hungary).

Cyclodextrins and Natrosol 250 HR solutions were purified using Zerolit DM-F mixed bed ion-exchange resin. Histidine was recrystallized from methanol–water (1:1) solution.

The solutes investigated were provided by laboratories of the Department of Organic Chemistry, Charles University. The formulae and numbering are given in Table I. Sample solutions of 2 mg/ml were prepared by dissolving each in *ca.* 1% Tris and were stored in dark bottles in a refrigerator.

Methods

Isotachophoretic experiments were performed using a Tachophor 2127 (LKB, Bromma, Sweden), equipped with a conductivity detector and a poly(tetrafluorethylene) capillary, modified for work under a nitrogen atmosphere. The sample solution was injected with a 10-μl microsyringe (Hamilton, Bonaduz, Switzerland). The operating conditions are given in Table II.

TABLE II

ELECTROLYTE SYSTEMS AND CONDITIONS FOR ITP

Parameter	*Condition*
Leading electrolyte	5 m*M* hydrochloric acid containing 0.4% HEC with His to pH 6.4
Terminating electrolyte	10 m*M* HEPES with Tris to pH 8.3
Capillary	(I) 230 mm × 0.55 I.D.; (II) 370 mm × 0.55 mm I.D.
Current	100 μA (for capillary I 5 min and for capillary II 7 min); for detection 50 μA
Thermostat temperature	18°C
Detection	Conductivity

TABLE III

$(h_i)_{rel}$ VALUES OF THE COMPOUNDS INVESTIGATED

The separation was effected using capillary I. $(h_i)_{rel} = (h_i - h_l)/(h_t - h_l)$, where h_i, h_l and h_t = step height of sample, leading electrolyte and terminating electrolyte, respectively.

Compound	*Leading electrolyte additive*									
	None	*4mM α-CD*	*2mM β-CD*	*4mM β-CD*	*2mM γ-CD*	*4mM γ-CD*	*2mM dimethyl-β-CD*	*4mM dimethyl-β-CD*	*2mM trimethyl-β-CD*	*4mM trimethyl-β-CD*
I	0.447	0.443	0.682	0.704	0.671	0.805	0.715	0.853	0.450	0.478
II	–	–	0.709	0.708	0.703	0.816	0,895	0.925	0.712	0.823
III	–	–	0.726	0.728	0.714	0.830	0.843	0.868	0.827	0.893
IV	–	–	0.756	0.789	0.695	0.809	0.882	–	0.475	0.551
V	–	–	0.794	0.824	0.737	–	–	–	–	–
VI	0.446	0.449	0.705	0.704	0.692	0.815	0.856	0.868	0.753	0.831
VII	0.396	0.402	0.663	0.670	0.521	0.714	0.765	0.869	0.397	0.404
VIII	0.444	0.478	0.782	0.785	0.747	0.744	0.875	0.878	0.664	0.753
IX	0.407	0.408	0.724	0.758	0.681	0.807	0.859	–	0.450	0.526
X	–	–	–	0.806	0.729	0.769	0.734	0.817	–	–
XI	0.420	0.420	0.765	0.782	0.703	0.760	–	–	0.475	0.533
XII	0.477	0.470	0.721	0.737	0.723	0.778	–	–	0.561	0.668
XIII	–	–	0.748	0.757	0.726	0.817	–	–	–	–
XIV	0.448	0.488	0.700	0.697	0.709	0.805	0.860	0.874	0.769	0.823
XV	–	–	0.695	0.692	0.713	0.833	0.854	0.858	0.807	0.861
XVI	0.437	0.442	0.683	0.684	0.691	0.808	0.760	0.765	0.648	0.731
XVII	–	–	0.701	0.703	0.752	0.842	0.784	0.808	0.490	0.558

RESULTS AND DISCUSSION

Choice of electrolyte system

The physico-chemical properties of bile acids, especially their low solubility in water and high molecular weight, complicate the choice of a suitable electrolyte system. The pK_a values of the investigated compounds lie between 5 and 7, depending on their structures. Preliminary experiments confirmed that the pH of the leading electrolyte must be higher than 5 to ensure sufficient dissociation and effective mobility. In the pH range 6–7 HEPES as one of the slowest anionic terminators works well and ensures a correct ITP migration order. In more alkaline electrolyte systems there is a lack of suitable slow migrating terminators.

Measurements with the electrolyte system described in Table II require the protection of the terminator against absorption of carbon dioxide. The amount of carbonate in the alkaline terminator increases during the measurement in opened electrolyte vessels. Therefore, it is necessary to preserve the terminator under nitrogen or to modify the pH of HEPES solution with barium hydroxide.

Effect of cyclodextrins on the resolution of the solutes studied

Data obtained for single solutes, expressed as relative step height [$(h_i)_{rel}$] values, are summarized in Table III. Almost half of the compounds studied do not migrate in an aqueous electrolyte system without CD because of precipitation in a neutral medium during the analysis. The same results were obtained when α-CD was added to the leading electrolyte. It was shown that, in agreement with theory, α-CD is not able to form stable complexes with bulky bile acid molecules and therefore the presence of α-CD in the leading electrolyte does not support the solubility of studied compounds in aqueous solution.

The situation changes when β-CD is used as an additive in the leading electrolyte. A strong retardation effect indicates stable complex formation. The problems caused by insufficient solubility of solutes are completely overcome and all of the compounds studied are able to migrate isotachophoretically and provide stable zones. In spite of the lower stability of inclusion complexes formed with γ-CD, the solubilization effect of this most spacious cyclodextrin is of the same efficiency as that of β-CD. Comparable results were obtained with further studied CD derivatives. Partially methylated dimethyl-β-CD, which forms the most stable inclusion complexes with the compounds studied, does not support, however, the solubility of solutes V and XIII and, moreover, the addition of trimethyl-β-CD does not effect the solubilization of solute X.

The results summarized in Table III give a general view of the possible resolution of the bile acids studied. Relatively high and similar $(h_i)_{rel}$ values obtained for measurements with the dimethyl-β-CD-modified leading electrolyte indicate the formation of strong inclusion complexes, the stability of which does not depend significantly on the structural differences of the solutes. Therefore, dimethyl-β-CD, although providing good solubilization effects, could not be used effectively for the resolution of the range of bile acids studied.

In contrast to dimethyl-β-CD, the structural differentiation of the compounds studied, using β-CD- or γ-CD-modified leading electrolytes, seems to be more efficient, the $(h_i)_{rel}$ values obtained for many solutes differing substantially. The ITP measure-

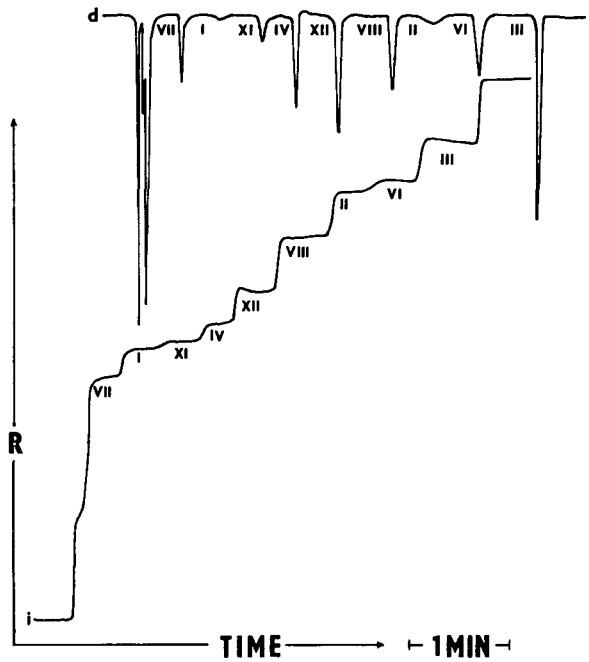


Fig. 1. Separation of bile acid mixture. Leading electrolyte containing 4 m*M* trimethyl-β-CD; capillary II (Table II). Numbering of acids as in Table I. *R* = Response of detector; *i* = conductivity; *d* = differential conductivity.

ments with a mixture of the compounds investigated, however, provided almost unresolved zones with poor boundary sharpness. The systems with β-CD and γ-CD, although providing satisfactory solubilization effects, do not have any practical significance for bile acids analysis.

The explanation of the loss of the separation power, which does not result simply from ITP experiments probably lies in the stoichiometry of the inclusion complex formation. It must be emphasized that the host–guest system utilized is very complicated. The bile acids studied are known as a special type of surfactants with the ability to aggregate in aqueous solution. Their fundamental cholane skeleton provides a number of binding sites for the complex-forming cyclodextrin. Therefore, we can hardly suppose that simple complex formation occurs. The statistical creation of a mixed polycentral inclusion complex, especially when the host cavity–guest interaction is not so tight, may substantially alter and complicate fast complex-forming equilibria established during the ITP run.

The largest $(h_i)_{rel}$ values and broadening effect for the compounds studied were observed with trimethyl-β-CD. We succeeded in resolving a mixture of nine bile acids using 4 m*M* trimethyl-β-CD in the leading electrolyte (Fig. 1). The resolution of the corresponding keto and hydroxy acids (solutes I, VII or II, III, VI, VIII) and the possibility of differentiation of nor-acids (solutes II, XII or VIII, XI) have practical significance especially for the monitoring of the synthesis. From the analytical point of view, the most interesting is the resolution of diastereomers II and III.

CONCLUSIONS

The experiments confirmed that the problem of the ITP of bile acids in aqueous electrolyte systems could be solved successfully by the addition of trimethyl-β-CD to the leading electrolyte, which proved to be useful for both the solubilization and the structural differentiation of the solutes studied. Other cyclodextrins tested, even if they gave an excellent solubilization effect, failed completely as structural discriminators.

The suggested aqueous electrolyte system, which could be used preferentially in a commercially available Perspex apparatus, enables a wide range of structurally related bile acids to be analysed without any pretreatment. The successful resolution of positional isomers and diastereomers confirmed the high resolving power of the suggested electrolyte system, which could be utilized especially for the analytical monitoring of bile acid synthesis.

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ANALYSIS OF SALMONELLA LIPOPOLYSACCHARIDES BY SODIUM DEOXYCHOLATE–POLYACRYLAMIDE GEL ELECTROPHORESIS

TETSUO KOMURO and CHRIS GALANOS*
Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg (F.R.G.)
(Received June 16th, 1988)

SUMMARY

Lipopolysaccharides (LPSs) were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium deoxycholate (DOC) or sodium dodecyl sulphate (SDS) and the results obtained compared. Two-dimensional PAGE of the S form of LPS revealed that, with SDS, bands which appeared to be single after the one-dimensional experiment were resolved into several bands after two-dimensional electrophoresis. In the presence of DOC, however, a diagonal of single bands was obtained in one-dimensional electrophoresis indicating optimum resolution. The high quality of resolution by DOC-PAGE was constant for amounts of LPS up to 20 μg. Finally, DOC-PAGE does not require boiling of the samples in DOC, which may be an advantage over SDS-PAGE.

INTRODUCTION

The lipopolysaccharides (LPSs) of Gram-negative bacteria are chemically heterogeneous mainly because of variations in the number of repeating units in the O-polysaccharide. Further heterogeneity may also result from the presence of an incomplete core and from a non-stoichiometric substitution of lipid A[1–5]. Among the different methods used to separate the many subclasses of molecules present in an LPS preparation, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)[1–3,6,7] is a simple useful tool. It is also very sensitive because the silver staining method[8] used detects less than 1 μg of LPS. In one-dimensional SDS-PAGE, separation of the S form of LPS usually results in a ladder-like banding pattern showing 30–40 gray and red doublet bands depending on the LPS used. However, two-dimensional SDS-PAGE showed an aberrant migration pattern with faster and slower bands, although each band in the first dimension appeared to be single. Further, in the case of the R form or LPS, SDS-PAGE revealed a few intense bands with a long smearing and/or tailing[6,9]. These findings suggested that SDS does not completely dissociate the LPS aggregates.

In the present study we investigated the effect of sodium deoxycholate (DOC) on the migration pattern of LPSs during PAGE. DOC is a well known surfactant and has often been used for dispersing LPSs because of its strong surface activity. It will

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be shown that DOC-PAGE is usefool tool for the molecular characterization of LPSs and that it has certain advantages over SDS-PAGE.

MATERIALS AND METHODS

Lipopolysaccharides

LPS from *Salmonella abortus equi* (S form) was isolated and purified as described[10]. The SR form of LPS from *Salmonella typhimurium* and the R form of LPS from *Salmonella minnesota* mutants, R60 (Ra), R345 (Rb), R5 (Rc), R7 (Rd_1), R3 (Rd_2), and R595 (Re), were isolated from bacteria and purified by the phenol–chloroform–light petroleum method[11]. All preparations were free of protein and other macromolecular contaminants.

SDS- and DOC-PAGE

Electrophoresis was performed with the buffer system of Laemmli and Favre[12], where, unless otherwise stated, separating gels were formed with 14% acrylamide and 1% SDS with a 4% acrylamide stacking gel. Samples were mixed 1:1 with sample buffer (containing 4% SDS) and boiled for 3 min. Aliquots of 1–10 μl were applied onto the gels. Electrophoresis was performed with Tris–glycine buffer containing 0.1% SDS, with a constant current of 18 mA until the tracking dye entered the separating gel and at 35 mA until the tracking dye reached the bottom of the gel. Pre-electrophoresis was not carried out.

In DOC-PAGE, unless otherwise stated, separating gels were formed with 14% acrylamide and 0.9% DOC with a 4% acrylamide stacking gel (containing 0.5% DOC). Samples were mixed 1:1 with sample buffer containing 1.0% DOC, 0.1 *M* Tris–glycine buffer, pH 6.8 and 20% glycerol and 1–2o μl aliquots were applied onto the gels. Where necessary, the samples were sonicated for 1 min to obtain complete dispersion. Some samples were boiled for 1 or 3 min to investigate the influence of boiling but unless otherwise stated, samples were not boiled. The buffer contained 0.25% DOC.

Two-dimensional electrophoresis was carried out as follows: a lane from the one-dimensional slab gel containing the S form of LPS was cut out immediately after the analysis and placed directly on the stacking gel of a new gel and subsequently electrophoresed in the second dimension as described above.

Molecular weight markers including phosphorylase (94 000 daltons), bovine serum albumin (67 000 daltons), ovalbumin (43 000 daltons), soy bean inhibitor (20 100 daltons) and lysozyme (14 400 daltons) were obtained from Bio-Rad Labs. Some gels were stained with Coomassie Brilliant Blue R-250 according to the conventional method. LPS bands were detected by the silver staining method of Tsai and Frasch[8].

RESULTS

Several *Salmonella* LPSs were subjected to electrophoresis in 14% polyacrylamide gel in the presence of SDS or DOC, and visualized by silver staining as described in Material and methods. The results obtained are shown in Fig. 1A and B. The SDS-PAGE migration patterns of these LPSs were in principle similar to those

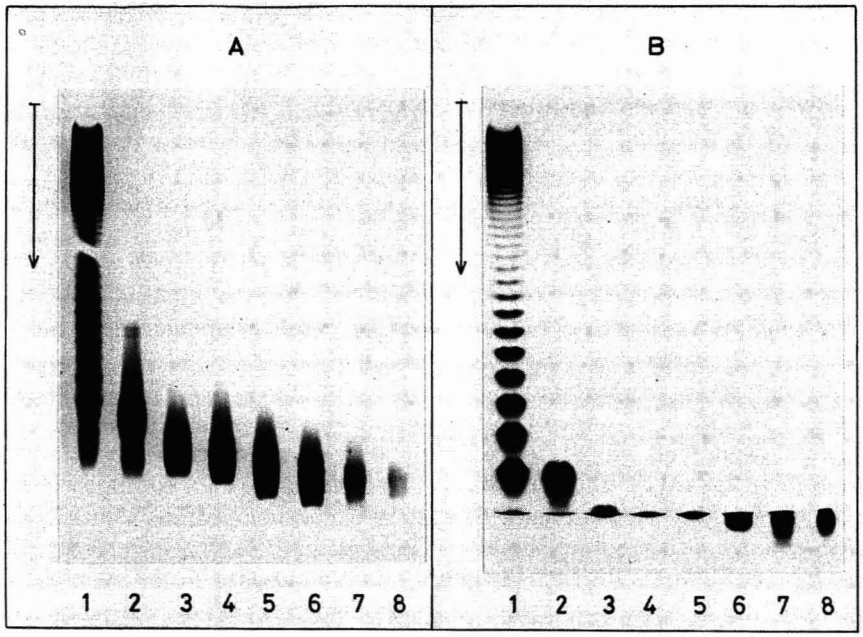

Fig. 1. Migration patterns of purified LPS in 14% polyacrylamide gels in the presence of SDS (A) or DOC (B). LPSs in the gels were detected by silver staining. The amounts of LPS applied in both A and B were 7 μg (S), 1 μg (SR, Ra, Rb, Rc, Rd_1 and Rd_2) and 3 μg (Re). Lanes: 1 = *S. abortus equi* S form; 2 = *S. typhimurium* SR form; 3–8 = *S. minnesota* Ra, Rb, Rc, Rd_2 and Re, respectively. The split of the gel in A is artificial.

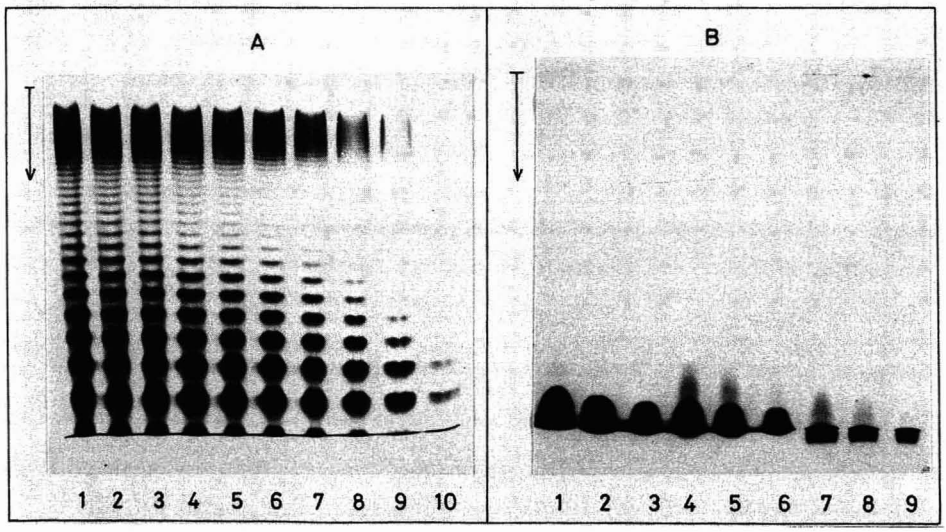

Fig. 2. DOC-PAGE of purified LPS from Salmonella strains. LPSs in the gels were detected by silver staining. Lanes: A: 1–10 = *S. abortus equi*, 20, 18, 16, 14, 12, 10, 8, 6, 4 and 2 μg, respectively; in B: 1–3 = *S. minnesota* Ra 9, 6 and 3 μg, respectively; 4–6 = *S. minnesota* Rc, 9, 6 and 3 μg, respectively; 7–9 = *S. minnesota* Re, 9, 6 and 3 μg, respectively.

reported previously by other investigators[2,3,6,8,9]. The DOC-PAGE migration patterns of the same LPSs were, however, apparently different from those of the SDS-PAGE. The S form of LPS of *S. abortus equi* exhibited approximately 32 regular bands with one compact dense band near the front of the gel, which was similar to the chemotype Ra (*S. minnesota* R-60). Three chemotypes, Rd (*S. minnesota* R7), Rd_2 (*S. minnesota* R3) and Re (*S. minnesota* R595), seemed to have two bands.

Further, the doublet pattern observed in SDS-PAGE was not seen in DOC-PAGE with any of the LPSs examined. In addition, the migration patterns were compact and very reproducible with amounts of LPS from 2 to 20 μg (Fig. 2A, B).

The relationship between the migration distance and the molecular weight among R chemotypes was more evident using a 17.5% polyacrylamide gel, as shown in Fig. 3.

For two-dimensional electrophoresis, one lane containing S LPS from the gel shown in Fig. 1A and B was excised before fixation and staining, and subsequently subjected to electrophoresis. The SDS-PAGE migration pattern obtained was similar to that reported previously by Hitchcock[13]. On the other hand, the two-dimensional DOC-PAGE showed the original migration pattern which was obviously a series of single bands forming a diagonal, as shown in Fig. 4.

In the case of SDS-PAGE, LPS samples were usually boiled for a few minutes to dissociate the LPS aggregates completely. To examine the effect of heating on the migration patterns of LPS, in the DOC-PAGE system, LPS samples were boiled for 1 or 3 min, but the migration patterns of all *Salmonella* LPS chemotypes examined under the experimental conditions used were not changed (data not shown). To examine the effect of DOC on the migration behaviour of proteins in DOC-PAGE, marker proteins with molecular weights of 14 400–94 000 daltons were electropho-

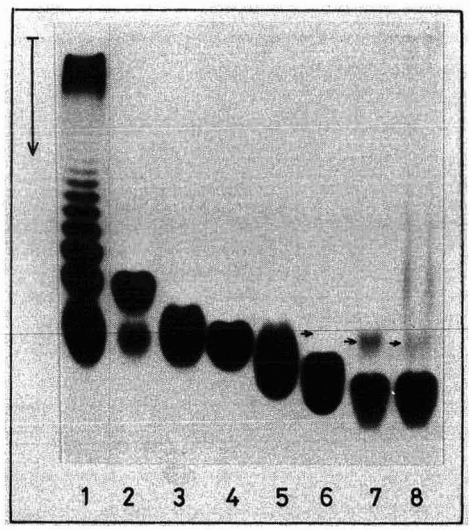

Fig. 3. Migration patterns of purified LPS in 17.5% polyacrylamide gel in the presence of DOC. LPSs were detected by silver staining. The amounts of LPS applied to the gel were 7 μg (lane 1: S) and 1 μg (lanes 2–8: SR, Ra, Rb, Rc, Rd_1, Rd_2 and Re). Electrophoresis was carried out at 4 mA for about 16 h. Arrows indicate weak bands which were not clear in the photograph.

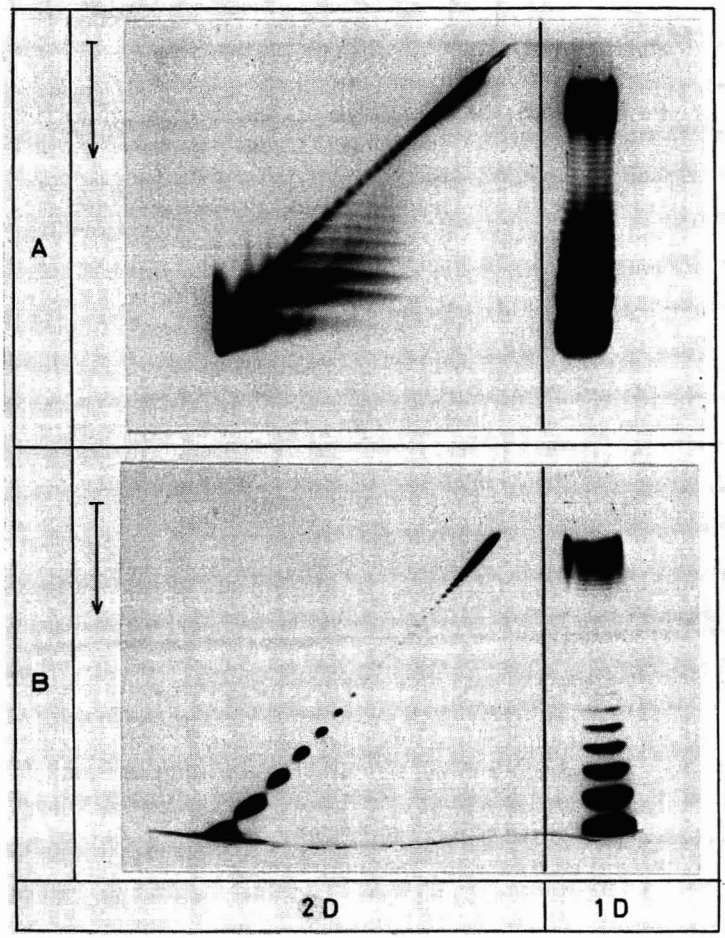

Fig. 4. Two-dimensional (2D) electrophoresis of purified LPS from *Salmonalla abortus equi* in the presence of SDS (A) or DOC (B). LPSs were detected by silver staining. In both A and B, 7 μg LPS included in each lane excised from the one-dimensional slab gel were subjected to two-dimensional electrophoresis in 13% polyacrylamide gels. The dark-staining region at the bottom of gel B is artificial due to incomplete removal of DOC during the washing procedure prior to staining. 1D = First dimension.

resed by both methods and the results obtained are shown in Fig. 5. The relative migrations of the different proteins to one another were similar in both systems but the bands obtained by DOC-PAGE were more compact than the corresponding ones in SDS-PAGE.

DISCUSSION

The separation of the S form of LPS by DOC-PAGE resulted in a ladder-like banding pattern, apparently reflecting the variation in the number of O antigen repeating units. This is generally consistent with the findings of previous studies[2,3,6,8,9] with the SDS-PAGE system except that, here, each band was single. The migration

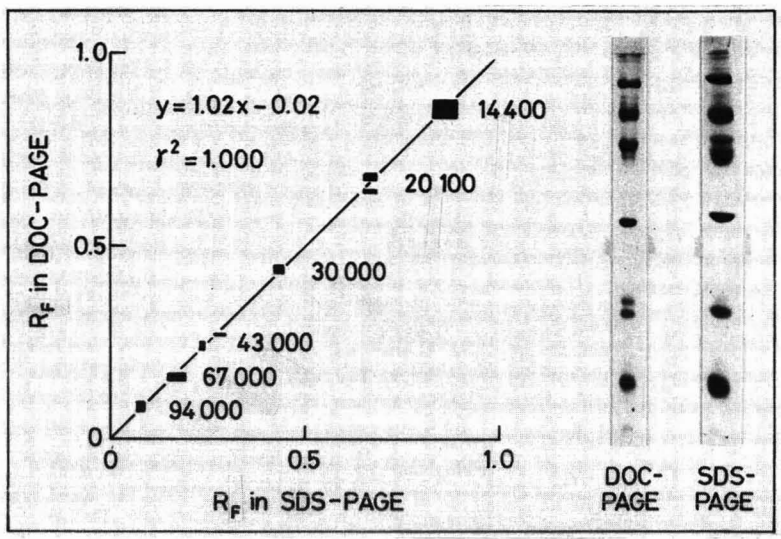

Fig. 5. Migration patterns of low-molecular-weight proteins in 13% polyacrylamide gel in the presence of SDS (A) or DOC (B). Proteins were stained by Coomassie Brilliant Blue R-250. The amounts of proteins applied to the gels were 10 μg in each case.

patterns of R chemotypes were characteristic and the migration distances of the main bands corresponded well to the expected molecular weights. In addition, each R chemotype LPS used in the present experiment migrated more quickly than or at least at nearly the some rate as the most rapid band of S chemotype LPSs, in contrast to previous reports[6,9]. In the SDS-PAGE system a common observation reported in several studies is that apparently single bands obtained after one-dimensional electrophoresis were resolved into several hands during two-dimensional electrophoresis. This problem was overcome by Hitchcock[13] by applying a pre-electrophoresis step on the gel prior to the one- and two-dimensional experiments. The aberrant migration pattern of LPS in the two-dimensional electrophoresis, which indicates an incomplete resolution during the one-dimensional experiment, was suggested to be due to the dissociation of SDS from LPS during electrophoresis, resulting in the formation of both slower and faster migration bands[13] and/or to be the result of the formation and dissociation of LPS multimers immediately before or during electrophoresis[14]. The migration pattern of LPS was also suggested to be influenced by the concentration of both, LPS and SDS[14].

The above problems were not encountered during DOC-PAGE. In the two-dimensional electrophoresis, the LPS separation was optimum, resulting in a diagonal series of single bands, without the need for pre-electrophoresis. Further, amounts up to 20 μg of the S form or 9 μg of the R form LPS had no apparent influence on migration patterns.

We believe that the concentration of 0.1% SDS often used in SDS-PAGE may not be high enough to prevent the formation of multimers, or to dissociate the LPS aggregates completely as suggested previously[14]. Average molecular weights of the

SDS-dissociated LPS obtained by the sedimentation equilibrium method were found to be larger than those of DOC-dissociated LPS[15].

Unlike SDS-PAGE, in the DOC-PAGE system, samples do not require boiling in sodium deoxycholate prior to electrophoresis, which may be an advantage especially when analyzing preparations of unknown structure. As shown here, DOC-PAGE may also be employed for protein analysis.

We believe that DOC-PAGE is a useful tool for analyzing the heterogeneity of LPS and has several advantages over SDS-PAGE. It should be mentioned however that the single bands obtained by DOC-PAGE do not necessarily represent an homogeneous population of identical LPS molecules. The presence of a microheterogeneity resulting from the presence or absence of a single low-molecular-weight substituent such as an ethanolamine residue or an acetyl or phosphate group may not be large enough to be discriminated by the present system. The characterization of single-band patterns in DOC-PAGE is now in progress.

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Note

Open split interfaces in capillary gas chromatography–mass spectrometry

Yield and quantitative aspects

N. W. DAVIES
Central Science Laboratory, University of Tasmania, P.O. Box 252C, Hobart, Tasmania 7001 (Australia)
(First received December 14th, 1987; revised manucript received June 9th, 1988)

One of the most common methods of coupling capillary gas chromatography (GC) columns to mass spectrometers is the open split interface first described by Henneberg *et al.*[1]. These devices usually employ a capillary restrictor tube which governs the amount of gas allowed into the mass spectrometer, with provision for venting any excess gas to atmosphere or a second detector. There is also usually provision for one or two "make-up" or "purge" gas lines to supplement column flow where necessary, to sweep dead volumes within the interface and to enable "cutting" of large GC peaks away from the mass spectrometer to avoid contamination[2–5]. Under these conditions the column exit is at atmospheric pressure and hence combined GC–mass spectrometry (MS) results can be directly compared with those from normal GC, since no change in retention times or resolution will have occurred for the same flow-rates. These interfaces also facilitate the rapid exchange of columns as the mass spectrometer is not directly exposed to atmospheric pressure, and enable columns requiring relatively high flow-rates to be used in conjunction with mass spectrometers of limited pumping capacity.

However, there are two related potential problems when open split interfaces are used in conjunction with isobaric pressure control of the GC column flow-rate and temperature programming, a combination found on several commercial instruments. The first is that if the interface is independently heated at a constant temperature, the "yield", defined as the proportion of any given compound that enters the mass spectrometer, depends on the elution temperature of the compound. This is evident since while the total flow-rate through the column will fall substantially as column temperature rises[6], the flow to the mass spectrometer will be fixed. As a consequence of this, it is possible for there to be insufficient gas at higher column temperatures if flow-rates are not established at the maximum operating column temperature, or appropriate allowances are not made. This results in an influx of air into the interface, which will be potentialy damaging to both the interface and the mass spectrometer.

Quantitative work based on standard curves of the analytes is not affected by the variation in yield provided the same temperature profile is used for standards and samples. Direct quantitative or semiquantitative comparisons of different peaks

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within a temperature programmed analysis will however require appropriate correction factors to be applied. Direct semiquantitative analysis of different compounds in a mixture by comparison of total ion currents is of course complicated by the different ionization cross sections of each compound. Nevertheless compounds in a homologous series such as alkanes will have a total ion current proportional to the total weight of sample and independent of the molecular weight of the individual species[7]. Useful semiquantitative data are then available from the mass spectrometer provided corrections for interface yield are made. If total ion current is indirectly determined by software from the summation of electron multiplier response for all ions in a given compound, then the dependence of the multiplier gain on ion size and structure further complicates direct comparisons. Notwithstanding the complications of total ion current, quantitative comparisons can be readily made using another detector monitoring the flow from the open split vent after appropriate corrections.

Operation in this mode also means that the yield to the mass spectrometer can only be maximized at the high temperature end of the analysis, causing losses in sensitivity by up to a factor of three for the earlier eluting components.

The following discussion presents the appropriate correction factors required to compensate for change in yield, and describes different methods for obtaining constant and maximum yield from an open split interface.

EXPERIMENTAL

The experimental results were obtained on an HP 5890A gas chromatograph with a 25 m × 0.32 mm I.D. fused-silica column installed, coupled to an HP 5970 mass selective detector with an open split interface. The interface was held at 270°C and the initial oven temperature (T_a) was 27°C. Flow-rates were measured at room temperature with a 1-ml soap bubble flow meter (Alltech) after the GC oven had equilibrated at the individual temperatures, with the average of three separate measurements being used in each case. The column flow-rate was measured only at 27°C (*i.e.* F_a), and the split vent flow-rate (F_{sa} and F_{sb}) at all indicated temperatures. F_m was determined from the difference between F_a and F_{sa} with no purge gas. F_p was determined from the difference between values of F_{sa} with the purge on and purge off.

RESULTS AND DISCUSSION

The schematic diagram shown in Fig. 1 illustrates an open split interface of the configuration described, with the flows used in the expressions below indicated. All flow-rates discussed are measured after equilibration with room temperature, so that the flux of gas can be compared fordifferent temperatures. Ignoring the very minor changes in column dimensions with increased temperature, the flow-rate F_b through the column at some elution temperature T_b (in Kelvin) will be[8]:

$$F_b = \frac{\eta_a T_a F_a}{\eta_b T_b} \tag{1}$$

where F_a is the original flow-rate at some temperature T_a, as measured after equilibration to room temperature, and η_a and η_b are the gas viscosities at T_a and T_b

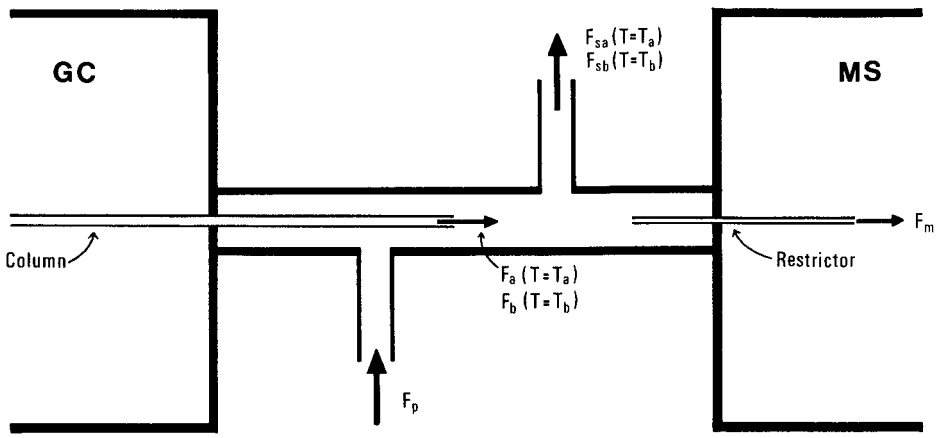

Fig. 1. Schematic diagram illustrating an independently heated open split interface with flows indicated.

respectively. The change in yield can be calculated from the following general case.

If the flow-rate going to the mass spectrometer is defined as F_m and the purge gas flow-rate is F_p, the initial percentage yield at T_a is

$$Y_a = \frac{100F_m}{F_p + F_a} \tag{2}$$

At temperature T_b the percentage yield becomes:

$$Y_b = \frac{100F_m}{F_p + \dfrac{\eta_a T_a F_a}{\eta_b T_b}} \tag{3}$$

Carrier gas viscosities can be calculated to within 1% for the normal temperature range of gas chromatography from the following relationship derived from empirical data[9]:

$$\eta = kT^{0.7} \cdot 10^{-7} \text{ kg m}^{-1} \text{ s}^{-1}$$

where T is the temperature in Kelvin and k is 3.60 for helium, 1.63 for hydrogen and 3.29 for nitrogen. Substitution in eqn. 3 gives:

$$Y_b = \frac{100F_m}{F_p + F_a(T_a/T_b)^{1.7}} \tag{4}$$

The ratio of the yields at T_b and T_a, which is the correction factor that integration results at each value of elution temperature T_b will have to be divided by for quantitative comparisons, is therefore:

$$Y_b/Y_a = \frac{F_p + F_a}{F_p + F_a(T_a/T_b)^{1.7}} \quad (5)$$

With no purge gas this reduces to:

$$Y_b/Y_a = (T_b/T_a)^{1.7} \quad (6)$$

As an illustration of the above effects, a change in temperature from 100 to 300°C results in slightly more than a doubling of the yield if no purge gas is employed. The same temperature change results in a 35% relative increase in yield when half the initial flow is from the purge line. The larger the proportion that the purge gas is of the total, the smaller the increase in yield.

Naturally if quantitative comparisons are made using a second detector monitoring the flow from the open split vent the correction will be opposite in direction. The ratio will be:

$$Y_{sb}/Y_{sa} = \frac{100 - Y_b}{100 - Y_a}$$

where Y_{sb} and Y_{sa} are the percentage of total flow going to the split vent at temperatures T_b and T_a respectively. Substitution of eqns. 2 and 4 and simplifying based on the fact that $F_p + F_a = F_{sa} + F_m$ where F_{sa} is the split vent flow-rate at oven temperature T_a, results in:

$$Y_{sb}/Y_{sa} = \frac{(F_p + F_a)[F_a(T_a/T_b)^{1.7} + F_{sa} - F_a]}{F_{sa}[F_p + F_a(T_a/T_b)^{1.7}]} \quad (7)$$

Integration results for each elution temperature T_b will need to be divided by this value for direct quantitative comparisons on a second detector.

Arithmetical yields of more than 100% in eqn. 4 correspond to an influx of air into the mass spectrometer. This situation is reached when

$$F_p + (T_a/T_b)^{1.7} F_a < F_m \quad (8)$$

Fig. 2 shows the comparison of calculated and experimental values for the yield ratios Y_b/Y_a and Y_{sb}/Y_{sa} for the examples listed. The discrepancy between experimental and calculated data ranged from −2.5 to +3.0% of the calculated yield ratio. During relatively rapid temperature programming the column may not be at equilibrium with the GC oven. Under these conditions there may be a somewhat greater discrepancy from calculated data than indicated here.

The yield will remain constant throughout a temperature programmed analysis only if the carrier gas is controlled by a mass flow controller, or if the interface is heated at the same rate as the GC column. The former method results in significant increases in average carrier gas velocity with increasing temperature[8], but would be suitable in many cases where it is desirable to maintain a constant and maximum yield. The latter method can be achieved either by independently heating the interface at the same rate

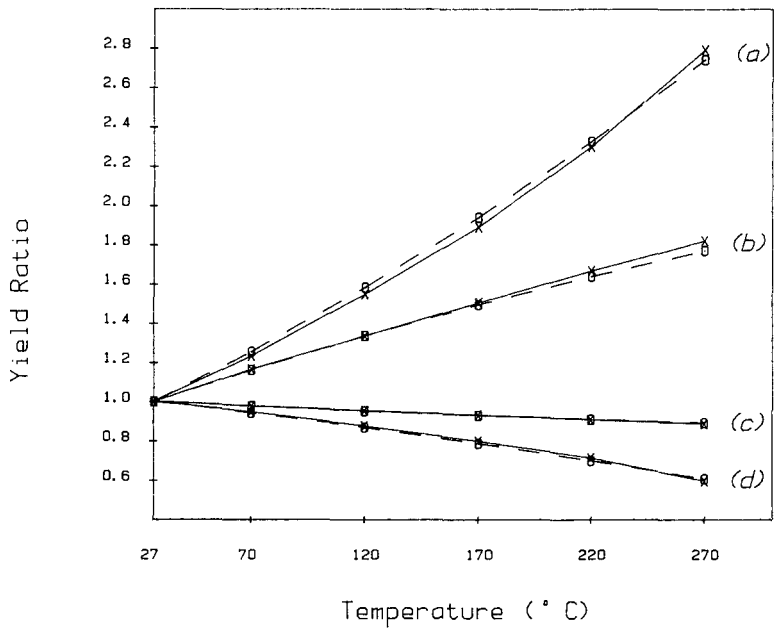

Fig. 2. Calculated data (O – – – O) and experimental data (× – – – ×) for yield ratios from an open split interface held at constant temperature during temperature programming of the GC oven, with isobaric control of flow-rates. (a) Y_b/Y_a, no purge gas, all values of Y_a. For experimental points F_a = 2.88 ml/min, Y_a = 18.4%. (b) Y_b/Y_a, F_p = 1.30 ml/min, F_a = 2.83 ml/min, Y_a = 12.4%. (c) Y_{sb}/Y_{sa}, same conditions as for (b), Y_{sa} = 87.6%. (d) Y_{sb}/Y_{sa}, no purge gas, same conditions as for (a), Y_{sa} = 81.6%.

as the GC oven, or by incorporating the interface in the GC oven. The yield will be maintaned at a constant value in the absence of purge gas since both the column and restrictor flows will be reduced by the same factor. Provided that any purge gas added is also isobarically controlled original yields will still be maintained across a temperature range. If only the purge gas is controlled by a mass flow controller there will be a decrease in yield with rising temperature.

Incorporating the interface in the GC oven is the simpler alternative, but increases the dead volume of the system and goes against the normal practice of placing the interface as close as possible to the mass spectrometer. However, since the dead volume is all on the high vacuum side it is rapidly swept out and should not result in any detectable broadening due to diffusion. A piece of deactivated fused-silica or glass-lined tubing (50 cm in length) as the final connection to the mass spectrometer will take less than 0.2 s to sweep out with a flow of 1.5 ml/min of helium[10].

A second advantage of incorporating the interface in the GC oven is that it can be removed simply and rapidly, allowing the option of direct coupling if required.

A disadvantage of having the open split interface remote from the mass spectrometer could be in the transfer of relatively involatile compounds, such as those boiling above about 500°C. The best results for these appear to be when the column or the restrictor tube go directly into the ion source[11]. This is despite the fact that vapour pressure in equilibrium with a glass wall is higher than that with a liquid phase, and

hence interface temperatures can in theory be lower than the column temperatue[2]. The "Joule–Thompson" cooling effect upon expansion of gas from the restrictor tube does not explain this provided either helium or hydrogen are used as carrier gases, since both have a negative Joule–Thompson coefficient resulting in heating of the gas upon expansion.

The combination of type of flow control and method of interface heating therefore depends on the analytical requirements. If maximum yield is not essential and quantitative comparisons are not important, or the appropriate correction factors are applied, then isobaric pressure control of column flow and an open split interface that is held at constant temperature in association with temperature programmed analyses presents no problems. If it is desired to yield almost 100% of the GC effluent to the mass spectrometer over a wide temperature range, or to maintain a constant yield of effluent to the mass spectrometer, then the method of flow control or the method of heating the interface is relevant when an open split interface is employed.

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Note

Purge-and-trap gas chromatographic method for residual volatiles in high-temperature polymers

JOSEPH W. LONG, RANDY W. SNYDER* and JAMES SPALIK

IBM Corporation, 1701 North Street/T43, Endicott, NY 13760 (U.S.A.)

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High-temperature polymers are finding increased usage in the electronics industry. Residual volatiles in these materials, either from processing chemicals or from solvents in the original formulation, can be detrimental during later processing operations, especially metallization or soldering. These processes may utilize temperatures in excess of 300°C, causing the evolution of residual solvents from the polymer. Processing of polymers prior to high-temperature operations must be optimized to minimize out-gassing of the polymer.

Historically, analysis of volatile materials in solid samples has been carried out by several techniques, including extraction with solvents,[1,2] solid injection methods,[3] and state[4] and dynamic[5,6] (purge-and-trap) headspace analysis. Of these techniques, the dynamic headspace analysis proved to be the method of choice for our analysis due to a number of factors described below. Dynamic headspace analysis involves purging of the sample with an inert gas and trapping volatile components by adsorption on a trap, often using a porous polymer such as Tenax® (Alltech, Deerfield, IL, U.S.A.). The volatile components are thermally desorbed from the trap and backflushed onto a chromatographic column for analysis. Advantages of the technique include: (i) sample preparation is not required, (ii) quantitative results can be obtained, and (iii) required temperature profiles can be modeled using the heatable cell.

Because of the low quantity of residual volatiles in our samples, the susceptibility of the polyimide to air oxidation, and the high temperatures required to simulate processing conditions, we found that the standard purge-and-trap accessories were not suitable. This note describes a custom-designed, gas-tight, purgable high-temperature cell for use as an attachment with the plumbing and electronics in a standard purge and trap accessory.

EXPERIMENTAL

Polyimide films, prepared from pyromellitic dianhydride–oxydianiline (PMDA/ODA) polyimide from E. I. du Pont de Nemours, sprayed on 36 mm square ceramic substrates, are used to demonstrate this technique. The polyimide is obtained as a solution of the polyamic acid in a mixed solvent of N-methyl-2-pyrrolidinone (NMP) and xylenes. The solution is applied to the ceramic substrates, imaging steps are carried out, the film is B-cured at approximately 200°C in air and finally C-cured

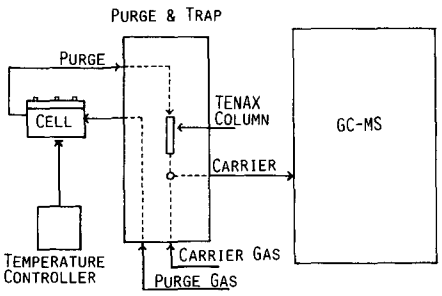

Fig. 1. Experimental setup for high-temperature purge-and-trap gas chromatography.

near 350°C in nitrogen. [For polyimide systems, a "B-cure" step is performed to partially cure the polymer (*ca.* 90%). This is followed by a high-temperature "C-cure" which completes the imidization reaction and performs some annealing of the polyimide]. After final C-cure, the films are approximately 8 μm thick. From the dimensions of the substrate and the film thickness we can determine an approximate polyimide film weight of 0.15 g, from which we thermally evolve about 1.5–15 μg of residual volatiles.

A Hewlett-Packard 5592B–gas chromatographic–mass spectrometric GC–MS system with a modified Hewlett-Packard 7675A purge-and-trap sampler was used for the residual volatiles analysis. The analysis setup is shown in Fig. 1. During development of the method, the mass spectrometer was operated in the total ion monitoring mode, using B-cured parts to obtain the mass spectra and to verify the volatile species. Because of the low levels of volatiles in the C-cured samples, the single ion monitoring (SIM) mode was utilized for these parts since it offers two to three orders of magnitude more sensitivity than the total ion mode.

Thermal desorption of the volatile materials was achieved through the use of a special heatable, gas tight sample holder that was designed and built for this analysis. A schematic of the cell is shown in Fig. 2. The cell employs cartridge type heaters, thermocouples for temperature monitoring, and liquid nitrogen cooling for rapid turnaround between samples. We found that a 0.015-cm soft copper gasket was the most effective gasket material for maintaining a good seal, between the top plate and the body of the cell, at the high temperatures required for the analysis. The cell was made entirely from aluminum for good heat conduction and had an entry port with septum to allow addition of an external standard.

Stand d sampling conditions

Sample purge conditions. Helium purge flow, 50 ml/min; total purge time, 35 min; temperature ramp, ambient to 360°C at 20°C/min; 0.05 μl hexane injected at 200°C.

Desorb conditions. Desorb time, 2 min; desorb temperature, 250°C; trap column, Tenax-GC.

GC Conditions. Helium carrier flow, 30 ml/min; injection port temperature, 250°C; oven temperature, 2 min at 100°C, ramp 15°C/min to 220°C; column, 1.8 m × 6.4 mm O.D. glass 6.6% Carbowax 20M on 80/20 Carbopak B.

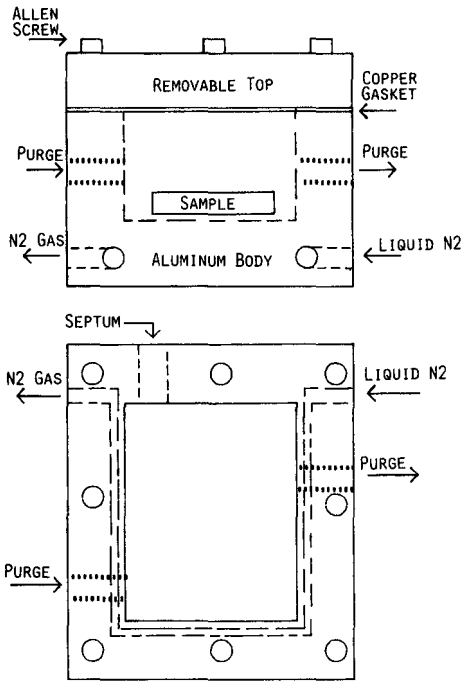

Fig. 2. Schematic of a heatable, gas-tight sample holder for high-temperature purge-and-trap gas chromatography. Holes for rod heaters and thermocouples are omitted for clarity. N2 = Nitrogen.

MS conditions. SIM window size, 0.10 a.m.u.; ion masses, 86.1, 91.1, 99.1; dwell times, 100, 100, 250 ms.

Sampling procedure

A C-cured polyimide film on ceramic was placed in the holder and the holder was checked for leaks. The sample cell was purged at room temperature for 10 min to remove all traces of air. The cell heater was then turned on and the hexane was injected when the cell temperature reached 200°C. After the purge cycle was completed the sample was desorbed from the Tenax column onto the GC column. The GC was held at 100°C through the end of the the 2-min desorb cycle, then ramped to 220°C. Upon completion of the desorb cycle the valve was opened from a liquid nitrogen cylinder to cool the cell to room temperature for the next sample.

The approximate time required to run a sample, including heating ramp, hold time at maximum temperature, and cool down was 1.5 h. Without the liquid nitrogen cooling this time would be extended by approximately 2–3 h.

RESULTS AND DISCUSSION

A chromatogram showing the sum of all the monitored ions (86.1, 91.1, and 99.1 a.m.u.) in a typical run is illustrated in Fig. 3. (Hexane is not normally observed in the polyimide samples and was injected in each run to normalize the data and

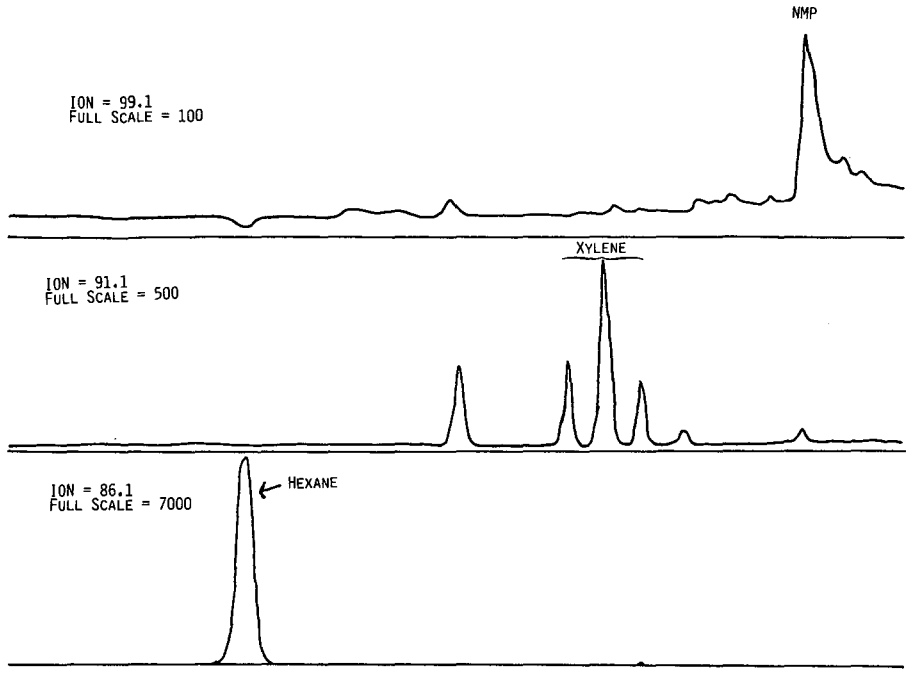

Fig. 3. Typical gas chromatogram of the residual volatiles in polyimide after C-cure plus the hexane standard. Chromatograms are plotted in the SIM mode of the mass spectrometer. (Retraced from actual chromatograms.)

account for potential leaks in the system.) NMP and xylene are major residual solvents (NMP is the solvent from the polyamic acid solution and xylene is used during processing) in the polyimide after C-cure. Some small peaks are also present in the SIM mode chromatograms that were not observed in the total ion monitored chromatogram due to differences in sensitivity but these were not investigated further. Our work focused on the relative amount of residual solvents left under varying process conditions and therefore was semi-quantitative. Quantification could be achieved by injecting standards of NMP and xylene in hexane.

CONCLUSIONS

The high-temperature purge-and-trap method can give good qualitative and semi-quantitative identification of volatiles that might evolve from the polymer films or porous materials during processing. For low levels of volatiles, the signal produced by this method is much larger than that that would be observed from a dynamic analysis. Quantitative measurement of the volatiles is possible by injecting an internal standard into the cell to be run simultaneously with the sample.

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Note

Automated analysis of the gas mixture from ethylene epoxidation by oxygen and nitrous oxide mixtures using gas chromatography

YOU-SING YONG*, K. JACOB SOERENSEN and NOEL W. CANT
School of Chemistry, Macquarie University, North Ryde, N.S.W. 2109 (Australia)
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The manufacture of ethylene oxide by the reaction of ethylene and oxygen over silver catalysts is a multibillion dollar business worldwide. There are continuing efforts to improve the process efficiency and understand the basic chemistry of the reaction. The products of the reaction include C_2H_4O and equimolar amounts of CO_2 and H_2O, together with unreacted C_2H_4 and O_2. Analysis requires quantitation of this five component mixture. This is usually achieved by gas chromatography using one (or two) column systems comprising porous polymers[1–3] supplemented in some cases by molecular sieves[4,5]. In recent work we have wished to study ethylene epoxidation using mixtures of O_2 and N_2O as the oxidant. The number of components is thus increased to seven since unreacted N_2O must be measured and N_2 is a product. Conventional systems are not capable of this. The automated system described below provides quantitation of all components in less than 15 min using a single injection. A particular feature is that the system is arranged so that H_2O and C_2H_4O are eluted first. This is superior to the usual arrangement in which they emerge last and tailing of the water peak is a problem[1,2]. This advantage is particularly pronounced when ethylene conversions in the reactor are low as is commonly the case in kinetic work.

EXPERIMENTAL

The heart of the system is formed by two electrically actuated multiport valves (Valco) located within the oven of a Shimadzu GC-8A gas chromatograph fitted with thermal conductivity detectors. The valves are driven according to a time schedule provided by a Shimadzu C-R3A programmable integrator. The arrangement of the valves and their time programming are shown in Fig. 1. The first valve has ten ports and is fitted with a 2-cm^3 gas sampling loop and two 3.2-mm I.D. columns. Column 1 is 1.5-m Porapak S and column 2 is 0.7-m Porapak QS. A third column (0.8-m 5A molecular sieve) is attached to the second valve (6-port) together with a stainless-steel Nupro metering valve (SS2-A) arranged to provide a similar pressure drop to the column. Column 1 is located in the chromatograph oven and maintained isothermally at 130°C and columns 2 and 3 are arranged external to the chromatograph, via 1/16-in. connecting tubes in an ice bath at 0°C.

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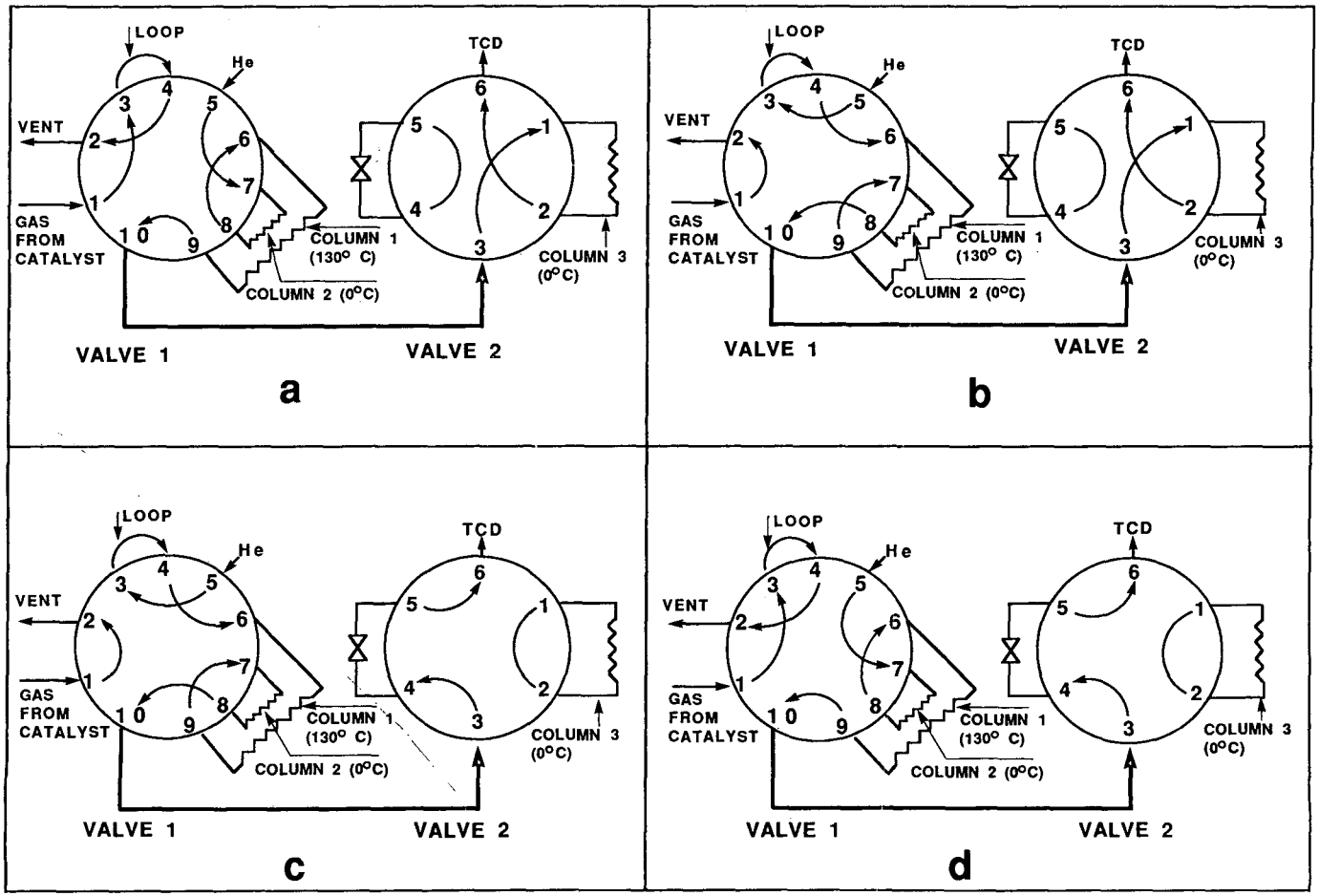

Fig. 1. Configuration of valves and columns during the different steps of a complete analysis: (a) prior to analysis; (b) start of analysis; (c) 20 s after start; (d) 90 s after start. TCD = Thermal conductivity detector.

RESULTS AND DISCUSSION

Prior to analysis (Fig. 1a) the gas stream from the catalytic reactor passes through the sample loop to the vent and the three columns are flushed in the order 2, 1, 3 with helium carrier gas (*ca.* 15 cm^3/min). On initiation of the time program (Fig. 1b) (by operation of the C-R3A integrator) the contents of the sample loop are swept first into the heated column 1 and then to columns 2 and 3 respectively. After 20 s (Fig. 1c), valve 2 is switched, trapping nitrogen and oxygen in the now isolated molecular sieve column 3. After 90 s (Fig. 1d), valve 1 is switched back to its starting position. At this time CO_2, N_2O and C_2H_4 are in column 2. On leaving this column they traverse column 1 again before proceeding to the thermal conductivity detector via the restrictor valve of valve 2. This takes longer than H_2O and C_2H_4O require to elute from their position on column 1. As a result of this arrangement the order of elution is H_2O (2.29 min), C_2H_4O (3.59), CO_2 (4.31), N_2O (5.06), C_2H_4 (7.08). After 10 min the time program reverts valve 2 to its original position allowing oxygen and nitrogen to elute from column 3 at elapsed times of 11.48 and 13.42 min respectively.

A full chromatogram is shown in Fig. 2 (top). In addition to the seven analy-

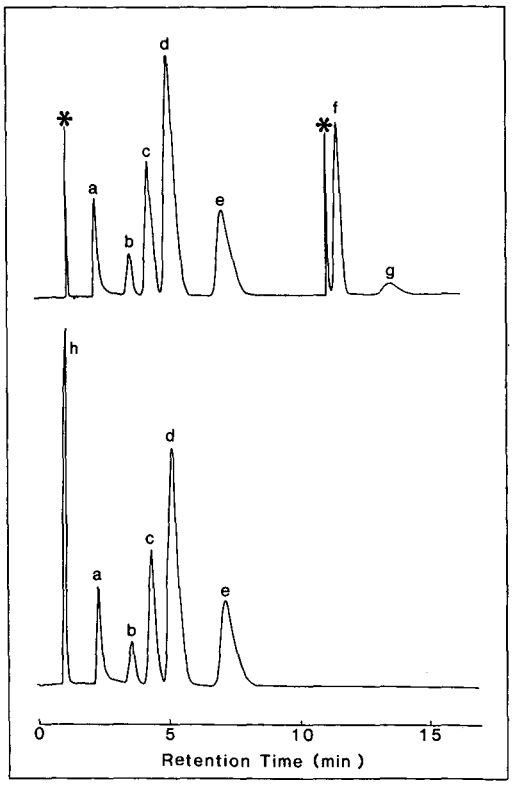

Fig. 2. Typical chromatograms of stream leaving reactor: (top) using all three columns; (bottom) using columns 1 and 2 only. Peaks: a = H_2O; b = C_2H_4O; c = CO_2; d = N_2O; e = C_2H_4; f = O_2; g = N_2; h = N_2 + O_2. The asterisks indicate peaks associated with pressure surges.

tical peaks, pressure surges associated with operation of valve 2 give rise to sharp apparent peaks at 1.21 and 11.05 min. These can be minimised by careful adjustment of the restrictor valve, but in any case cause no difficulty since they do not overlap with component peaks and their areas are easily eliminated by programming of the integrator. As can be seen the system provides baseline separation of all seven components and integration was possible with high reliability. Repeat analysis of the same mixture agreed to within $\pm 1\%$.

If separate quantitation of nitrogen and oxygen is not required (as when oxygen alone is used as oxidant) then a truncated program in which valve 2 is retained in the position corresponding to Fig. 1c and d throughout is possible. Nitrogen and oxygen are then eluted as a composite peak with retention time of 0.98 min whilst the position of the remaining elutants are unchanged. As shown in Fig. 2 (bottom) the analysis is then complete in 8 min.

The overall catalytic system incorporates an additional 4-port manually operated valve to enable direct routing of gas mixtures through the sampling loop bypassing the catalyst (which can then be pretreated as required prior to use). Initial determination of response factors for each component were carried out with carefully made up gas mixtures containing one or more of the components in helium and flowing on bypass. This calibration was routinely checked with a precise mixture of 5.08% CO_2 and 3.00% C_2H_4O in helium (CIG Ltd.). The loop size used was chosen to enable accurate analysis of mixtures in which each component made up 0.1–30% of total flow at atmospheric pressure with helium as the balance gas. Ten-fold smaller or larger pressures could be readily handled by adjustment of the loop size. The system has proved ideal for the purpose required and been in continuous use for many months apart from occasional regeneration of the molecular sieve column.

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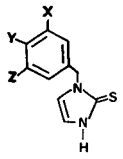
Note

Regio-selective derivatization of 1-[(3,5-difluorophenyl)methyl]-1,3-dihydro-2H-imidazole for gas chromatographic analysis

TED K. CHEN* and DAVID THORNTON
Department of Analytical Chemistry, Smith Kline & French Laboratories, P.O. Box 1539 (L960), King of Prussia, PA 19406-0939 (U.S.A.)
(First received December 23rd, 1987; revised manuscript received July 4th, 1988)

The difficulties involved in effecting derivatization at a specific atom of a heterocycle where reaction at another site is also possible, constitute a major problem in heterocyclic chemistry[1]. The problem is particularly difficult in derivatization for quantitative chromatogrpahic analysis since in this application, the cardinal requirement of the derivatization protocol is the preferential and quantitative conversion of the analyte to a single stable product for analysis. Recently, we required a method for the gas chromatographic (GC) analysis and impurity profiling of bulk 1-[(3,5-difluorophenyl)methyl]-1,3-dihydro-2H-imidazole [SK&F 102698 (1)], the prototype molecule of a new class[2,3] of orally active antihypertensive whose effects are mediated by the novel mechanism of dopamine β-hydroxylase inhibition[4]. We report here our strategy for the regio-selective derivatization of compound 1 with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) where regio-selectivity was stereochemically induced. Derivatization of compound 1 with other silylating and alkylating reagents gave either incomplete conversion or formed multiple products making it unsuitable for the impurity profiling of the drug substance.



(1) X = F Y = H Z = F
(2) X = H Y = CF_3 Z = H
(3) X = H Y = Br Z = H
(4) X = H Y = CH_3 Z = H

MATERIALS AND METHODS

Reagents

Pentafluorobenzyl bromide, MTBSTFA (with and without 1% *tert.*-butyldimethylsilyl chloride), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (both with and without 1% trimethylsilyl chloride) were purchased from Regis (Morton Grove, IL, U.S.A.). Acetonitrile was dried before use. Compound 1 and its 6-trifluoromethyl- (2), 6-bromo- (3) and 6-methyl- (4) analogues were obtained from Smith Kline & French Laboratories. The purity of compound 1 was independently established to be >99.5% by high-

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performance liquid chromatography weight assay comparison with primary reference compound.

Derivatization procedure

To a solution of 14 mg sample in 750 μl acetonitrile placed in a 4-ml reaction vial with PTFE-lined cap, 50 μl of pyridine and 250 μl of MTBSTFA or pentafluorobenzyl bromide were added. The reaction vial was then stoppered under dry nitrogen and heated at 75°C for 2 h. After cooling to room temperature, an aliquot of the reaction mixture was injected into the gas chromatograph. Reactions with BSTFA and MSTFA were similarly carried out and heated for 2 as well as 4 h.

Chromatography. GC was performed either using a HP 5880 or HP 5970 gas chromatograph equipped with capillary injection system and flame ionization detectors. The HP 5880 gas chromatograph was equipped with a dual column injection system consisting of a DB-5 (25 m × 0.5 μm film thickness) and a DB-17 column (30 m × 1 μm film thickness). The DB-5/DB-17 columns were held at 100°C for 2 min and then temperature-programmed to 270°C at 4°C/min. Hydrogen was used as the carrier gas. Chromatography with a DB-210 column (15 m × 0.32 μm film thickness) was performed using a HP 5970 gas chromatograph either at 175°C isothermal with helium as the carrier gas or held at 100°C for 2 min and then temperature-programmed to 210°C at 4°C/min using hydrogen as the carrier gas (linear velocity, 39 cm/s at 175°C). The injection port temperature was held at 220°C in both chromatographs. Data were acquired and processed using a Beckman CIS-CALS system.

Gas chromatography–mass spectrometry (MS). GC–MS analysis were performed in the electron impact mode on a Finnigan 700 ion trap. Full-scan spectra from *m/z* 42 to *m/z* 550 were recorded with a scan time of 1.0 s/scan. The derivatized samples were analyzed on a 30 m × 0.32 mm I.D. fused-silica capillary column with a DB-17 bonded phase coating using similar GC conditions as above.

RESULTS AND DISCUSSION

GC analysis of the underivatized compound 1 gave a severly tailing peak making the direct analysis of compound 1 unsuitable for impurity-profiling and quantitation of impurities that may be present at 0.1% level or less since impurities may be hidden under the broad drug peak. Attempts at silylation of compound 1 with BSTFA and MSTFA (both with and without 1% trimethylsilyl chloride as catalyst) gave consistently only *ca.* 97% conversion, the remaining 3% starting material most probably being derived via hydrolysis of the more hindered and inherently less stable thio-trimethylsilyl ether[5,6]. Alkylation with pentafluorobenzyl bromide yielded a mixture of N- and S-alkylated products resulting from the tautomerization of compound 1 in the reaction solution along with some unreacted starting material. These difficulties are in accord with earlier reports of similar problems encountered in attempting the direct analysis of polar imidazoles[7] and selective derivatization of tautomeric heterocycles[1].

We theorized that for steric reasons, introduction of a bulkier *tert.*-butyldimethylsilyl group using MTBSTFA may be regio-selectively effected at the N- rather than S-site. The formation of a stable[8] N-*tert.*-butyldimethylsilyl derivative as dem-

onstrated in the derivatization of adenine nucleotides[9] would also encourage such a conversion. This was indeed what we found. Compound 1 was preferentially and quantitatively converted to a single N-*tert.*-butyldimethylsilyl derivative with excellent chromatographic behavior. With thick film (0.5 and 1 μm film thickness) and long (25

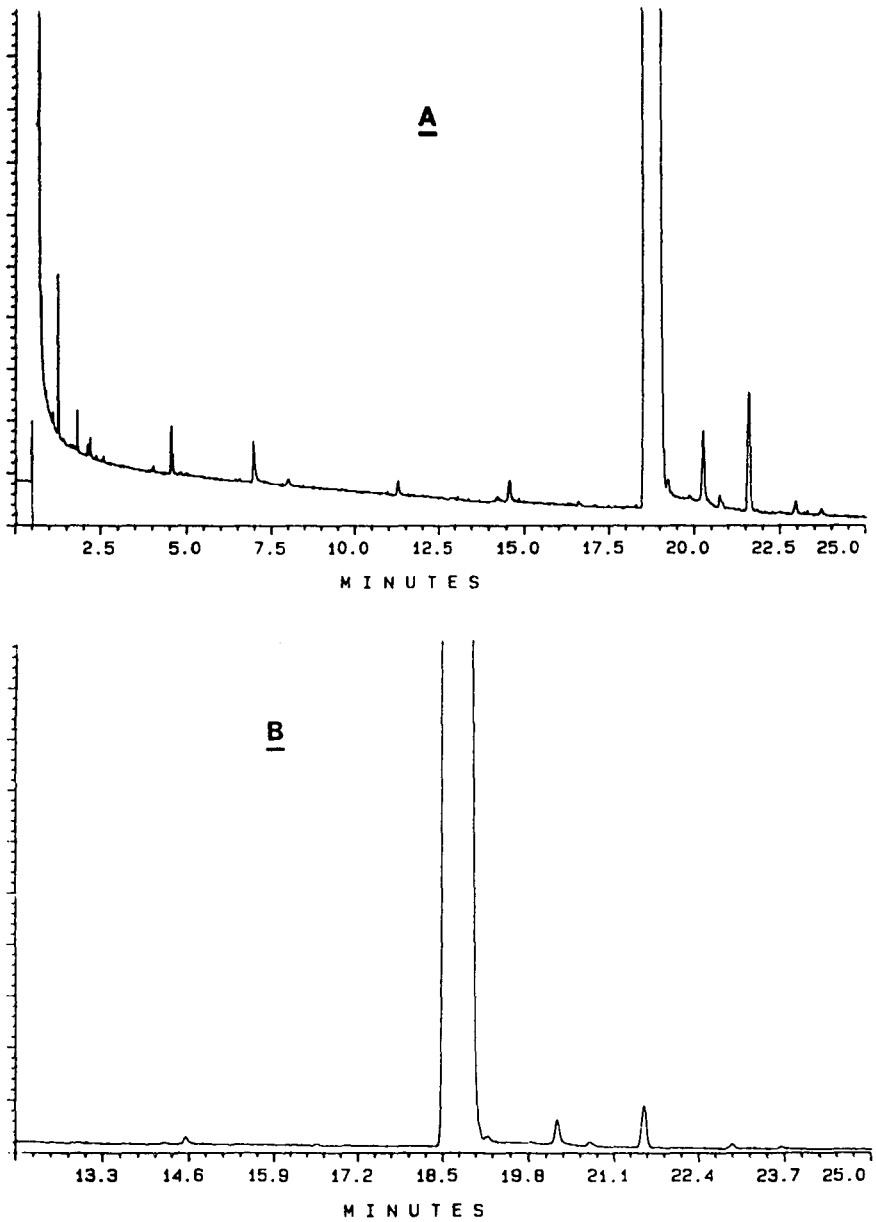

Fig. 1. Gas chromatogram of N-*tert.*-butyldimethylsilyl derivative of compound 1: (A) whole chromatogram; (B) expanded chromatogram.

m and 30 m) DB-5 or DB-17 columns respectively, the derivative exhibited some thermal degradation. This was overcome by performing the assay on a thin film (0.32 μm) and short (15 m) DB-210 stationary phase at lower isothermal temperature without sacrificing resolution of other minor impurities (Fig. 1). Regio-selectivity was also achieved with the 6-trifluoromethyl- (2), 6-bromo- (3) and 6-methyl- (4) analogues of compound 1 in a similar manner.

Chemical derivatization followed by GC continues to play an important role in meeting the analytical needs of biotechnology and medicine. The present results suggest that the aforementioned strategy may be employed with other similarly structured heterocycles in their development as potential drug candidates. The approach is attractive since in addition to stereochemically-induced regio-selectivity, the *tert.*-butyldimethylsilyl group also confers the characteristic $M^+ - 57$ fragment in GC–MS studies[10].

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Note

High-performance liquid chromatographic assay of imazodan, methylparaben and propylparaben in imazodan injection

TARA GERIA

Analytical Research, Consumer Product, Warner-Lambert Company, Morris Plains, NJ 07950 (U.S.A.)

and

WEN-HAI HONG* and ROBERT E. DALY

Analytical Research, Product Development, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, 170 Tabor Road, Morris Plains, NJ 07950 (U.S.A.)

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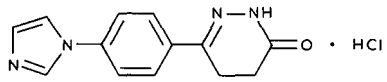
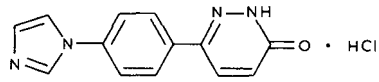
Imazodan hydrochloride, 4,5-dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone hydrochloride (I) is a new cardiotonic[1], which was formulated as capsules and injection, and has been used in the clinical trials. The injection contains preservatives, antioxidant and citrate buffer. 6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone (II) exists as a process contaminant in the bulk drug and is also as a potential degradation product, which is structurally very similar to that of imazodan with an additional unsaturation in the pyridazinone ring.



Imazodan hydrochloride (I)



6-[4-(1H-Imidazol-1-yl)-phenyl]-3(2H)-pyridazinone (II)

Reversed-phase high-performance liquid chromatography (HPLC) has been successfully used in pharmacokinetic studies for imazodan[2]. However, no separation between imazodan and II was mentioned. Methylparaben and propylparaben are commonly used preservatives in many cosmetic and pharmaceutical formulations. Their HPLC analyses have often been cited[3–13]. This report presents a reversed-phase HPLC assay of imazodan in an injection and the data validating it. The method allows simultaneous determination of imazodan, a potential degradation product (II), and two preservatives, methylparaban and propylparaban.

EXPERIMENTAL

Chemicals and reagents

Unless otherwise specified all chemicals and reagents were reagent grade and obtained form Fischer Scientific (Springfield, NJ, U.S.A.). Compounds I and II were synthesized at Parke-Davis Pharmaceutical Research Labs. Methylparaben and Pro-

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pylparaben were USP Reference Standards. Water was double-distilled and deionized.

Apparatus

The chromatographic system consisted of a Series 4 HPLC pump (Perkin-Elmer, South Plainfield, NJ, U.S.A.). An autosampler (Perkin-Elmer ISS-100 autosampler equipped with a 20-μ loop), a reversed-phase C_{18} column (Altex ODS column, 4–6 μm, 15 cm × 4.6 mm I.D., Beckman, San Ramon, CA, U.S.A.), a variable-wavelength UV detector (Perkin-Elmer LC-95 spectrophotometer, set at 267 nm), and a computing integrator SP-4270, Spectra-Physics, San Jose, CA, U.S.A.).

Mobile phase

The mobile phase consisted of acetonitrile–0.05 *M* ammonium phosphate buffer adjusted to pH 3.0 with 1 *M* phosphoric acid–water (38:2:60, v/v). It was filtered through a 0.45-μm membrane filter, or equivalent, and degassed before use. The flow-rate was 0.6 ml/min.

Standard stock solution

(A) Compound II stock solution. A known quantity of II monohydrate reference standard, equivalent to about 20 mg of II base, was accurately weighed and quantitatively transferred to a 100-ml volumetric flask. The standard was dissolved in and brought to volume with mobile phase. A 5.0-ml aliquot was diluted to 50.0 ml with mobile phase and mixed.

(B) Imazodan–compound II stock solution. A known quantity of imazodan hydrochloride monohydrate reference standard, equivalent to about 40 mg of imazodan was accurately weighed and quantitatively transferred to a 50-ml volumetric flask. A 2.0-ml aliquot of the standard stock solution A was added, mixed to dissolve the standard, brought to volume with mobile phase, and mixed.

(C) Propylparaben stock solution. About 16 mg of propylparaben USP Reference Standard was accurately weighed and quantitatively transferred to a 50-ml volumetric flask. The standard was dissolved in, brought to volume with mobile phase, and mixed.

(D) Methylparaben–propylparaben stock solution. About 29 mg methylparaben USP Reference Standard was accurately weighed and quantitatively transferred to a 100-ml volumetric flask. A 10.0-ml aliquot of the standard stock solution C was added, mixed to dissolve the standard, brought to volume with mobile phase, and mixed.

Standard preparation

A 5.0-ml aliquot each of the standard stock solutions B and D was pipetted into a 100-ml volumetric flask, brought to volume with mobile phase, and mixed.

System suitability

Six successive injections of the standard preparation should provide a relative standard deviation (R.S.D.) of not greater than 2.0%. The number of theoretical plates should not be less than 2300 for imazodan. The resolution between imazodan and compound II should be greater than 1.5. If system suitability is not obtained,

adjust the parameters, clean or replace the column, if necessary, until system suitability is achieved.

Assay preparation

A volume of 2.0 ml of the 2-mg/ml injection was pipetted into a 10-ml volumetric flask, or 2.0 ml of the 5-mg/ml injection into a 25-ml volumetric flask, brought to volume with mobile phase and mixed. A 5.0-ml aliquot was further diluted to 50.0 ml using the same medium. An appropriate aliquot was filtered through a 0.45-μm membrane filter, or equivalent, discarding the first 5 ml.

Procedure

Separate 20-μl aliquots of the Standard and Assay preparations were injected into the chromatograph and the chromatograms recorded.

Calculation

The quantity of imazodan, compound II, methylparaben, or propylparaben was calculated according to the following formulae.

For the 2 mg/ml injection

$$\text{mg/ml of imazodan, methylparaben, or propylparaben} = (R_u/R_s)C(50/5)(10/2) = 50C(R_u/R_s)$$

$$\%\ \text{compound II} = (R_u/R_s)C(50/5)\ (10/2)(100/W) = 5000(R_u/R_s)(C/W)$$

For the 5 mg/ml injection

$$\text{mg/ml of imazodan, methylparaben, or propylparaben} = (R_u/R_s)C(50/5)(25/2) = 125C(R_u/R_s)$$

$$\%\ \text{compound II} = (R_u/R_s)C(50/5)(25/2)(100/W) = 12500(R_u/R_s)(C/W)$$

in which R_u and R_s are the peak areas for imazodan, compound II, methylparaben, or propylparaben obtained from the Assay and Standard preparations, respectively, C is the concentration, in mg/ml, of imazodan, compound II, methylparaben, or propylparaben in the Standard preparation, and W is the quantity, in mg/ml, of imazodan found in the injection.

RESULTS AND DISCUSSION

Method validation

Recovery and precision. The method was validated by spiking known amounts of imazodan, compound II, methylparaben, and propylparaben into a placebo at 80–120% of label claim, except for compound II which was at 80–120% of the specification limit of 0.1% of imazodan. Average recoveries of 100.7% (R.S.D. 0.7%) for imazodan, 99.5% (R.S.D. 3.3%) for compound II, 100.5% (R.S.D. 0.5%) for meth-

TABLE I

RECOVERY DATA OBTAINED FROM SPIKED PLACEBO USING REVERSED-PHASE HPLC

Sample No.	*Label claim (%)*	*Recovery (%)*			
		Imazodan	*Compound II*	*Methylparaben*	*Propylparaben*
1	80	101.3	103.4	101.1	99.0
2	80	101.3	102.0	101.1	98.6
3	100	100.6	102.0	100.1	100.4
4	100	101.8	103.8	100.3	100.9
5	100	101.3	97.1	101.3	101.6
6	100	100.1	96.7	100.8	101.2
7	100	99.7	95.2	101.4	101.5
8	100	100.4	102.2	101.0	100.5
	Average	100.7	99.5	100.8	101.0
	R.S.D. (%)	0.7	3.3	0.5	0.5
9	120	99.3	98.0	98.6	99.2
10	120	99.8	96.6	99.8	99.4

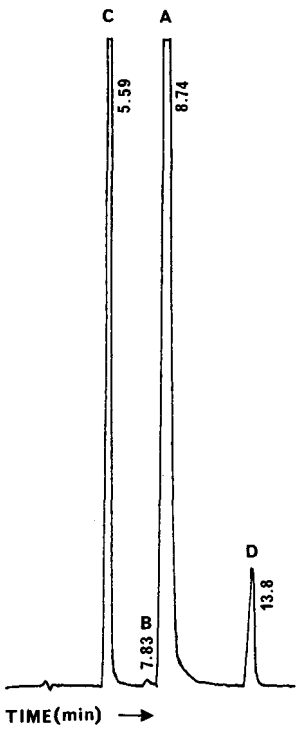

Fig. 1. A representative chromatogram obtained from a standard preparation containing imazodan (A), compound II (B), methylparaben (C), and propylparaben (D). Amount injected: 20 μl.

ylparaben, and 101.0% (R.S.D. 0.5%) for propylparaben, by peak area were obtained at 100% of label claim (Table I). The excipients do not interfere with the recovery. Recoveries were quantitative and precise. Although the precision for compound II exceeds 2.0%, an R.S.D. of 3.3% is thought to be acceptable at such a low level as 0.04 μg/ml.

Selectivity. The proposed reversed-phase HPLC method affords baseline separation of imazodan from compound II and two preserevatives, methylparaben and propylparaben. Fig. 1 presents a typical chromatogram obtained from a standard preparation. As seen, imazodan, compound II, methylparaben, and propylparaben are well resolved, with resolution factors of 2.1 between imazodan and compound II, 4.9 between imazodan and methylparaben, and 8.3 between imazodan and propylparaben, respectively.

Linearity. The calibration curves (peak area *vs.* concentration) were constructed at least with five different concentrations. The curves were linear in the range of 10–80 μg/ml for imazodan (10, 20, 40, 50, 60 and 80 μg/ml; $r^2 = 0.9998$), 0.01–0.08 μg/ml for compound II (0.01, 0.02, 0.05, 0.06 and 0.08 μg/ml; $r^2 = 0.9999$), 7–29 μg/ml for methylparaben (7.2, 14.4, 18.8, 21.6 and 28.8 μg/ml; $r^2 = 0.9997$), and 0.8–3.2 μg/ml for propylparaben (0.8, 1.6, 2.0, 2.4 and 3.2 μg/ml; $r^2 = 0.9997$).

System suitability

Six replicate injections of a standard preparation containing 50 μg/ml of imazodan, 0.05 μg/ml of compound II, 18 μg/ml of methylparabwen, and 2 μg/ml of propylparaben gave R.S.D. values of 0.4% for imazodan, 3.2% for compound II, 0.8% for methylparaben, and 0.5% for propylparaben. The number of theoretical plates calculated according to ref. 14 were 3300 for imazodan and 3100 for compound II. The resolution factor between imazodan and compound II was 2.1. The tailing factors calculated according ref. 14 were 1.4 for imazodan and 1.6 for compound II.

Limit of detection

The limit of detection for compound II was found 0.02% in imazodan.

TABLE II

ANALYTICAL DATA OBTAINED FROM REVERSED-PHASE HPLC ASSAY OF IMAZODAN INJECTION

Sample	*Assay results (mg/ml)*			
	Imazodan	*Compound II**	*Methylparaben*	*Propylparaben*
Imazodan injection, lot A				
Initial	4.86	0.04	1.69	0.18
6 Months at 37°C	4.79	0.09	1.57	0.14
6 Months at 45°C	4.68	0.12	1.52	0.10
Imazodan injection, lot B				
Initial	4.90	0.05	1.69	0.17
6 Months at 37°C	4.96	0.07	1.63	0.15
6 Months at 45°C	4.86	0.08	1.59	0.14

* As % of imazodan.

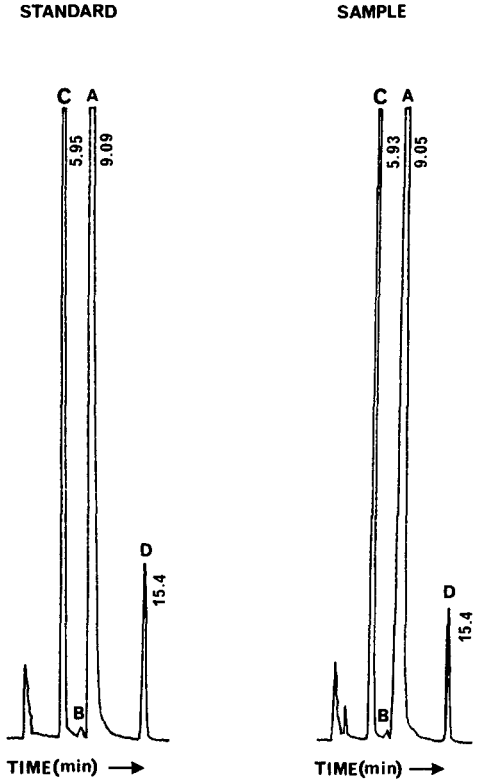

Fig. 2. Typical chromatograms obtained from the analysis of imazodan injection, lot A, 6 months at 37°C.

Dosage form analysis

Table II presents the assay data on two lots of imazodan injection, stored upright for up to 6 months at 37°C and 45°C, respectively. The contents of compound II were all within the 0.1% tentative specification, except for lot A at 6 months/45°C which gave 0.12%. Fig. 2 shows the representative chromatograms obtained from the analysis of lot A stored upright for 6 months at 37°C. No interference from excipients was detected.

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Note

High-performance liquid chromatographic determination of conjugated estrogens in tablets

ROBERT W. TOWNSEND, VOLKER KEUTH, KORAL EMBIL, GOTELIND MULLERSMAN, JOHN H. PERRIN and HARTMUT DERENDORF*

College of Pharmacy, Box J-494, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610 (U.S.A.)

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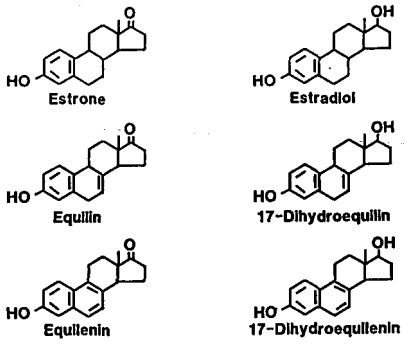
Conjugated estrogens are defined in the USP XXI[1] as a mixture of sodium estrone sulfate and sodium equilin sulfate obtained synthetically or from equine urine. They may contain other conjugated estrogenic substances, such as equilenin, estradiol, 17-dihydroequilin or 17-dihydroequilenin. Conjugated estrogens contain 50–63% of sodium estrone sulfate and 22.5–32.5% sodium equilin sulfate. The analytical method for the determination of conjugated estrogens was recently changed from a colorimetric method (USP XX) to a gas chromatographic (GC) method (USP XXI) after hydrolysis. This method is very time consuming, and it was our goal to explore the use of high-performance liquid chromatography (HPLC) for the determination of conjugated estrogens in tablets. Such methods had been described for the HPLC analysis of the estrogens after acid hydrolysis[2] and dansylation[3–5] or formation of the 2,4-dinitrohydrazine derivatives[6]. In the present study we developed an HPLC procedure for the direct analysis of the conjugated estrogens without any hydrolysis and compared the results to those obtained after application of a sulfatase hydrolysis.



EXPERIMENTAL

Materials

All estrogens as well as their sulfate esters were gifts from Embil Pharmaceuticals (Istanbul, Turkey) and used as supplied. The sulfate standards are mixed with tromethamine in a ratio of 60:40. Prednisolone hemisuccinate was a gift from Hoechst (Frankfurt, F.R.G.). Sulfatase was obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents and solvents used were of analytical grade. Conjugated estrogen tablets were supplied by Embil Pharmaceuticals (Istanbul, Turkey).

Instrumentation

The following instruments were used for the HPLC assay: high pressure pump, Constametric III (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), variable-wavelenght UV detector (LDC/Milton Roy), manual injection valve 190 (Negretti, Southampton, U.K.) with a 20-μl or 50-μl loop, integrator Model 3390 A (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a DuPont Zorbax ODS column, 15 cm × 4.6 mm I.D. (6 μm packing) with a DuPont 5 cm × 4.6 mm I.D. guard column filled with Zorbax ODS (DuPont, Wilmington, DE, U.S.A.).

HPLC assay of estrogens after hydrolysis

Chromatography. The mobile phase consisted of acetontrile–water (40:60) to which 0.5 ml/l of phosphoric acid (85%) was added. The flow-rate was 1.0 ml/min. UV detection was performed at 280 nm.

Sample preparation. A quantity of conjugated estrogens, accurately weighed and equivalent to approximately 2 mg of total conjugated estrogens was obtained from the powder of eight finely crushed conjugated estrogens tablets. Acetate buffer (pH 5.2, 15 ml) and barium chloride (1 g) were added. The tubes were shaken and a sulfatase enzyme preparation equivalent to 2500 units was added. The samples were agitated for 20 min in a 50°C water bath and left overnight at room temperature. Then 15 ml ethylene dichloride were added and the tubes were shaken and centrifuged at 1500 *g* for 10 min. A 10-ml volume of the organic layer was removed and dried with anhydrous sodium sulfate. From the dried solution 5 ml are evaporated under nitrogen and reconstituted in 1 ml mobile phase. Of this solution 20 μl were injected into the HPLC system.

Calibration curves. For the calibration curves stock solutions of the estrogen sulfates were diluted and treated in the same way as the tablet extracts.

Direct HPLC assay of estrogen sulfates

Chromatography. The mobile phase consisted of acetontrile (400 ml), methanol (350 ml), water (1000 ml) and tetrabutylammonium hydroxide (13 g). The pH of this mobile phase was adjusted to pH 3.0 with phosphoric acid. The flow-rate was 1.0 ml/min. UV detection was performed at 214 nm.

Sample preparation. One tablet was pulverized and extracted with 8 ml deionized water. The sample was shaken for 10 min and centrifuged for 15 min. at 1500 *g*. The supernatant liquid was transferred in a clean tube and centrifuged again for 15 min at 1500 *g*. To 1 ml of supernatant liquid was added: 0.5 ml 0.1 *M* acetate buffer (pH 3), 0.5 ml internal standard (prednisolone hemisuccinate, 100 μg/ml in aceto-

nitrile–water, 50:50), 0.2 ml tetrabutylammonium chloride (10 mg/ml) and 5 ml chloroform. The samples were shaken for 10 min and centrifuged for 10 min. The upper aqueous layer was removed by aspiration and the chloroform was transferred into a clean tube and dried under nitrogen at room tempeerature. The samples were reconstituted in 200 μl mobile phase. Of the samples 20 μl were injected into the HPLC system.

Standard solutions. The stock solution contained 500 μg/ml sodium estrone sulfate and 12.5 μg/ml sodium equilin sulfate in water. A 1-ml volume of this solution was treated the same way as 1 ml of the tablet supernatant liquid above.

Calibration curves. Dilutions of stock solutions of all investigated compounds were made in the range of 1 to 500 μg/ml. Calibration curves were obtained by plotting peak height ratios of the estrogens to the internal standard *versus* concentrations of the estrogens. Equations were calculated by the least square method using linear regression.

RESULTS

Chromatography

As can be seen from Fig. 1A chromatographic separation of estrone, equilin and equilenin after enzyme hydrolysis can be achieved with retention times of 11.5, 10.5 and 8.5 min, respectively. Fig. 1B shows the direct chromatographic separation of the five steroids from their sulfate esters. Baseline separation was obtained for all compounds. Since the sulfate esters of 17α-dihydroequilin and 17α-dihydroequilenin were not available, their location in the chromatogram (Fig. 1B) could not be determined. Assuming a constant relationship between the retention times of the estrogens and their corresponding conjugates the capacity factors (k') of the substances can be used to predict the retention times of the not available sulfates of 17α-dihydroequilenin and 17α-dihydroequilin. According to this calculation the peaks of both substances will elute between the peaks of estrone and sodium equilenine sulfate (17α-dihydroequilenin sulfate, 13.7 min; 17α-dihydroequilin sulfate, 16.6 min). For both methods the calibration curves obtained from these chromatograms were linear up to 500 μg/ml with correlation coefficients of 0.998.

Sample preparation

The sample preparation for the direct HPLC assay is much simpler and less time consuming than the enzymatic hydrolysis procedure. However, it was not possible to chromatograph aqueous tablet extracts directly as other tablet ingredients interfered with the estrogen peaks. So a clean-up step had to be added. The estrogen sulfates were extracted as tetrabutylammonium ion pairs with chloroform. This procedure resulted in clean chromatograms that allowed the quantification of the steroids in the investigated tablets.

Reproducibility

Accuracy and precision of the assay were evaluated by repetitive analysis on five different days of the three estrogen esters in different concentrations (Tables I and II). These solutions were subjected to the complete sample preparation procedures for the two methods described above. The results indicate better accuracy and precision for the direct method.

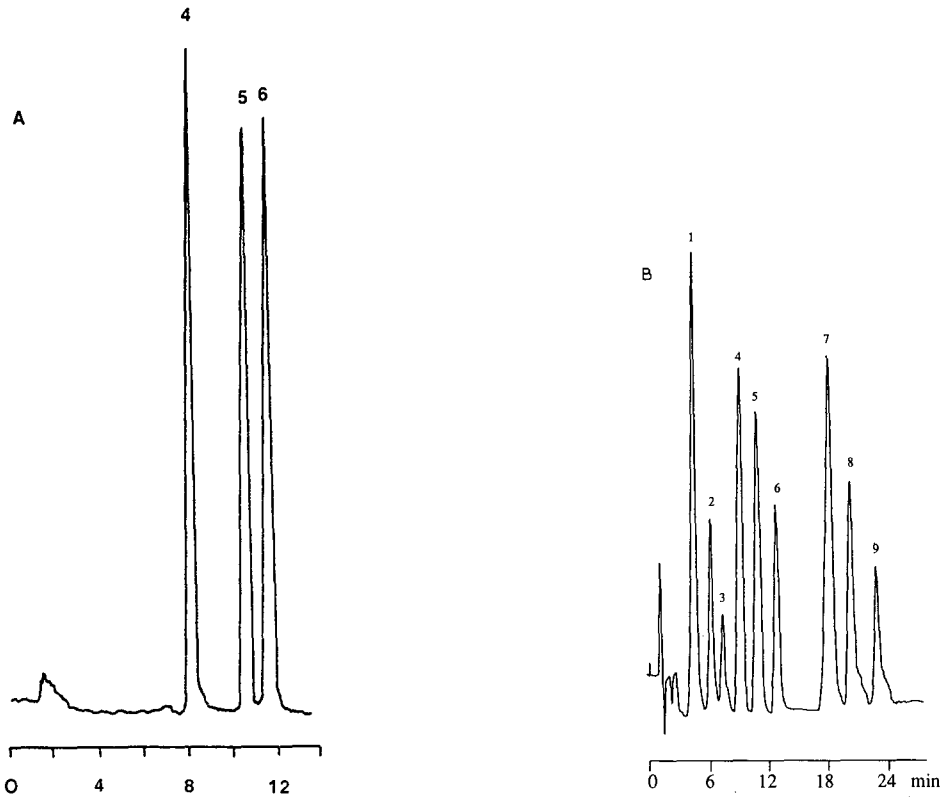


Fig. 1. (A) Chromatogram of equilenin (4), equilin (5) and estrone (6) after hydrolysis of the respective conjugates. UV detection was done at 280 nm. (B) Chromatogram of 17α-dihydroequilenin (2), 17α-dihydroequilin (3), equilenin (4), equilin (5), estrone (6), sodium equilenin sulfate (7), sodium equilin sulfate (8) and estrone sulfate (9) after extraction. Prednisolone hemisuccinate (1) was used as internal standard. UV detection was done at 214 nm.

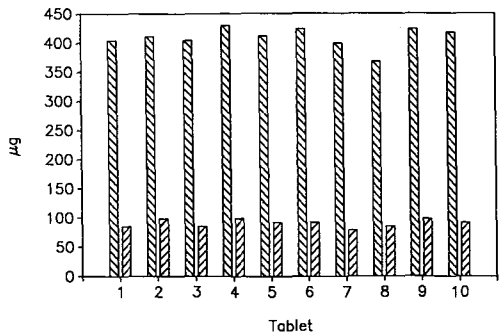


Fig. 2. Content uniformity of estrone sulfate (left bars) and equilin sulfate (right bars) in ten tablets using the direct HPLC method without hydrolysis.

TABLE I

ACCURACY AND PRECISION OF THE HPLC ASSAY AFTER ENZYME HYDROLYSIS

C.V. = Coefficient of variation.

Concentration (μg/ml)	*Assayed (μg/ml)*					*Mean (μg/ml)*	*C.V. (%)*
Sodium estrone sulfate							
10.0	10.6	12.2	6.8	8.3	9.9	9.6	21.8
50.0	53.7	55.7	43.3	51.6	44.2	49.7	11.3
100.0	91.4	88.9	86.9	111.1	95.2	94.7	10.2
250.0	231.3	226.6	244.5	243.4	258.9	240.9	5.2
500.0	510.9	507.7	496.2	496.2	511.4	504.5	1.5
Sodium equilin sulfate							
10.0	10.1	10.0	9.1	14.4	12.6	11.2	19.5
50.0	44.5	51.3	48.9	58.1	61.6	52.9	13.1
100.0	100.6	97.5	90.1	89.8	97.6	95.1	5.1
250.0	264.4	250.5	262.7	256.5	229.0	252.6	5.7
500.0	491.9	492.6	491.4	513.3	469.1	491.7	3.2
Sodium equilenin sulfate							
10.0	11.1	10.6	9.9	10.2	9.1	10.3	8.6
50.0	52.8	57.8	51.5	51.3	48.2	52.2	6.3
100.0	128.2	113.9	104.4	102.5	107.9	111.4	9.3
250.0	260.5	257.9	259.9	248.7	257.6	256.9	1.8
500.0	476.5	520.9	493.5	497.2	493.4	496.3	3.2

TABLE II

ACCURACY AND PRECISION OF THE DIRECT HPLC ASSAY

Concentration (μg/ml)	*Assayed (μg/ml)*					*Mean (μg/ml)*	*C.V. (%)*
Sodium estrone sulfate							
1.0	1.1	1.1	1.2	1.3	1.1	1.2	7.7
10.0	10.0	10.2	10.2	9.8	9.8	10.0	2.0
25.0	24.4	25.3	24.6	25.3	26.4	25.2	3.1
50.0	49.1	49.4	48.7	50.3	49.2	49.3	1.2
100.0	100.6	103.9	101.0	93.8	102.1	100.3	3.8
Sodium equilin sulfate							
1.0	0.9	0.9	1.1	1.2	0.9	1.0	14.1
10.0	9.8	8.8	10.0	8.6	10.0	9.4	7.2
25.0	23.2	27.0	27.8	26.1	24.9	25.8	7.0
50.0	49.8	50.0	49.1	53.5	47.9	50.1	4.2
100.0	96.9	102.1	101.1	100.6	95.2	99.2	3.0
Sodium equilenin sulfate							
1.0	1.0	1.1	1.0	0.8	0.9	1.0	11.9
10.0	10.4	10.1	9.8	9.7	9.2	9.8	4.6
25.0	25.0	25.3	24.1	25.5	25.7	25.1	6.3
50.0	49.1	49.9	48.1	52.0	50.6	49.9	3.0
100.0	103.5	95.9	101.9	98.7	100.3	100.0	3.1

Content uniformity

To apply the described assay ten tablets were analyzed for estrone sulfate and equilin sulfate. With the enzymatic method 406 ± 27 (mean ± S.D.) μg estrone sulfate and 112 ± 5 μg equilin sulfate were measured, whereas the direct method gave 410 ± 18 μg estrone sulfate and 91 ± 7 μg equilin sulfate. Fig. 2 shows a graphic representation of the results.

DISCUSSION

The described direct HPLC assay is simpler and less time consuming than the GC method specified in the USP or the described HPLC analysis of free estrogens after enzymatic hydrolysis of the conjugates. It is also more specific and sensitive since it analyzes the parent steroid ester and not its hydrolysis product. Therefore it is recommended that this approach to the analysis of conjugated estrogens be considered in future revisions of the USP.

ACKNOWLEDGEMENTS

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Note

Rapid preparative-scale purification of peptides derived from caseins using mass ion-exchange chromatography

L. C. CHAPLIN*,* and A. T. ANDREWS
Biotechnology and Enzymology Department, AFRC Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2 9AT (U.K.)
(First received March 4th, 1988; revised manuscript received May 24th, 1988)

There is an increasing interest in the study of functional properties of peptides produced by enzymic digestion of proteins[1–4]. In order to study the properties of peptides in a systematic way it is necessary to work with highly purified peptides since it is reported that small amounts of impurities can drastically effect functionality (*e.g.* emulsifying properties[5]).

Peptides are often prepared in large quantities using trichloroacetic acid (TCA) or solvent precipitation techniques[6–8]. Conventional gel filtration[7,8] and ion-exchange chromatography[9] are also extensively used together with reversed-phase high-performance liquid chromatographic (HPLC) or fast protein liquid chromatographic (FPLC) techniques[10–12]. The HPLC/FPLC techniques are more suitable for preparing highly purified peptides in milligram amounts[12].

Large-scale purification of peptides is notoriously time consuming and a rapid, reproducible, large-scale preparative method is needed. The LKB mass ion-exchange cartridges provide one solution. This article describes the preparation of large amounts of highly purified acidic and basic peptides using DEAE-ZetaPrep 250 and SP-ZetaPrep 250 cartridges respectively. The acidic peptide is fragment 1–52 [β(1-52)] derived from β-casein by the action of α-chymotrypsin and its sequence has now been confirmed[13]. The basic peptide described is fragment 1–23 [α_{s1}(1-23)] of α_{s1}-casein obtained by enzymic digestion with chymosin. Peptide α_{s1}(1-23) has been prepared previously[14].

EXPERIMENTAL

Materials

All chemicals and biochemicals were from BDH (Poole, U.K.) unless stated otherwise. Bovine chymosin (rennin) (product No. R7751, E.C. 3.4.23.4), and α-chymotrypsin (type II, E.C. 3.4.21.1) were obtained from Sigma (Poole, U.K.).

Instrumentation

The LKB ZetaPrep 15 disks, ZetaPrep 250 mass ion-exchange cartridges, the

* Present address: Protein Chemistry Department, Celltech Ltd., 216 Bath Road, Slough, SL1 4EN, U.K.

0021-9673/88/$03.50

Frac-100 fraction collector and the laser densitometer were all from Pharmacia LKB Biotechnology (Milton Keynes, U.K.). A Perkin-Elmer PE-552 UV–VIS spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.) was used for monitoring the progress of the chromatographic separations.

Preparation and purification of acidic and basic peptides from total casein

Production of the acidic peptide β(1-52). Hammarsten casein (sodium caseinate, BDH), (100 g) was dissolved in 0.1 *M* sodium phosphate buffer, 0.02% (w/v) sodium azide, at pH 7.0 to a volume of 4 l. α-Chymotrypsin (1 mg/ml in the same buffer) was added to give 4.4 Sigma units of enzyme per gram of casein. The hydrolysis mixture was incubated at 37°C for 24 h. It was then heated to 90°C for 15 min in a boiling water bath to inactivate the enzyme.

The peptides were precipitated by addition of solid TCA to give a final concentration of 12.5% (w/v). After standing for 1 h at 4°C the precipitate was collected by centrifugation at 2000 *g* and 4°C for 15 min. It was washed five times by suspension and recentrifugation with acetone, air-dried and then dissolved in deionized water with the addition of dilute ammonia to adjust the pH to 7.0, and freeze-dried.

Determination of elution conditions for purification of β(1-52) using ZetaPrep 15 disks. DEAE- and QAE-ZetaPrep 15 disks were used to optimise elution conditions required for purification of β(1-52). Each disk was treated similarly. They were equilibrated with 0.05 *M* Tris–HCl, pH 7.0. The digest (0.75 g, prepared as above) was loaded onto the disk and eluted using a step-wise gradient with 2 × 20 ml portions of the same buffer for each step, containing increasing amounts of sodium chloride from 0.2 to 1.0 *M* in 0.05 *M* increments. A flow-rate of 5 ml/min was used throughout each experiment. The absorbance of the eluates was measured at a wavelength of 235 nm in 1-cm quartz cells. Polyacrylamide gel electrophoresis (PAGE), in $T = 20\%$, $C = 4\%$ gels [where T is the total acrylamide monomer concentration and C the percentage of cross-linker (Bis) in T] using the buffer system of Andrews[15], was used to monitor the progress of the separation and to assess purity. For the latter, gels were scanned with a laser densitometer (LKB Ultroscan) and the peak area of interest expressed as a percentage of the sum of the areas of all peaks present.

Large-scale purification of β(1-52) using the DEAE-ZetaPrep 250 anion-exchange cartridge. The DEAE-ZetaPrep 250 cartridge was pre-equilibrated with 1 l of 0.2 *M* Tris–HCl at pH 7.3 and equilibrated with 5 l of 0.05 *M* Tris–HCl (pH 7.0) using a flow-rate of 40 ml/min. Crude freeze-dried acidic peptide (18 g) prepared as described above was dissolved in 600 ml 0.05 *M* Tris–HCl, 0.15 *M* sodium chloride, pH 7.0. It was filtered through a Whatman No. 4 filter paper, in order to remove any undissolved material, before loading the sample onto the cartridge. The sample was loaded at a flow-rate of 10 ml/min to allow for maximum absorption of sample and, when loaded, the flow-rate was increased to 40 ml/min for elution. The first 500 ml of eluate was saved separately, then the sample components were eluted batch-wise using 0.05 *M* Tris–HCl buffer (pH 7.0) containing 0.29, 0.45 or 1 *M* sodium chloride. The volume of buffer used at each salt concentration was 2 l and 25-ml fractions were collected throughout the experiment. The absorbance of the eluate was measured in 1-cm quartz cells at a wavelength of 235 nm.

The fractions containing the purest peptide material (the 0.45 *M* sodium chloride fraction) were diluted to 0.22 *M* in sodium chloride by adding 0.05 *M* Tris–HCl

(pH 7.3) and recycled through the cartridge. Similar conditions to the first run were used but with smaller increments in salt concentration (0.22, 0.30, 0.35 and 0.45 *M* sodium chloride) to elute the peptide. The purest fractions were collected and the acidic peptide was isolated by TCA precipitation. It was washed five times by suspension and recentrifugation with acetone, air-dried, then redissolved in water with the addition of dilute ammonia to give pH 7.0, and freeze-dried.

Production of the basic peptide α_{s1}(1-23). Hammarsten casein (50 g) was dissolved in 0.05 *M* sodium acetate buffer (pH 6.4) to a volume of 5 l and the pH was readjusted to pH 6.4 with 5 *M* sodium hydroxide. Chymosin (1 mg/ml in the same buffer) was added to give 3.7 Sigma units of enzyme per gram of casein. The hydrolysis mixture was incubated at 37°C for 18 h. The enzyme was inactivated by heating to 90°C in a boiling water bath for 15 min. After cooling, deionized water was added to give a final volume of 12.5 l and a buffer concentration of 0.02 *M* sodium acetate.

Determination of the elution conditions required for the purification of α_{s1}(1-23) using ZetaPrep 15 disks. An SP-ZetaPrep 15 disk was equilibrated with 40 ml of 0.05 *M* sodium acetate at pH 6.4. The casein digest [100 ml, 1% (w/v), prepared as above] was loaded onto the disk and 200 ml of 0.05 *M* sodium acetate was passed through it. Then the peptide was eluted with 6 × 20 ml portions each of the same buffer containing 0.5 and 1 *M* sodium chloride. A flow-rate of 5 ml/min was used throughout the experiment. The eluate was collected and the progress of the separation monitored by acid PAGE using a method based on that of Reisfeld *et al.*[16] and as described by Maurer[17], in $T = 14.7\%$, $C = 0.67\%$ gels. Peptide purity was assessed by densitometry of PAGE patterns as above.

Large-scale purification of α_{s1}(1-23) using the SP-ZetaPrep 250 cation-exchange cartridge. The SP-ZetaPrep 250 cartridge was pre-cycled with 1.3 l of 0.1 *M* trisodium phosphate followed by 0.25 l of 0.1 *M* acetic acid. For final equilibration 5 l of 0.02 *M* sodium acetate at pH 6.4 was used. The digested casein containing the basic peptide (12.5 l) prepared as described above was loaded onto the cartridge at a flow-rate of 40 ml/min. The cartridge was then eluted stepwise with two 1-l volumes of 0.02 *M* sodium acetate (pH 6.4) followed by 2-l portions at each step of the same buffer containing 0.05 to 0.5 *M* sodium chloride increasing in 0.05 *M* increments. Fractions (25 ml) were collected and the absorbance of the eluate was monitored in 1-cm path length quartz cells at a wavelength of 214 nm.

The appropriate fractions were pooled and the peptide precipitated by the addition of solid TCA to give 12.5% (w/v). It was collected, washed in acetone and freeze-dried as described for the isolation of the crude acidic peptide.

RESULTS AND DISCUSSION

Small-scale experiments using ZetaPrep 15 disks were used in order to determine which salt concentrations were required for optimal separation of the two peptides. Using the DEAE-ZetaPrep disk it was evident that the acidic peptide β(1-52) eluted at a salt concentration of 0.3–0.4 *M* sodium chloride. The QAE-ZetaPrep 15 disk did not give a better separation of the peptide and therefore a DEAE-ZetaPrep 250 cartridge was used for scaling-up the purification of this peptide. The basic peptide α_{s1}(1-23) eluted in the 0.5 *M* sodium chloride fraction when separated on the SP-ZetaPrep 15 disk, together with one main impurity (most likely to be *para*-κ-

casein from the cleavage of residues 105–106 in κ-casein by chymosin[18]). For the large-scale separation of α_{s1}(1-23), shallower increments of sodium chloride were used with the SP-ZetaPrep 250 cartridge.

Most peptides obtained by the enzymic hydrolysis of caseins with proteinases are acidic. The casein digest containing the acidic peptide β(1-52) was loaded onto the DEAE-ZetaPrep cartridge at a salt concentration of 0.35 *M* sodium chloride which, from the preliminary ZetaPrep disk experiments, was found to be just below the salt concentration at which the acidic peptide of interest elutes. In this way the sample loading can be increased, as some of the less acidic peptides are not retained by the matrix of the cartridge while the required peptide and the more acidic peptides are. The loading capacity of the DEAE-ZetaPrep 250 cartridge for casein peptides is approximately 15 g. Peptide β(1-52) eluted mainly in the 0.45 *M* sodium chloride fraction during the first cycle through the cartridge. This method produced a peptide with a purity of approximately 85% as monitored by densitometric scanning of polyacrylamide gels. In order to obtain a purer peptide, the fractions containing the required peptide were recycled through the cartridge, using smaller increments in salt concentration. During the second cycle through the cartridge the peptide eluted in the

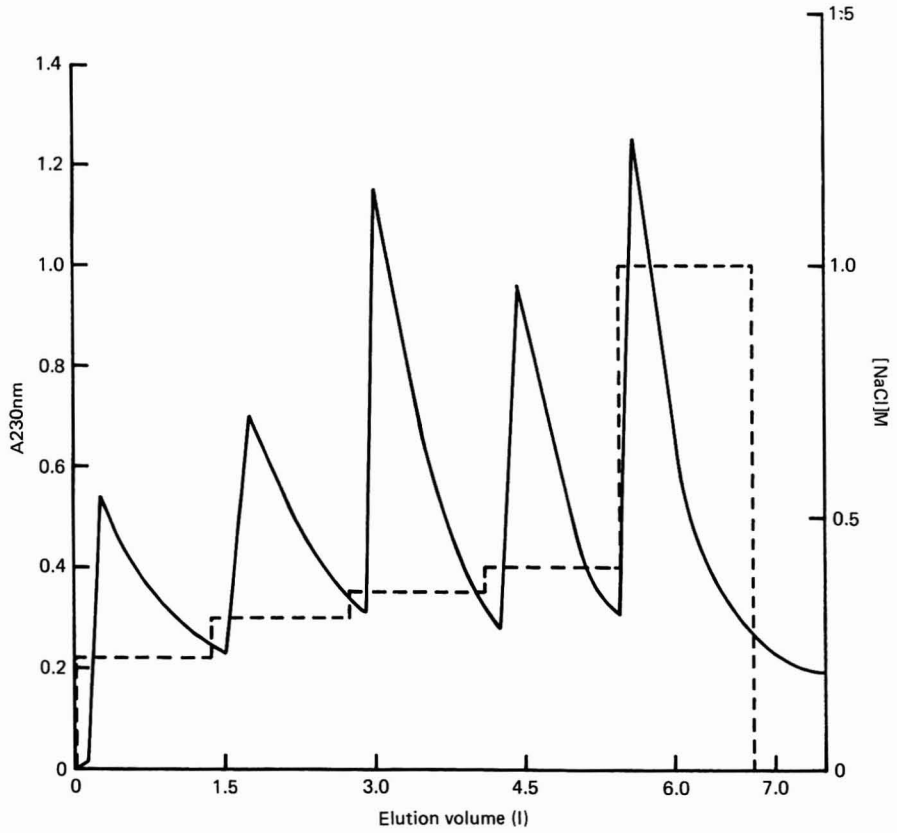

Fig. 1. Elution profile obtained for the separation of 15 g total casein digested with α-chymotrypsin on a DEAE-ZetaPrep 250 cartridge. For run conditions see text.

0.3 and 0.35 *M* sodium chloride fractions. Three chromatographic separations of this type produced a total yield of β(1-52) of 3.9 g with a purity of approximately 95%. Fig. 1 shows the elution pattern obtained for the preparation of the peptide β(1-52) using the DEAE-ZetaPrep 250 cartridge, during the second cycle through the cartridge.

The majority of peptides in the total casein digest being acidic, will pass straight through the SP-ZetaPrep 250 cation-exchange cartridge. For this reason it is easier to obtain the basic peptide in a pure form in one run than it is the acidic one. The yield of basic peptide α_{s1}(1-23) with a purity of more than 95% obtained from two separate chromatographic runs, without recycling, using this method was 2.01 g.

Fig. 2 shows PAGE separation of the peptides obtained using DEAE- and

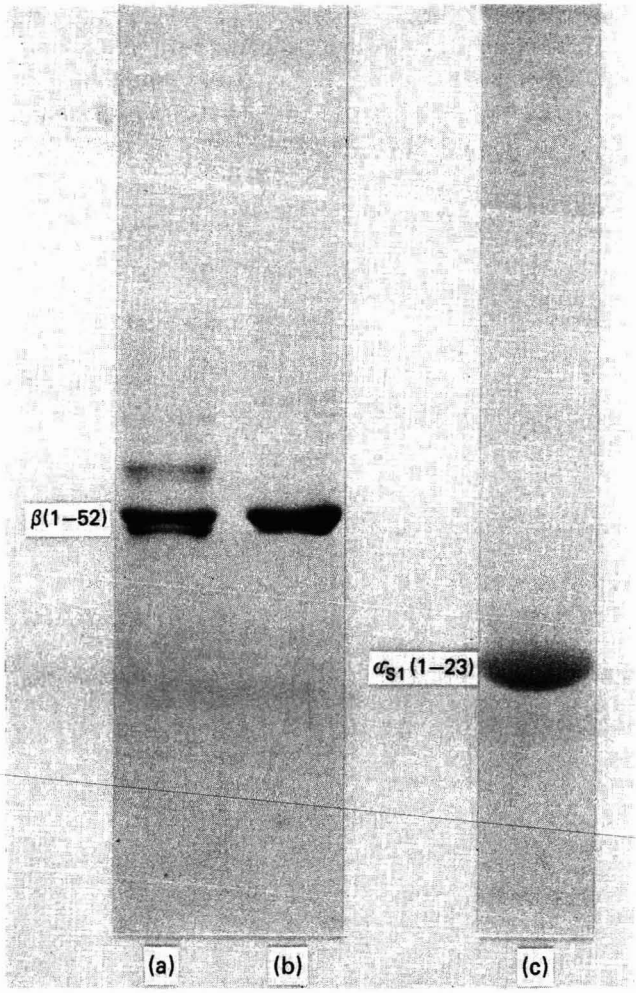

Fig. 2. PAGE patterns obtained for peptides prepared using LKB ZetaPrep 250 mass ion-exchange cartridges. (a) Acidic peptide β(1-52) obtained after one cycle through the DEAE cartridge. (b) The same peptide after recycling through the cartridge (T = 20%, C = 4% for the PAGE gel). (c) Basic peptide α_{s1}(1-23) obtained after one cycle through the SP cartridge (T = 14%, C = 0.67% for the acid PAGE gel).

SP-ZetaPrep 250 cartridges. Fig. 2a shows the acidic peptide β(1-52) obtained from the first cycle through the cartridge (approximately 85% pure) and Fig. 2b shows the purity of the peptide when it is recycled through the cartridge (approximately 95% pure). Fig. 2c shows the basic peptide α_{s1}(1-23) obtained from one passage through the SP-cartridge (>95% pure), run on an acid polyacrylamide gel.

This method shows considerable advantages over conventional ion-exchange chromatography. It is fast and convenient with run times being in minutes rather than hours. LKB mass ion-exchange cartridges should be applicable to the separation of large quantities of highly purified peptides and proteins from a wide variety of sources. Due to their high loading capacities they may be particularly suitable for an initial step in more complex purification regimes of peptides and proteins. We have shown that conditions optimised on a small scale with ZetaPrep disks can be directly transposed to the much larger cartridges, with virtually unchanged separation results, in spite of the scaling-up by a factor of about 150, so it seems reasonable to suppose that even larger cartridges would perform similarly under the same conditions of pH, buffer and ionic strength. Cartridges with larger capacities than used in this work and capable of binding 500–1500 g of material are now available from Anachem*.

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* ZetaPrep cartridges are no longer marketed by Pharmacia LKB but they can be purchased from Anachem (Luton, U. K.)

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Note

Determination of the polymeric light stabilizer Tinuvin 622 in polyolefins

W. FREITAG* and R. WURSTER
Central Analytical Department, FO 3.32, Ciba-Geigy AG, Postfach, CH-4002 Basle (Switzerland)
and
N. MADY
Analytical Research Department, Ciba-Geigy Corp., Ardsley, NY (U.S.A.)
(First received April 8th, 1988; revised manuscript received June 16th, 1988)

Plastics cannot be used in practical applications unless they are protected by several additives such as antioxidants, UV absorbers and light stabilizers. These additives protect the polymer against degradation during the manufacturing process and through long periods of usage. Since the introduction of hindered amine light stabilizers (HALS) as commercial products in the early 1970s, numerous studies on the mechanism by which HALS stabilize polymers against the effect of light irradiation were published[1–8]. Concurrently, the analysis of commercially known HALS such as Tinuvin 770 and Tinuvin 144 has been well established by chromatographic and spectroscopic techniques[9–11]. However, since the introduction of polymeric-type HALS such as Chimassorb 944 and Tinuvin 622, the need arose for a good quantitative method of determining the level of polymeric HALS in polymers. Satisfactory methods have so far been developed for the determination of Chimassorb 944[12–14] in polymers. However, attempts to determine the concentration of Tinuvin 622 in polymers[15–17] were either unspecific or required large-scale equipment such as an FT-NMR spectrometer. This paper presents a method that is specific for the determination of Tinuvin 622 with no interference from other known additives or pigments. The method is accurate, reproducible and yields good recoveries.

PRINCIPLE OF THE METHOD

This paper describes the sample preparation and a chromatographic method for the determination of Tinuvin 622 in polyolefins. The first part of the method consists in dissolving the polyolefin in boiling toluene followed by the addition of an alcoholic solution of tetrabutylammonium hydroxide. The polymeric light stabilizer Tinuvin 622 is thereby saponified to 4-hydroxy-2,2,6,6-tetramethyl-1-piperidineethanol ("diol") (see Fig. 1).

Saponification by tetrabutylammonium hydroxide is the first step in the transesterification procedure described[18] for the preparation of methyl esters from polyester resins. As we were interested in the analysis of the "diol" rather than in the

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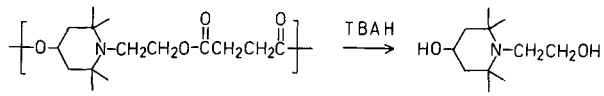

Tinuvin 622 "diol"

Fig. 1. Saponification of Tinuvin 622 by tetrabutylammonium hydroxide (TBAH).

succinic acid moiety of Tinuvin 622, the derivatization of the succinic acid to the corresponding methyl ester was omitted.

The addition of the alcoholic solution of the saponification reagent precipitates simultaneously the polyolefin matrix. The "diol" is then quantified using either an isocratic or a gradient high-performance liquid chromatographic (HPLC) system on an amino-modified phase for separation. The advantage of the gradient system is the separation of any matrix interference if it occurs. The method can be used to determine Tinuvin 622 in polyolefins in the concentration range 0.05–1%, which represents the usual application concentration.

EXPERIMENTAL

Materials

Tetrabutylammonium hydroxide (25% in methanol) was obtained from Fluka. Toluene and ethanol were of analytical-reagent grade and were used without further purification. *n*-Hexane (from Fluka) was of HPLC grade. All solvents used for HPLC analysis were vacuum degassed or purged with helium before use. 4-Hydroxy-2,2,6,6-tetramethyl-1-piperidineethanol ("diol") was obtained from Ciba-Geigy (Additives Division).

Apparatus and chromatographic conditions

The HPLC system consisted of a Waters M-45 pump, a Kratos Spectroflow 773 UV detector, a Rheodyne 7010 injection valve and a Hewlett-Packard 3390A integrator. The analytical column used was made of stainless steel (250 × 4.6 mm I.D.) packed with Nucleosil NH_2, 10 μm (Macherey, Nagel & Co.) and operated at ambient temperature. The injection volume was 20 μl. The mobile phase, pumped at a flow-rate of 2 ml/min, was *n*-hexane–ethanol–toluene (88:11:1, v/v). The ratio of *n*-hexane *vs.* ethanol has to be adapted to the quality of the column. The detector was operated at 225 nm.

In the event of matrix interferences, a linear solvent gradient of *n*-hexane–ethanol (from 1% to 16% ethanol in 12 min) was used. Advantageously, the gradient system was run in cycles; *e.g.*, after an equilibration period of 17 min at 1% ethanol the gradient was started again.

The injection time depends on the dead volume between solvent mixing and the injection valve. It may vary depending on the construction, *e.g.*, pulse damper. The injection was preferably carried out just in front of the baseline rise (see Fig. 3); in our example the sample was injected at the start of the gradient.

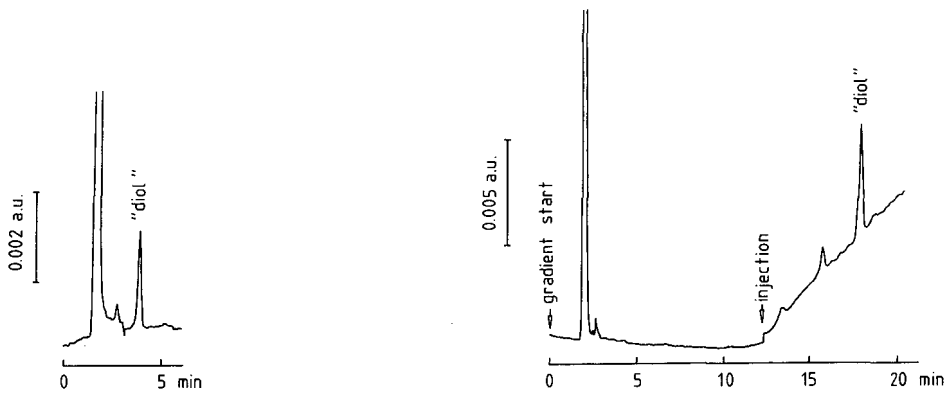

Fig. 2. Chromatogram of a low-density polyethylene sample containing 0.2% of Tinuvin 622 under isocratic conditions (see chromatographic conditions); detection at 227 nm.

Fig. 3. Chromatogram of a high-density polyethylene sample containing 0.2% of Tinuvin 622 with the solvent gradient (see chromatographic conditions); detection at 225 nm.

Calibration solution

The calibration solution contained 20 mg of the monomeric "diol" per litre of toluene–ethanol (1:1, v/v).

Procedure

Approximately 1 g of polyolefin sample was weighed and dissolved in 50.0 ml of toluene by refluxing. The solution was cooled to about 95°C. Slowly, 50.0 ml of ethanol containing 2 ml of tetrabutylammonium hydroxide (25% in methanol) were added through the top of the reflux condenser, whereupon the polymer precipitated. After cooling to room temperature a 2-ml aliquot of the supernatant liquid was filtered through a filter of 0.45 μm pore size. The filtrate was suitable for injection. Fig. 2 shows a typical chromatogram of the isocratic method; Fig. 3 is an example of the gradient method.

The calculation of the concentration of the "diol" was performed by the external standard method. The concentration of Tinuvin 622 was calculated from the concentration of the "diol" by multiplication by a factor of 1.41.

RESULTS AND DISCUSSION

To test the accuracy and reproducibility of the method, additions of 0.20% of Tinuvin 622 were made to blank polypropylene and high-density polyethylene before the dissolution step. The analysis of Tinuvin 622 resulted in a recovery of 100–105% in this concentration range. The relative standard deviations for an addition of 0.2% of Tinuvin 622 to each of six samples of blank high-density polyethylene and of blank polypropylene were 1.4% and 3.0%, respectively.

The advantage of the method is that (i) it is sufficiently specific and to our knowledge other additives, *e.g.* pigments, do not interfere, (ii) it is accurate and reproducible and (iii) because of the transformation of the polymeric structure into a well defined monomer with a narrow peak shape, the sensitivity is high.

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CHROM. 20 751

Note

Determination of the polymeric light stabilizer Chimassorb 944 in polyolefins by reversed-phase high-performance liquid chromatography

W. FREITAG
Central Analytical Department, FO 3.32, Ciba-Geigy AG, Postfach, CH-4002 Basle (Switzerland)
(First received March 28th, 1988; revised manuscript received June 6th, 1988)

To protect polyolefins from the degrading action of light, the addition of light stabilizers is necessary[1]. For the protection of thin materials such as fibres or films, light stabilizers of both low volatility and low extractability had to be developed. The polymeric amine Chimassorb 944 (Fig. 1), one of the commercially most important light stabilizers for polyolefins, meets these requirements[2] because of its relatively high molecular mass. Because of its polymeric structure the determination of Chimassorb 944 in a polyolefin matrix is more difficult than that of a monomeric stabilizer. Quantification by UV spectroscopy[3] at 245 nm after extraction into dilute sulphuric acid proved to be reliable, similarly to the use of IR spectroscopy[4,5]. More specific methods include pyrolysis–gas chromatography[6] and high-performance liquid chromatography (HPLC) using a size-exclusion column with a flame ionization detector[7].

This paper describes a specific reversed-phase HPLC method requiring only standard HPLC equipment. It consists in dissolving the polyolefin in refluxing toluene and after precipitation of the polyolefin, analysis of the filtrate using a C_{18} reversed-phase column and UV detection at 240 nm. The method is applicable to the determination of the stabilizer in virgin polymer.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Spectra-Physics 8700 pump, a Spectra-Physics 8780 XR autosampler equipped with a 50-μl injection loop, a Kratos Spectroflow 773

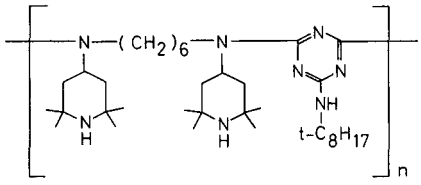

Fig. 1. Chimassorb 944.

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UV detector operated at 240 nm with a sensitivity of 1.0 a.u.f.s. and a Spectra-Physics 4270 integrator equipped with a replay module for baseline subtraction and re-integration. The analytical column was made of stainless steel (125 × 4.6 mm I.D.), packed with Nucleosil C_{18} (5 μm) from Macherey, Nagel & Co. and operated at room temperature.

Solvents and mobile phase

Toluene, methanol (for sample preparation) and acetone were of technical quality. Diethanolamine was obtained from Merck. Tetrahydrofuran (Fluka) and methanol (Merck) were of HPLC quality. The mobile phase consisted of solvents A and B: (A) tetrahydrofuran–water–acetone (900 ml + 100 ml + 1 ml); (B) tetrahydrofuran–methanol–diethanolamine (900 ml + 100 ml + 1.5 g). The gradient was a linear gradient from 100% A to 100% B within 7 min, then 100% B for 10 min; after 7 min of re-equilibration with 100% A, the next injection was started.

Procedure

A 0.9–1.1 g polyolefin sample was weighed into a flask and dissolved in 25 ml of toluene by refluxing for about 40 min with stirring. After cooling to about 60°C, 75 ml of methanol were added through the top of the reflux condenser. The suspension was allowed to cool to room temperature and filtered under vacuum through a Büchner funnel. The filter cake was washed with 10 ml of toluene. The combined filtrate was evaporated to dryness and the residue dissolved in exactly 5 ml of tetrahydrofuran.

A calibration solution for HPLC was prepared by dissolving 25 mg of Chimassorb 944 in 50 ml of tetrahydrofuran. This solution served as an external standard solution.

RESULTS AND DISCUSSION

For a convenient HPLC determination of a polymeric mixture such as Chimassorb 944, it is desirable that the peaks of the individual homologues elute as

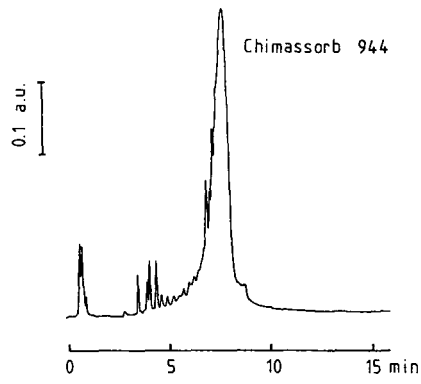

Fig. 2. Chromatogram of an extract of polyethylene stabilized with 0.3% of Chimassorb 944. Injection volume: 50 μl; UV detection at 240 nm, without baseline correction. Column: C_{18}, 5 μm. The peak was integrated in the region from 5 to 10 min. For other conditions see Experimental.

closely as possible to form a narrow peak. On the other hand, HPLC conditions that lead to a very narrow peak may not provide a sufficient separation from other additives of similar structure such as Chimassorb 119. The chromatogram shown in Fig. 2 is typical for the above-described conditions.

During the evaluation of these conditions it was found that diethanolamine must be absent from the mobile phase at the beginning of the gradient in order to reduce the peak width. The presence of a small amount of acetone in solvent A helped to reduce the baseline shift, which itself depends on the quality of the solvents. For the evaluation of the peak area, which was used for quantification by the external standard method, it was helpful to subtract the baseline before integration; for reduced accuracy the peak height could be used for calculation.

The method can be used to determine 0.05–0.5% of Chimassorb 944 in polyolefins. To test the accuracy and reproducibility of the method, 0.25% of Chimassorb 944 was added to each of six samples of base-stabilized polypropylene: the mean recovery was 96% with a relative standard deviation of 2.2% ($n = 6$). The recovery of 0.05% of Chimassorb 944 added to polypropylene was 83%. The method was also tested for low-density polyethylene. Other additives that may be present in the polyolefin such as phenolic antioxidants, processing stabilizers and UV absorbers are eluted without retention and, therefore, do not interfere.

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CHROM. 20 813

Note

High-pressure size-exclusion chromatography of chlorinated and unchlorinated aquatic fulvic acids using a polyacrylamide gel

JESSICA St. AUBIN JACOBS, RUSSEL F. CHRISTMAN and J. DONALD JOHNSON*
Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, Box 7400, Rosenau Hall, 201-H, Chapel Hill, NC 27599 (U.S.A.)
(First received November 17th, 1987; revised manuscript received May 30th, 1988)

The formation of chlorinated organic compounds during drinking water chlorination is well documented[1–5]. Attempts to identify these byproducts have been only moderately successful. Low-molecular-weight volatile and acidic compounds such as chloroform and trichloroacetic acid have been shown to account for a small fraction of the total chlorine incorporation under drinking water conditions[5–7]. Under more vigorous laboratory conditions these compounds have been shown to account for one-fourth to one-half of the total organic halogen (TOX) produced[8,9]. In addition, chlorinated water has been shown to be mutagenic[8,10–13]. While some of the more volatile mutagenic components have been identified, the contribution of identified volatile products to the total does not account for a significant portion of the observed mutagenicity[8]. This fact suggests that much of the mutagenicity may be non-volatile and part of the TOX fraction[8,11–13]. The non-volatile TOX, because of its nature, remains largely unidentified. This fraction has been postulated to be of high molecular weight (macromolecuar)[14,15] and/or very polar[16]. This work was initiated to attempt a characterization of these non-volatile TOX fractions.

Naturally occurring organic matter, primarily humic substances, are the organic precursors for TOX formation in drinking water through oxidation and substitution by free chlorine. Aquatic humic substances, which impart the yellow-brown color to natural waters, are usually subdivided into three fractions based upon solubility in acid and base[17]. Fulvic acid is the most hydrophilic and has the lowest molecular weight of the three fractions. Furthermore, fulvic acid accounts for the largest percentage of the dissolved organic carbon in surface waters attributable to humic substances[18]. For this reason, isolated aquatic fulvic acid was chosen as the model precursor in this study of chlorination byproducts.

Humic substances are polymeric and as such can be characterized by their molecular weight distribution. Since humic substances are condensation products of natural product material they are not likely to polymerize in a consistent manner. The source from which they are derived influences their composition as well as their molecular weight. The conformation (size, shape and degree of aggregation) of humic substances in solution is also influenced by the large number of functional groups contained in the molecule[19]. Methods commonly used for molecular weight determinations of humic substances include low-pressure Sephadex (Pharmacia) gel filtra-

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tion[7,20–25], ultrafiltration[15,26], small-angle X-ray scattering[27], and high-pressure size-exclusion chromatography (HPSEC) including high-pressure gel filtration on silica based supports[15,28–30], and high-pressure gel permeation[31]. Despite the wide range of reported values, fulvic acid is generally found to be of low molecular weight (<2000)[27].

HPSEC was chosen because it would allow the fulvic acids to be separated for further characterization. An aqueous mobile phase was sought because water is the natural solvent of fulvic acid. Because low-pressure SEC is time consuming and yields poor resolution, a support which could withstand increased pressures to improve resolution and speed the separation was sought. Rigid silica based supports hold up well to the high pressures, but any free silanol units are possible sites for interaction with the many functional groups of fulvic acid. It was for this reason that a non-silica support was chosen.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1084B liquid chromatograph equipped with an auto-injection system, variable-wavelength detector and fraction collector was employed. The oven temperature was maintained at 30°C. A Waters Model 410 differential refractometer was used to monitor elution of non-UV absorbing standards. TOX measurement utilized the Dohrmann MCTS-20 microcoulometric titration system. Total organic carbon (TOC) was measured with a Beckman 915B TOC analyzer.

Columns

The packing material consisted of a 10-μm particle size, highly cross-linked polyacrylamide gel with a pore size distribution to separate polysaccharides between 100 and 10 000 dalton. The 30 cm × 7.5 mm I.D. stainless-steel column was prepacked and sold as PL-Aquagel 10 μm by Polymer Labs. (Shropshire, U.K.). A precolumn of the same material (5 cm × 7.0 mm I.D.) was used after the injector.

Reagents

All mobile phases were made using water prepared from either a Corning LD-2a water purification system or a Millipore Milli Q system. Sodium tetraborate decahydrate was soxhlet extracted with diethyl ether for 24 h prior to use. All mobile phases were vacuum filtered using a 0.2-μm Nylon-66 (DuPont) membrane filter and degassed.

Compounds used to calibrate the size-exclusion system are listed in Table I. Aquatic fulvic acid was isolated from Singletary Lake, NC, U.S.A., by the modified procedure of Thurman and Malcolm[18,32]. Sodium hypochlorite (4–6% laboratory grade, Fisher Scientific) was diluted 1:10 with purified water and neutralized to pH 7 with phosphoric acid. Phosphoric acid, sodium hydroxide, sodium arsenite and all salts used in the mobile phases were reagent grade.

Procedures

Sample preparation. Fulvic acid was dissolved to 50 ppm (as fulvic acid) in purified water. The pH of this fulvic acid solution was adjusted to 7, followed by

TABLE I

CALIBRATION STANDARDS

A = Sigma; B = Aldrich; C = Fisher Scientific; D = Mallinckrodt; E = Burdick & Jackson Labs.

Standard	*Molecular weight*	*Supplier*	*Detection*
Dextran	17 900*	A	RI
Dextran	9000*	A	RI
Polyethylene glycol	8000*	B	RI
Polyethylene glycol	3400*	B	RI
Tannic acid	1701**	A	UV (254 nm)
Polyethylene glycol	1000*	B	RI
1,2,4,5-Benzenetetracarboxylic acid	254	B	UV (254 nm)
1,3,5-Benzenetricarboxylic acid	210	B	UV (254 nm)
Trichloracetic acid	163	A	UV (210 nm)
Dichloroacetic acid	129	C	UV (210 nm)
Acetic acid	60	D	UV (210 nm)
Acetone	58	E	UV (263 nm)

* Average molecular weight.

** Molecular weight information was not supplied by Sigma. The value listed is only an estimate for this material[44].

addition to the diluted preneutralized sodium hypochlorite to produce initial Cl_2 to C molar ratios of 1.14, 0.63, and 0.065. Residual chlorine was measured by the DPD ferrous titrimetric method[33]. The reaction proceeded for 24 h in darkness at ambient temperature. pH was kept near 7 by addition of small amounts of acid or base. Reactions were quenched by addition of sodium arsenite after final residual chlorine analysis. Samples were freeze concentrated, vacuum filtered (0.5-μm Nylon-66 membrane filters) and pH adjusted to 9.2.

Sample analysis. Calibration standards were prepared with mobile phase as diluent. Chlorinated and unchlorinated fulvic acid were eluted with 0.01 *M* borate buffer (pH 9.2) at a flow-rate of 0.5 ml/min. Six fractions were collected for TOX and TOC analysis. Calibration was accomplished by the peak position method[34]. Table I lists the detection systems used in calibrating the system.

RESULTS AND DISCUSSION

Column and mobile phase selection

Choice of the size-exclusion column was made after careful comparison of commercially available non-silica HPSEC columns compatible with aqueous mobile phases. Size exclusion of humic materials has classically been done using Sephadex as the support medium. Sephadex, however, contains some terminal carboxylic acid groups which serve as sites for cation exchange[24], has an affinity for aromatic and heterocyclic compounds[35], and is too soft to hold up to the pressures encountered in high-pressure liquid chromatography. Only grade G-25 (molecular weight < 5000) is rigid enough to be used in high-pressure systems, but only up to a pressure of 150 p.s.i.[34]. In addition, solute-support interactions are much more pronounced with the

small pore sized, highly cross-linked grades such as G-25[36]. Bruchet *et al.*[7] used low-pressure Sephadex G-25 gel filtration with distilled water elution to characterize surface and chlorinated waters and found that most of the TOX was found in the low-molecular-weight fraction (< 1000). However, it is difficult to interpret his results since calibration information was not provided. Liao[37] used low-pressure SEC of degraded and undegraded aquatic humic materials on Amberlite XAD-8, a semi-polar macroporous methylmethacrylate polymer (Rohm and Haas, Philadelphia, PA, U.S.A.), and obtained relatively good separation although a polarity effect was seen. Neither Sephadex nor XAD-8 were available commercially as HPSEC columns.

Bio-Gel P (Bio-Rad, Richmond, CA, U.S.A.) is a polyacrylamide gel commonly used in SEC. It is produced by free radical polymerization via the vinyl functional groups of acrylamide (a monovinyl monomer) and N,N′-methylene bisacrylamide (a divinyl monomer which serves to cross-link the polymer chains). Polyacrylamide does not contain a net charge, although the amide functional groups do display acidic properties which convey the gel's hydrophilicity. Consequently, hydrogen bonding between the amide functional groups and basic proteins[38], heterocyclic compounds[39], and substituents on aromatic compounds[40] has been shown to occur. Despite these possible drawbacks, polyacrylamide was chosen since it was commercially available as a high-pressure support (up to 1500 p.s.i.), its pore size distribution would yield a separation in the range desired, and it had not been unsuccessfully used in such an application. It was also felt that a mobile phase could be found to minimize interactions[23].

A number of different aqueous mobile phases were sequentially tested using elution behavior of model compounds as indicators of mobile phase performance. A mobile phase was sought to minimize aromatic adsorption, ion-exclusion and ion-exchange interactions between the solutes and support. Deionized-distilled water was tried first since it represents the simplest possible eluent. However, resolution was extremely poor and retention was highly dependent upon sample ionic strenght. Increasing the ionic strength of the mobile phase to low values with neutral salts markedly improved resolution and retention of acids (*i.e.*, reduced exclusion) but aromatic adsorption was still a problem. Further increase of ionic strength only served to increase retention of the acids beyond the inclusion volume without elimination of aromatic adsorption. Low pH elution resulted in severe retention of acids and also didn't overcome the problem with aromatics.

Elution as the ammonium salts was also unsuccessful. Swift and Posner[23] suggested that larger size amines (*e.g.*, Tris, triethylamine and ethylamine) reduce or eliminate aromatic adsorption by complexing with the active sites in the gel matrix. As weak bases, they also tie up ion-exchange sites. However, addition of organic modifiers was to be avoided if TOC balances were to be done. Swift and Posner[23] also suggested that use of a 0.025 *M* borate buffer would result in elimination of aromatic interactions, although they found some humic acid fractions were still adsorbed on the polyacrylamide. Borate is known to form borate esters of polysaccharides through interaction with the vicinal OH groups[41]. Sephadex, which is a polydextran gel, is likely to interact with borate in this manner. Polyacrylamide, however, contains amide functional groups which probably interact through hydrogen-bond formation with the borate. Low ionic strength (0.0001 *M*, pH 9.0) borate was first tried and it appeared to overcome the aromatic adsorption but exclusion of acids (early elution)

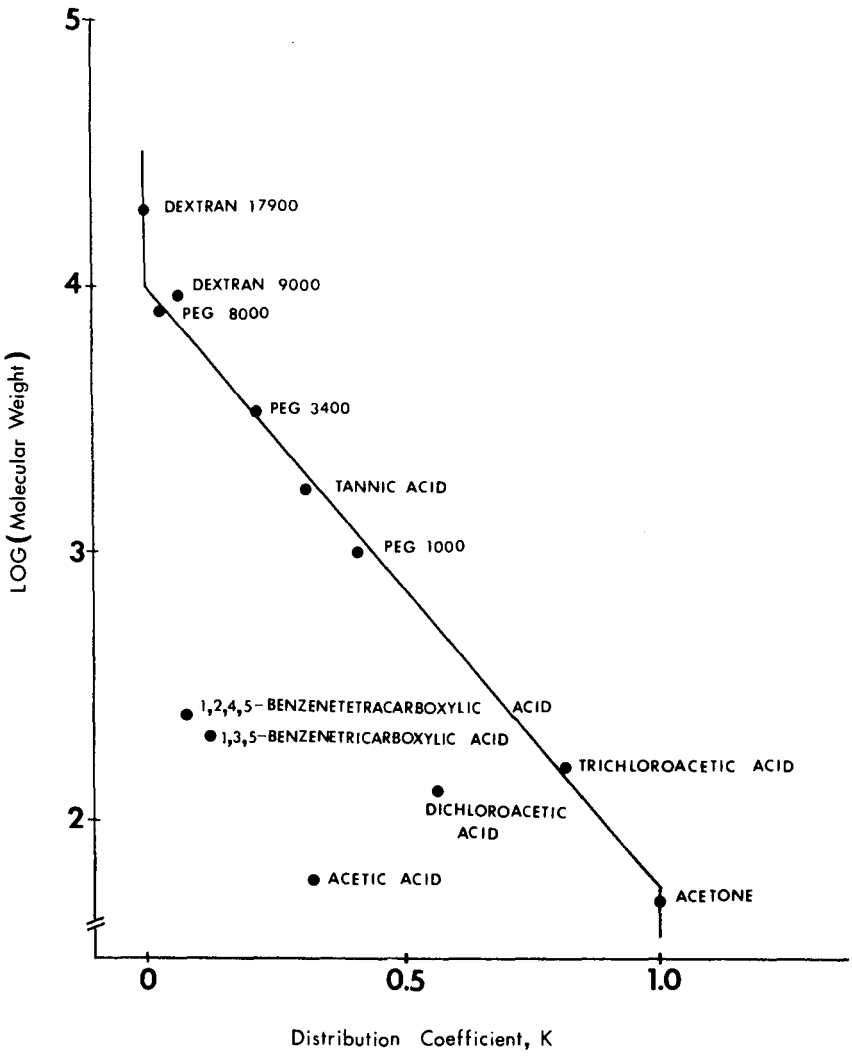

Fig. 1. Calibration curve. Distribution coefficient, K, is defined as $(V_R - V_0)/(V_i - V_0)$, where V_R = retention volume; V_0 = void volume; V_i = inclusion volume.

was still a problem. Since trichloroacetic acid is a major product of fulvic acid chlorination, it was desired that it elutes along the calibration curve, and this was achieved with a borate concentration of 0.01 M (pH 9.92). Fulvic acid did not appear to adsorb under these conditions.

The calibration curve is shown in Fig. 1. Unavailability of humic-like standards prompted the use of narrow-molecular-weight-range dextrans and polyethylene glycols. Low-molecular-weight degradation products of fulvic acid chlorination were used to calibrate the lower end of the range. From the calibration curve it is seen that in addition to the desired elution based on molecular weight, exclusion of acids was

occurring. The series acetic acid, dichloroacetic acid, and trichloroacetic acid is seen to elute in the order of increasing acid strength, independent of molecular weight. This order of elution was seen with all mobile phases tested and could not be overcome by increasing mobile phase ionic strength as suggested by Williams[36]. These results compare with those of Streuli[40] who found trichloroacetic acid and acetic acid to elute in the same order on bio-Gel F-2 using dilute sodium chloride elution. Ion exclusion also occurred with the benzenecarboxylic acids. As with Sephadex, the charge density is higher in the low-water-regain gels due to the larger percentage of cross-linking and perhaps this is why ion exclusion is such a problem with this highly cross-linked polyacrylamide[36].

Since all standards as well as chlorinated and unchlorinated fulvic acid eluted within the total column volume, it was decided to use the 0.01 *M* borate buffer as mobile phase. The obvious deviations from true size-exclusion behavior using this system along with the evidence that silica supports also are inappropriate may be an indication of the unsuitability of humic materials as solutes for SEC[24,42].

Application to chlorinated aquatic fulvic acid

The non-purgeable organic halide (NPOX) produced in the reaction of Singletary Lake fulvic acid with aqueous chlorine was separated, using this high-pressure size-exclusion system, into six fractions based upon apparent molecular weight and characterized for TOX. The NPOX distribution and UV absorbance at 254 nm were observed while varying the initial Cl_2 to C molar ratio of the reaction. Only NPOX was measured since the purgeable fraction was lost in sample preparation and since most of the unidentified and all macromolecular or highly polar TOX is in the non-purgeable fraction.

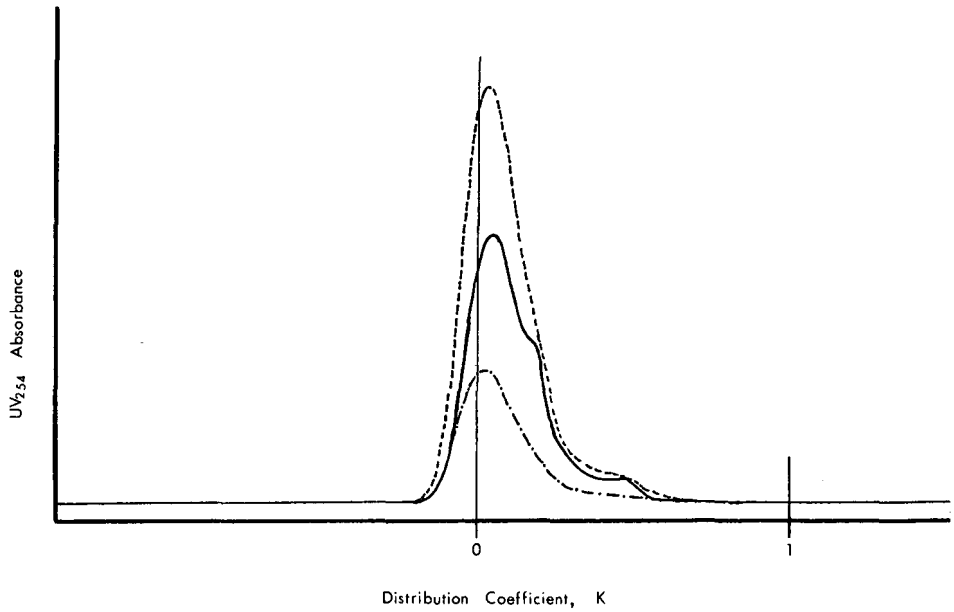

Fig. 2. UV absorbance at 254 nm as a function of distribution coefficient. – · – · –, Fulvic acid control, 20 μl (4.2 μg C); – – –, Cl_2:C = 0.065, 50 μl (13 μg C); ———, Cl_2:C = 0.63, 100 μl (18.8 μg C).

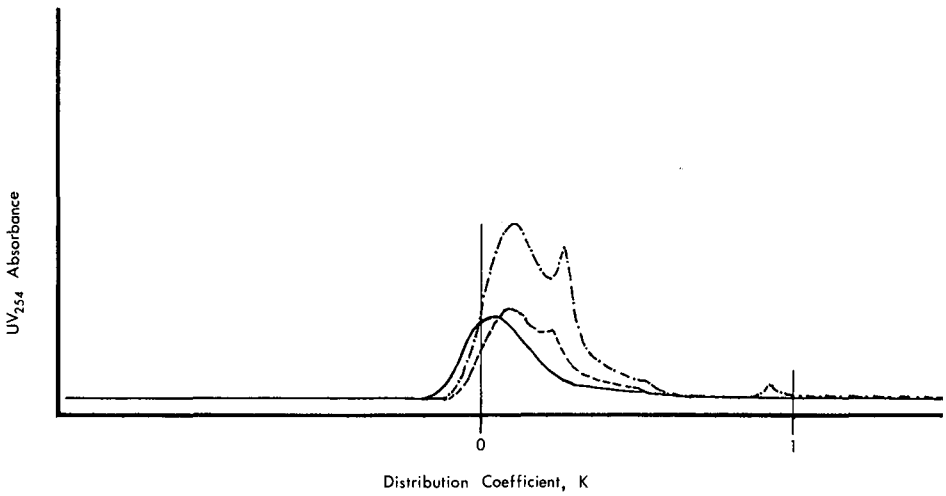

Fig. 3. UV absorbance at 254 nm as a function of distribution coefficient. – · – · –, Cl_2:C = 1.14, 100 μl (19.6 μg C); – – – –, Cl_2:C = 1.14, 50 μl (9.8 μg C); ———, fulvic acid control, 20 μl (4.5 μg C).

The effect of chlorination on the chromatogram was seen to be three-fold: (1) decreased peak height or sample absorption at 254 nm (color); (2) shift to lower $M_{max.}$; and (3) change in peak shape. In Fig. 2 it is seen that the UV absorption was less for Cl_2:C = 0.63 than for Cl_2:C = 0.065 even though less of the Cl_2:C = 0.065 sample was applied. In Fig. 3 it is seen that UV absorbance of the Cl_2:C = 1.14 sample was even less. The shift to apparently lower-molecular-weight compounds is also seen. Finally, the peaks change from mononodal (no chlorination) to approximately trinodal with increasing chlorination.

As illustrated in Fig. 3, despite changes in peak shape, peak position did not vary with sample size —a criterion Swift and Posner[23] suggest is necessary for a valid SEC system. A second criterion is that all sample must elute within the total column volume and this was also seen to occur. The final criterion is that re-injection of fractionated material must result in distinct peaks at the expected $M_{max.}$. As shown in Fig. 4, re-injection of fractions 1–3 of the Cl_2:C = 1.14 sample did result in distinct peaks. However, the maxima, except for fraction 1, occurred at volumes earlier than expected. This makes interpretation of the third criterion difficult.

Because the size-exclusion system was strongly affected by ion exclusion, any decreases in $M_{max.}$ could be due to increasing polarity of the reaction products, degradation to smaller molecules, or both. At low Cl_2 to C molar ratios where little oxidation occurs, decreases in $M_{max.}$ due to degradation probably will be small since substitution or addition of chlorine to a molecule will increase its molecular weight. At high Cl_2 to C molar ratios where small chlorinated acids are the majority of identifiable chlorine-containing products, a shift to apparently low-molecular-weight chlorinated products was expected. However, since these chlorinated acids tend to be excluded by the support material, TOX of apparently high molecular weight may actually be these low-molecular-weight chlorinated acids.

The organic halide content of the fractionated material is listed in Table II. It is

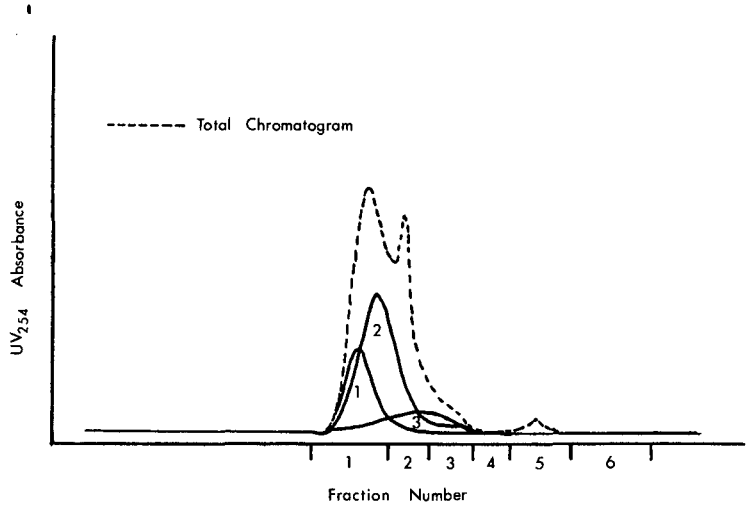

Fig. 4. Reinjection of fractions 1, 2 and 3. Cl_2:C = 1.14.

seen that as initial Cl_2:C was increased, greater percentages of the TOX were found in the later eluting fractions, especially fraction 5. Trichloroacetic acid [pK_a = 0.65 (ref. 43)] eluted within this fraction and so this trend is in agreement with individual product quantifications[6,9,16]. Dichloroacetic acid [pK_a = 1.29 (ref. 43)] eluted within fraction 3, but probably cannot account for all of fraction 3 TOX. There is too much TOX present in fractions 1–3 to be accounted for by the other small chlorinated acids. Therefore, this TOX (fractions 1–3) is likely to be larger weak acids or truly macromolecular in nature. If any basic TOX (*e.g.*, organic chloramines) was produced it was likely to elute late in the chromatogram as a result of adsorption although the borate should have minimized this.

The stability of the column material at pH 9.2 was investigated since the manufacturer suggests an upper stability limit of pH 10. Under the anaerobic conditions and low operation temperature (30°C) of the chromatography, hydrolysis of the polyacrylamide should have been minimal. Analysis of the borate buffer for TOC both

TABLE II

TOTAL ORGANIC HALOGEN CONTENT OF FRACTIONATED CHLORINATED SINGLETARY LAKE FULVIC ACID

Fraction	*Apparent molecular weight*	*Molecular weight at peak*	*Distribution coefficient range*	*%TOX per fraction*		
				Cl_2:C = 1.14	*Cl_2:C = 0.63*	*Cl_2:C = 0.065*
1	Exluded–3400	9420	−0.186–0.206	16.1 ± 0.5	20.6 ± 0.5	34.3 ± 1.8
2	3400–1230	2050	0.206–0.402	23.2 ± 0.9	23.4 ± 0.03	19.0 ± 0.5
3	1230–380	685	0.402–0.629	13.0 ± 0.1	14.2 ± 0.6	14.3 ± 2.4
4	380–138	229	0.629–0.825	6.0 ± −	4.3 ± 0.08	9.6 ± 0.1
5	138 −	62	0.825–1.13	39.2 ± 3.3	35.8 ± 0.4	16.5 ± 0.4
6	− −	−	1.13–1.55	2.4 ± 0.2	1.7 ± 0.4	6.3 ± 4.8

before and after the column indicated that column bleed was not occurring to any significant degree in the absence of sample. The TOC of the recovered sample, however, was found to exceed the TOC injected by two to three times. This discrepancy appeared to be independent of whether the sample was chlorinated or not. Since dissolved carbon dioxide was purged prior to TOC analysis, it appears that the high carbon values might be due to interactions between the sample and the polyacrylamide, possibly leading to hydrolysis of the polymer. The apparent column bleed was neither constant nor uniform, thus precluding TOC balances. There was no apparent effect on the sample chromatogram at 210 or 254 nm, however. Chromatograms were very reproducible and retention volumes did not decrease with use. Therefore there is no reason to believe the results of the TOX measurements of fractionated samples were adversely affected.

CONCLUSION

Despite the fact that this system is not a true size-exclusion system it does provide useful descriptive information in its application to chlorinated and unchlorinated fulvic acid. The results obtained are consistent with the hypothesis that increasing the chlorine dose serves to break up the macromolecular structure of the fulvic acid and produce smaller, more polar chlorinated products. The failure of this and previous attempts to separate humic substances solely by size enhances the hypothesis of Wershaw and Pinckney[24] that these substances do not fulfill the requirements necessary for solutes in size exclusion.

ACKNOWLEDGEMENTS

We express our thanks to J. Ed. Hall for his help with high-pressure liquid chromatography and its interpretation and to James Hemby for preparation of the fulvic acid samples. This research was supported by the United States Environmental Protection Agency via Cooperative Agreement CR 810532-01 to the University of North Carolina (A. A. Stevens, Project Officer).

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Note

Liquid column chromatography using thin-layer chromatographic silica gel

DAVID J. BEVERIDGE, DUNCAN M. HARRISON and ROBERT M. McGRATH*
Department of Biochemistry, University of the Witwatersrand, P.O. WITS 2050, Johannesburg (South Africa)
(First received May 4th, 1988; revised manuscript received June 25th, 1988)

Thin-layer chromatographic (TLC) grade silica gel has already been used in column form to separate intermediates in aflatoxin biosynthesis but was packed dry[1,2]. The method depends on capillary attraction to pull the solvent through the column similar to solvent movement on a TLC plate. No large externally applied forces are required to either push or pull the solvent. However, in some cases, particularly when the solvent is alkaline, and in spite of precautions such as dampening the silica gel with solvent before packing, the gel cracks and channels during development. To obviate this problem we packed the column wet, as in traditional column chromatography, reasoning that the continual evaporation from the bottom of the column would create the capillary forces needed to move the solvent through the gel. This is necessary because the TLC-grade silica gel is so fine that the solvent cannot move through it under gravity or indeed under applied moderate pressure.

EXPERIMENTAL

The column design is shown in Fig. 1 and is self explanatory. It was packed with a slurry of TLC silica gel 60 GF_{254} (Merck, Darmstadt, F.R.G.) prepared by weighing an amount of dry gel equal to the volume of the column multiplied by the factor 0.37 and vigorously shaking it with the column volume of solvent. The slurry was briefly and carefully degassed at a water vacuum pump before being poured all at once into the column. The silica was allowed to settle and the solvent to run until a little remained to cover the gel, when the end of the column was sealed with a household cling wrap.

The sample was loaded by dissolving it in volatile solvent, adding 1 to 3 g of silica gel and taking to dryness in a rotary evaporator. The free running dry gel with sample was then poured onto the column. A little eluent, bearing Sudan blue dye (fat soluble) to act as indicator for the front, was carefully added to the dry load sufficient just to mobilise it. Next, glass wool was pushed on top of the loaded column and eluent added carefully before inserting the reservoir into the top of the column which was left to develop.

Separation was deemed complete when the Sudan blue just exited from the column. At this point the silica gel was extracted from the column using a suitable plunger onto a sheet of Whatman 3MM chromatography paper.

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The various components could be detected under short-wave length UV light and simply sliced out to be eluted with a suitable solvent or the silica column could be sliced into equal portions and each extracted with methanol for TLC analysis. The extruded gel must not be allowed to dry otherwise it collapses into a fine powder.

Flow-rate depended on the column diameter, volatility and mobility of the solvent system. The mobility of a solvent system can readily be gauged on a TLC plate and slow moving systems, such as propanol and butanol, could not be used as described here. Very suitable solvents include methanol, ethanol, ethyl acetate and light petrolum (b.p. 80–100°C).

RESULTS AND DISCUSSION

Fig. 1 shows the separation of 125 mg of a chloroform extract of acidified mycelium and medium of a culture of *Penicillium cyclopium* known to produce cyclopiazonic acid mycotoxins. The silica gel column measured 30 × 2.5 cm and required 8 h for development. Fig. 2 shows a TLC analysis of the separation obtained on this column of silica gel. The R_F differences between the original material (lane 8) and separated material (lanes 0–7) are presumably due to the amount loaded and also to the difference in environment each component causes for the others.

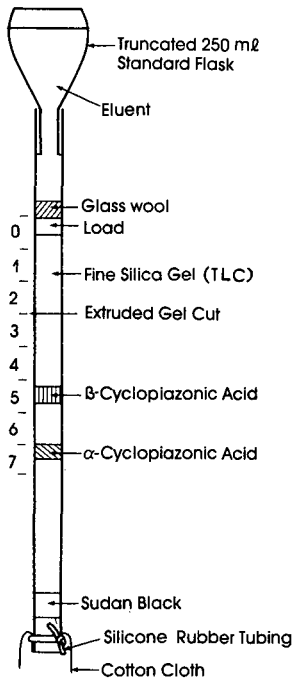

Fig. 1. A line drawing of a wet-poured TLC-grade silica gel 60 GF_{254} 30 × 2.5 cm, with an extract (125 mg) from *P. cyclopium* partially separated using ethyl acetate–methanol–conc. ammonium hydroxide (70:25:5, v/v/v) as solvent. The numerals indicate the cut sections of the extruded gel that were extracted with methanol and analysed as described in Fig. 2.

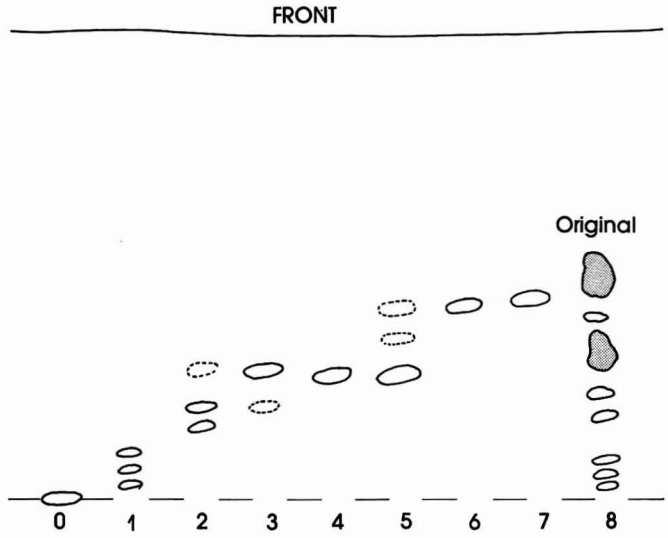

Fig. 2. A line drawing copy of the analysis by Merck silica gel 60 GF_{254} TLC plates of the column chromatography described in Fig. 1. The mobile phase was ethyl acetate–methanol–conc. ammonium hydroxide (60:35:5, v/v/v) and the components detected under UV at 254 nm.

Fig. 3 shows a similar investigation by TLC of the results achieved in the separation of the components of nutmeg oil on a gel column 25 × 1.2 cm using light petroleum (b.p. 80–100°C)–diethyl ether (80:20, v/v). Here the time required for separation was only 3 h, and even so a fast moving component was lost. It was this loss that prompted the use of a fat-soluble dye as a marker for the front and for column performance. The components were located under UV at 254 nm and cut from the extruded gel to be eluted with methanol.

The relationship between the R_F values on TLC plates and columns was investigated (the column R_F values were calculated using Sudan blue as the reference

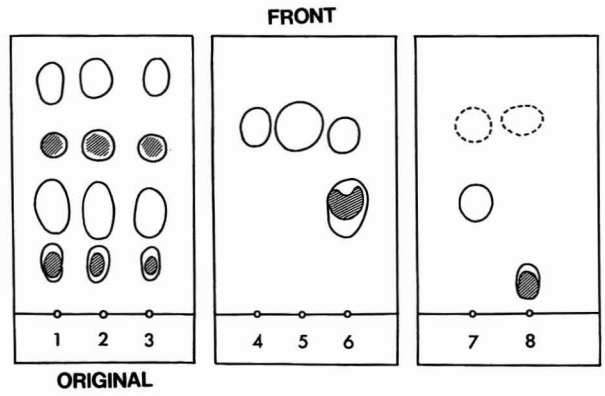

Fig. 3. A line drawing copy of the analysis by Merck silica gel F_{254} high-performance TLC plates of the column chromatography of the constituents of the oil of nutmeg. The mobile phase was light petroleum (b.p. 80–100°C)–diethyl ether (80:20, v/v). The shaded area represents iodine uptake and the clear area is absorption at 254 nm-

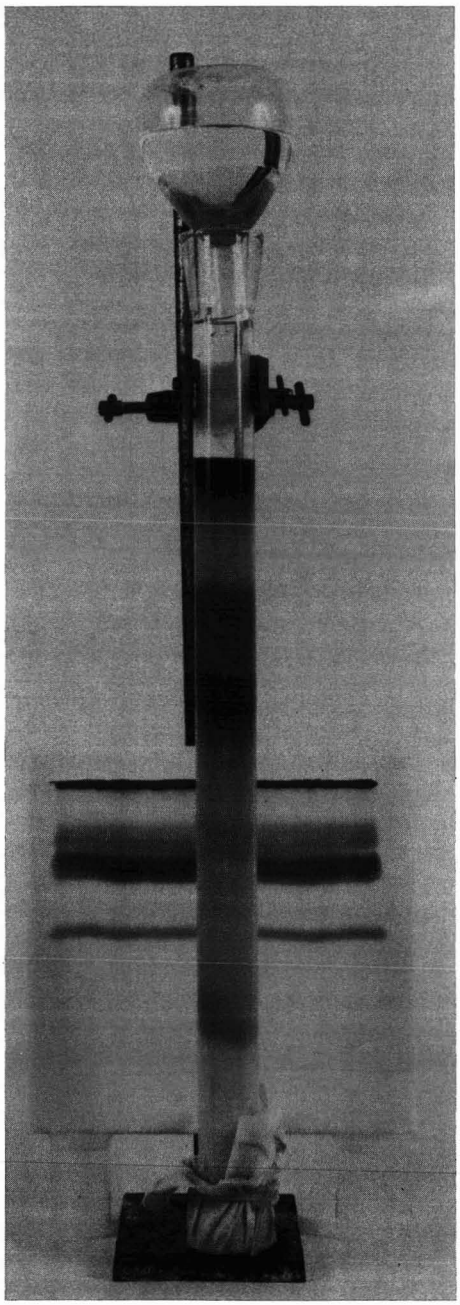

Fig. 4. The separation of the dyes of 175 mg black ink on a column of TLC-grade silica gel 60 GF_{254} (38 × 2.5 cm) and of 17.5 mg black ink on a preparative layer chromatographic plate (20 × 20 × 0.2 cm) of the same silica gel. The mobile phase was ethyl acetate–methanol–conc. ammonium hydroxide (60:35:5, v/v/v).

TABLE I

R_F VALUES OBTAINED BY TLC OF THE SECONDARY METABOLITES OF *P. CYCLOPIUM* ON HIGH-PERFORMANCE SILICA GEL PLATES (10 × 10 cm) AND ON 400 × 25 mm COLUMNS OF TLC-GRADE SILICA GEL PACKED WET

Abbreviations: A = α-cyclopiazonate; B = β-cyclopiazonate; C = cycloacetoacetyltryptophanyl; D and E = unknown.

	R_F values	
	Ethyl acetate–methanol–conc. ammonium hydroxide	
	70:25:5, v/v/v	*60:35:5, v/v/v*
Columns	A(0.57), B(0.46), C(0.28), D(0.10), E(0.05)	A(0.80), B(0.67), C(0.39), D(0.15), E(0.10)
Plates	A(0.39), B(0.38), C(0.18), D(0.06), E(0.03)	A(0.44), B(0.32), C(0.22), D(0.09), E(0.05)
Average ratio of R_F (column) to R_F (plate)	1.60 ± 0.18	1.87 ± 0.15

front) and the results are shown in Table I. While the R_F values are not the same between plate and column, a reasonably constant ratio is maintained. Thus, unlike traditional column chromatography on coarser gels, one can fairly accurately predict where the metabolite should be located. One can use TLC plates to investigate a mixture for the best eluent and translate this directly into a column technique. Though we have described an isocrfatic use, the column technique should lend itself to stepwise elutions.

The effectiveness of the method is portrayed in Fig. 4 where the dyes of black ink are shown separated on a TLC silica gel column and, for comparison, on a preparative layer chromatographic plate (20 × 20 × 0.2 cm) of the same silica gel, *i.e.* 60 GF_{254}; the column was loaded with 175 mg ink compared to 17.5 mg on the plate. Though the ink load on the column was ten times that on the preparative plate the volume of silica gel used in the column was 190 ml compared to 80 ml on the plate. Thus the ratio of load to silica gel was 0.92 for the column and only 0.22 for the plate, and yet the plate was being used almost to its maximum capacity. The reasons for the difference probably lie in the ease of loading of the column, which gave a more uniform band, and the longer development time for the column (16 h) compared to the plate (2 h).

The main advantages of this wet packing compared to dry packing of columns are that it is easier to do, it gives more reproducible results, and can be used for alkaline solvent systems. On the other hand it cannot be used for low-mobility solvent systems when dry packing can. And copmpared to preparative layer chromatography it is cheaper, easier to load, simpler to reclaim the separated components, and calcium sulphate can be omitted since no binding is needed, and it takes a heavier load of sample.

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CHROM. 20 736

Note

Rapid radio thin-layer chromatography for assay of lipase-catalyzed esterification and interesterification reactions

RICARDO SCHUCH
Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, 05508 São Paulo (Brazil)
and
KUMAR D. MUKHERJEE*
Bundesanstalt für Fettforschung, Institut für Biochemie und Technologie —H. P. Kaufmann-Institute— Piusallee 68, D-4400 Münster (F.R.G.)
(First received May 3rd, 1988; revised manuscript received June 20th, 1988)

Radiochemical methods for the assay of enzyme-mediated lipid transformations are well established techniques in lipid laboratories[1–3]. Especially, isotopic derivative techniques of analysis are well known in the field of lipids[3–8]. Radiochromatographic techniques have been shown to be convenient for monitoring and assaying lipase-catalyzed interesterification reactions[9]. Lipase activity has been determined by the aid of radio thin-layer chromatography (TLC), using microplates coated with silica gel containing sodium carbonate[10]. This method facilitates a rapid separation and quantification of fatty acids, which are retained at the origin of the chromatogram as their sodium salts, from the various acylglycerols, which migrate together close to the solvent front.

We describe here a rapid radio TLC method for monitoring and assaying lipase-catalyzed esterification and interesterification of lipids using plates coated with sodium carbonate/silica gel. This technique enables an accurate analysis of as many as 20 samples on a single plate.

EXPERIMENTAL

Materials

Glycerol, adsorbents for TLC and analytical grade reagents were from E. Merck (Darmstadt, F.R.G.). Oleic acid and lipid standards for TLC were from Nu-Check-Prep (Elysian, MN, U.S.A.). [1-^{14}C]Oleic acid, 59.7 μCi/μmol, was from Amersham Buchler (Braunschweig, F.R.G.) and toluene scintillator from Packard Instruments (Downers Grove, IL, U.S.A.). Distilled solvents were used throughout. "Lipase G", an immobilized lipase preparation from *Penicillium* sp., was a product of Amano Pharmaceutical (Nagoya, Japan).

Methods

Oleic acid (0.4 μmol), containing a known amount (1 μCi) of [1-^{14}C]oleic acid, and 0.4 μmol of glycerol were mixed with Lipase G (5% of the weight of the reac-

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tants), and the reaction mixture was stirred at 50°C. Samples (10 μl) were withdrawn from the reaction mixture after 20, 40, 60, 100 and 200 min, the reaction products extracted with hexane and Lipase G was removed by centrifugation.

The reaction products were fractionated by TLC on glass plates (20 cm × 20 cm), coated with silica gel H containing 5% (w/w) sodium carbonate[10]. The chromatoplates were subdivided into narrow lanes (9 cm high × 2 cm wide). Each of the lanes was initially spotted with about 10 μl of an aqueous solution of methanol (50%, v/v) on the start line at about 0.5 cm from one edge. Lipids were then applied to the start line and water was evaporated by heating the plate briefly (10–15 s) on a hot plate kept at 90°C. The chromatoplates were developed using diethyl ether–hexane–methanol (60:40:3, v/v/v), to a height of about 9 cm, which requires only 5–6 min. The chromatogram was stained with iodine vapour, the fractions corresponding to triacylglycerols (as trioleoylglycerol), diacylglycerols (as dioleoylglycerols), monoacylglycerols (as monooleoylglycerols) and fatty acids (as sodium oleate) were scraped off, and the radioactivity present in each fraction was deteremined in a Tri-Carb C 2425 liquid scintillation spectrometer (Packard Instruments), using toluene scintillator. The amount of oleic acid esterified with glycerol was calculated from the total radioactivity due to the [1-^{14}C]oleoyl moieties incorporated into various acylglycerols, *i.e.*, monooleoylglycerols, dioleoylglycerols and trioleoylglycerol.

RESULTS AND DISCUSSION

The technique described here involves retention at the start line of the chromatogram of the fatty acids as their sodium salts by reaction with sodium carbonate,

Fig. 1. Chromatogram showing the fractionation of lipid classes by TLC on sodium carbonate/Silica Gel H. The chromatogram was developed using diethyl ether–hexane–methanol (60:40:3, v/v/v) up to a height of 9 cm. The fractions are (from origin to solvent front): fatty acids (as sodium salts), monoacylglycerols, 1,2- (2,3-) plus 1,3-diacylglycerols and triacylglycerols.

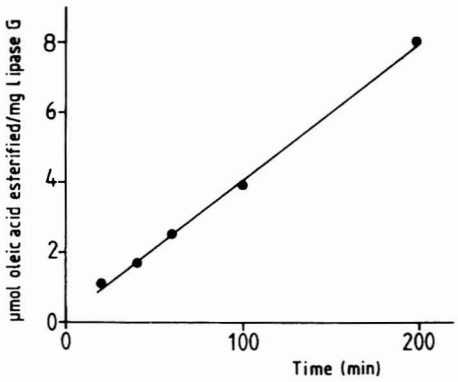

Fig. 2. Time course of esterification of [1-^{14}C]oleic acid with glycerol catalyzed by lipase G.

which is present in the absorbent, in a similar manner to that described earlier[10]. However, the present method enables a rapid yet clear-cut separation of unesterified fatty acids and each of the different classes of acylglycerols that are likely to occur in products of lipase-catalyzed esterification and interesterification reactions.

The salts are formed from the fatty acids only if water is applied to the start line. To ensure that they do not migrate from the start line during development of the chromatoplate, water must be totally removed by heating the plate briefly at 90°C. Fig. 1 shows that fatty acids (in form of sodium oleate) are clearly retained at the start line. Moreover, other lipid classes are distinctly separated by this technique. Since the two halves of one single 20 cm × 20 cm plate may be subdivided into as many as 20 lanes (9 cm × 2 cm, each) and the time of development is very small (5–6 min), the technique is especially convenient for the rapid assay of a large number of samples used to monitor lipase-catalyzed esterification and interesterification reactions.

The method described has been applied to the assay of several lipase-catalyzed reactions using radioactively labelled reaction partners. As a typical example, Fig. 2 shows the time course of the esterification of [1-^{14}C]oleic acid with glycerol catalyzed by an unspecific lipase from *Penicillium* sp. (lipase G).

The standard deviation of the method, calculated from separate determinations, was found to be 0.43 for a mean value of 11.43 µmol oleic acid esterified to glycerol (variance = 0.18; coefficient of variation = 3.77%). The method described should also be applicable to the assay of chemically catalyzed hydrolysis, esterification and interesterification reactions.

ACKNOWLEDGEMENTS

Financial support provided to R. S. by Fundaçao de Amparo à Pesquisa do Estado de São Paulo, FAPESP, São Paulo, Brazil, and Deutscher Akademischer Austauschdienst, DAAD, Bonn, Federal Republic of Germany, is gratefully acknowledged. This work was carried out as a part of a research project financed by Bundesministerium für Ernährung, Landwirtschaft und Forsten, Bonn, F.R.G.

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CHROM. 20 764

Note

Thin-layer chromatography of flucythrinate residues

PARM PAL SINGH* and RAM PARKASH CHAWLA

Department of Entomology, Punjab Agricultural University, Ludhiana-141 004 (India)

(Received June 20th, 1988)

Flucythrinate, (*RS*)-α-cyano-3-phenoxybenzyl-(*S*)-2-[4-(difluoromethoxy)phenyl]-3-methylbutyrate, is a synthetic pyrethroid pesticide of recent origin having an high level of activity against insect, mite and tick pests. A scan of *Analytical Abstracts* from January 1981 to April 1988 and reviews covering the literature on pesticide analysis[1–3] published from October 1980 to December 15, 1986 revealed that no method for thin-layer chromatographic (TLC) determination of this compound is available. As TLC is a useful technique for confirmation of residues tentatively identified in environmental and biological samples by gas or high-performance liquid chromatography[4–7], experiments were conducted to develop a TLC method for flucythrinate by using silver nitrate incorporated in alumina G plates as a chromogenic reagent and the findings are now reported.

MATERIALS AND METHODS

TLC equipment

This comprised 20 cm × 20 cm glass plates, a slurry applicator, a TLC drawing board (Perfit), developing tanks (Kontes) and an 120-W UV lamp without filter (Toshniwal Instruments).

Solvents and other chemicals

Alumina 60 G neutral, Type E (Merck), benzene, hexane (light petroleum fraction, b.p. 67–70°C) and silver nitrate (BDH), unless otherwise specified, were of analytical quality.

Reference standard

Flucythrinate (PAY-OFF®) of 97.8% purity was obtained from American Cynamid (Princeton, NJ, U.S.A.).

TLC procedure

TLC plates of thickness 0.25 mm were prepared from a slurry of alumina G in 0.4% (w/v) aqueous silver nitrate[8]. The coated plates were air dried and then activated at 110°C for 45 min. On the plates cooled to room temperature, suitable aliquots of solutions containing flucythrinate were spotted. The plates were developed to about 10 cm using hexane–benzene (45:55, v/v) as a solvent, removed from the developing

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tank, air dried and irradiated with ultraviolet light for 20 min (*Caution*: benzene is toxic and considered to be a tumour-producing agent. Skin contact and breathing of its vapour should be avoided[9]).

RESULTS AND DISCUSSION

By following the procedure described, flucythrinate appeared as a single brownish yellow spot on a white background. Its R_F value varied slightly with changes in room temperature and was 0.29 ± 0.01 (mean ± S.D. of six determinations) on a day when the laboratory temperature was 28.8°C. Ubiquitous organochlorine contaminants like DDT, benzene hexachloride and polychlorinated biphenyl residues as well as other halogenated synthetic pyrethroids (permethrin, cypermethrin, fenvalerate and deltamethrin) are also detectable by this technique[8,10] and can therefore interfere with the estimation of flucythrinate. However, under the experimental conditions employed, these compounds had R_F values ranging from 0.50 to 0.99 and did not affect the determination of flucythrinate.

While 200 ng of flucythrinate can be observed as a faint spot, 500-ng amounts of the compound gave distinct and easily discernible spots by this method. Though Sundararajan and Chawla[10] detected only 1000–2000 ng of certain halogenated synthetic pyrethroids by using previously recommended chromogenic reagents like phosphomolybdic acid[11] and palladium chloride[12], they reported a sensitivity of 50 ng each for permethrin, cypermethrin, fenvalerate and deltamethrin by using aluminium oxide G plates impregnated with silver nitrate as a visualizing agent. The lower sensitivity of flucythrinate by using this chromogenic reagent may be due to the brownish yellow colour of its spots which gave less contrast with the background than the black spots observed for certain synthetic pyrethroid compounds by Sundararajan and Chawla[10] as well as by us when these compounds were cochromatographed with flucythrinate. It may be noted that halogenated compounds are visualized in silver nitrate–ultraviolet light due to the formation of silver halides. While flucythrinate is a fluorinated compound, permethrin, cypermethrin and fenvalerate contain chlorine atoms, whereas deltamethrin is a bromide-substituted derivative. Hawley[13] has reported that silver chloride and silver bromide darken and finally turn black when irradiated. However, silver fluoride only darkens and remains brownish yellow on exposure to light.

As 1 g equivalent of soil, tomato fruit and plant extracts cleaned-up by the procedure described by Sundararajan and Chawla[10] were spotted without darkening of the background or production of streaks, the limit of determination of flucythrinate by this TLC method is 0.5 $\mu g\ g^{-1}$.

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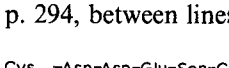
Erratum

J. Chromatogr., 443 (1988) 285–298
p. 294, between lines 7 and 8 the following structure should be inserted:



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journal of chromatography news section

NEW BOOKS

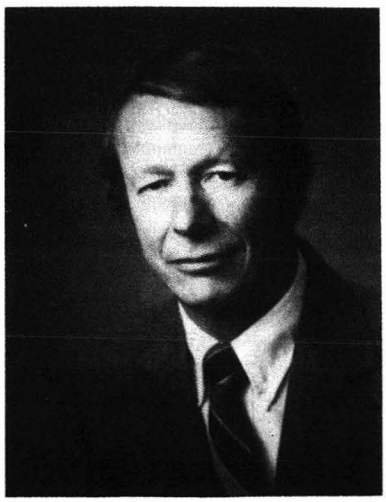
Introduction to microscale high-performance liquid chromatography, edited by D. Ishii, VCH Verlagsgesellschaft, Weinheim, 1988, XII + 208 pp., price DM 118, £ 41.45, ISBN 3-527-26636-4.

Automatic methods of analysis, by M. Valcárcel and M.D. Luque de Castro, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, XII + 560 pp., price US$ 131.50, Dfl. 250.00, ISBN 0-444-43005-9.

Electrochemical detection techniques in the applied biosciences, Volume 1, Analysis and clinical applications, edited by G.A. Junter, Ellis Horwood, Chichester, 1988, *ca.* 300 pp., price *ca.* US$ 101.70, ISBN 07458-0494-2.

Downstream processes: Equipment and techniques, edited by A. Mizrahi, Wiley, Chichester, New York, 1988, *ca.* 380 pp., price *ca.* US$ 117.50, ISBN 08451-3207-5.

AWARD



THE CHROMATOGRAPHIC SOCIETY 1988 MARTIN AWARDS

The first of the 1988 Martin Awards has been made to J. Calvin Giddings. He was born in American Fork, UT, U.S.A., on September 26th, 1930 and studied at Brigham Young University in Salt Lake City where he received his B.S. degree in 1952. Two years later, his researches into chemical kinetics, quantum chemistry and chromatography, conducted under the aegis of Henry Eyring, were rewarded with a Ph.D. He then went to the University of Wisconsin to work on the theory of flames with J.S. Hirschfelder. In 1957, he returned to the University of Utah as assistant professor in chemistry, became associate professor in 1959, research professor in 1962 and, finally, was installed as professor of chemistry in 1966.

The scope of his interests and activities is enormous — first, and foremost, is an extensive contribution to the understanding of many of the fundamentals of chromatography itself; in addition he has worked on the unification of separation theory, the theory of diffusion and last, chemical kinetics. The development of new separation methodologies, techniques for measuring diffusion coefficients and macromolecular separations are all well-documented in the literature under his name. His researches culminated in the invention of field flow fractionation and, as if that was not enough, his lifelong love of skiing and mountaineering prompted an involvement in snow and avalanche physics. A natural concern for the environment has seen much effort directed toward research into, and education, in respect of the world around us and he has contributed much to problems of conservation.

As well as having been a member of the Advisory Board of *Analytical Chemistry* for several years he is associated with many publications editorially, most notable is his direction of the series "*Advances in Chromatography*", now in its 28th volume.

His honours are legion — they include the ACS Award in Analytical Chemistry (twice), the Tswett Medal, the Dal Nogare Chromatography Award, the ACS Award in Separation Science and Technology and Honorary Degrees and Lecture Awards bestowed upon him by many Universities.

He plays as hard as he works, for his spare time is devoted to a diversity of activities — river running, mountaineering, skiing and hiking. He was President of the American Whitewater Affiliation, 1972–1977 and, in 1975, organised an expedition which achieved the first successful exploration and descent of the upper canyons of Peru's Apimurac River, the source and head-waters of the Amazon.

ANNOUNCEMENT OF MEETINGS

WINTER CONFERENCE ON FLOW INJECTION ANALYSIS, ORLANDO, FL, U.S.A., JANUARY 5–7, 1989

The Winter Conference on Flow Injection Analysis (FIA) will be held at the Embassy Suite Hotel in Orlando, FL, U.S.A., January 5–7, 1989.

The purpose of this conference is to bring together a wide forum of FIA practitioners from industrial and government laboratories, from academia — including students, as well as vendors of FIA equipment so that all participants can interact in an informal way in a convenient location.

The advanced registration fee is US$ 175 (after December 15, US$ 215; cancellation fee US$ 40; student discount, 50%)

For further details, contact: Gary D. Christian or Jaromir Ruzicka, Department of Chemistry, BG-10, University of Washington, Seattle, WA 98195, U.S.A., or Gil E. Pacey, Department of Chemistry, Miami University, Oxford, OH 45056, U.S.A.

10th INTERNATIONAL SYMPOSIUM ON CAPILLARY CHROMATOGRAPHY, RIVA DEL GARDA, ITALY, MAY 22–25, 1989

The 10th International Symposium on Capillary Chromatography will be held at the Palazzo dei Congressi in Riva del Garda, Italy, May 22–25, 1989. The format and atmosphere will be similar to previous meetings in Hindelang, Riva del Garda, Gifu and Monterey, respectively.

The scientific programme will cover basic and practical aspects of micro separation techniques such as: capillary gas chromatography; capillary liquid chromatography; capillary supercritical fluid chromatography; and capillary electrokinetic chromatography; with emphasis on: stationary phases and columns; instrumentation (sampling, detection, multidimensional and ancillary techniques); and applications (petrochemistry, environment, biomedical food and drug analysis, etc.). The symposium will consist of: review papers by leading scientists on the latest developments; invited papers by young scientists; submitted papers presented in poster sessions in order to achieve intensive discussion. Parallel discussion sessions on special topics will serve to augment the formal presentation.

In "workshop type seminars" scientists of the instrument manufacturers will present and discuss the latest developments in capillary instrumentation.

Authors intending to submit papers for the symposium will be required to adhere to the following schedule: abstract (300 words) before December 15, 1988 (notification of acceptance will be mailed to the authors by December 31, 1988); manuscripts ready for direct reproduction before February 1, 1989, to the address given below.

A book of the symposium proceedings will be available at the registration desk. The papers will be published in *Journal of High Resolution Chromatography and Chromatographic Communications* after the usual reviewing.

In conjuction with the symposium there will be an exhibition of capillary chromatography instruments and accessories.

For further details contact: Dr. P. Sandra, Laboratory for Organic Chemistry, University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent, BELGIUM.

9th INTERNATIONAL CONFERENCE ON COMPUTERS IN CHEMICAL RESEARCH AND EDUCATION, RIVA DEL GARDA, ITALY, MAY 28–JUNE 2, 1989

The ICCCRE conferences have become a major meeting point of leading specialists in computer chemistry. The IXth conference will be devoted to the following main topics: classical chemometrics, expert systems in chemometrics, synthesis design, reaction prediction, structure elucidation, computer graphics, intelligent instrumentation and automated laboratory equipment, molecular modelling, supercomputers in chemistry, personal computers and work stations, drug design and QSAR, data aquisition, data banks, computers in protein design, and computer chemistry in industry. Invited keynote and other lectures will cover the whole spectrum of topics. A parallel poster session will be on display during the whole conference period.

The IXth ICCCRE will be held in Riva del Garda, at the north end of the Lake Garda, one of the most beautiful natural areas of the world and only a short drive from Verona and Venice.

For further information, contact: Professor Dr. Mario Marsili, IXth ICCCRE, Piazza Gondar 14, 00199 Roma, Italy.

32nd IUPAC CONGRESS, STOCKHOLM, SWEDEN, AUGUST 2–7, 1989

The 32nd International Congress of Pure and Applied Chemistry will be organized by the Swedish National Committee for Chemistry of The Royal Swedish Academy of Sciences.

The scientific programme will be mainly devoted to seven areas of chemistry. Sessions on these areas will take place both concurrently and sequentially. Prominent chemists from all over the world will be invited to present plenary and keynote lectures.

Participants are encouraged to contribute lectures as well as posters. The subject matter of the posters is not necessarily limited to the areas mentioned below.

The seven sessions of the Conference are as follows: Large-scale separation of biological macromolecules, cells and particles; Atmospheric and marine chemistry; Chemical communication and interaction between organisms; Solid-state chemistry of inorganic materials, frontiers in the chemistry, structure and dynamics of macromolecules; Electron-transfer reactions; and Chemistry and biochemistry of bile acids.

For further details, contact: IUPAC, c/o Stockholm Convention Bureau, P.O. Box 6911, S-102 39 Stockholm, Sweden. Tel.: (46) 8230990, telex: 11556, FAX: (46) 8348441.

SHORT COURSE

CHROMATOGRAPHIC FUNDAMENTALS, KENT, OH, U.S.A., DECEMBER 5–9, 1988

Kent State University's Chemistry Department in cooperation with Spectra-Physics will present a course on Fundamentals of Chromatographic Analysis, December 5–9, 1988. This is the sixth year this programme is offered.

The objective of the programme is to provide an overview of chemical separations by chromatographic methods. The course will cover liquid, gas and thin-layer methods emphasizing these techniques as complementary rather than competing processes. The lecturers will present the theory relevant to liquid, gas and thin-layer chromatography and describe the latest instrumentation advances. Examples of testing methods that can be applied to everyday laboratory problems will be discussed. Laboratory periods will allow for hands-on experience with various HPLC, GC, GC–MS and TLC instrumentation. Dr. Roger K. Gilpin, Chairman of Chemistry at Kent State University, Dr. Neil D. Danielson, Associate Professor at Miami University, Mr. Ronald L. Lewis, a consultant and Dr. Afaf Wensky of Spectra-Physics will be the lecturers for the week.

Information on this course can be obtained from: Carl J. Knauss, Chemistry Department, Kent State University, Kent, OH 44242, U.S.A. Tel.: (216) 672-2327.

PUBLICATION SCHEDULE FOR 1988

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. 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.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

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