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devoted to the application of microtechniques in all branches of science

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Gas Chromatography Carbonate Microassay of Calcified Tissue. L. TORRES AND J. MATHIEU, *Laboratoire de Chimie Analytique, Ecole Nationale Supérieure de Chimie, 118, route de Narbone, 31007 Toulouse Cedex, France.* C. GODINOT AND G. BONEL, *Laboratoire de Chimie des Solides et des Hautes Pressions, 38 rue des 36 Ponts, 31078 Toulouse Cedex, France.*

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The stability constants β_1 and β_2 of simple Cu(II) complexes with methoxyacetic, phenylacetic, and cyclohexylacetic acid were determined spectrophotometrically and compared with the stability of composite complexes containing 2,2′-bipyridyl as the first ligand and the above mentioned acid as the second ligand.

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Microchem. J. **26**, 174–180 (1981).

Graphite–Silver Diethyldithiocarbamate as a New Potentiometric Sensor for Titration of Some Metals, Halides, Thiols, and Sulfonamides. SAAD S. M. HASSAN AND M. M. HABIB, *Department of Chemistry, Faculty of Science, Ain Shams University, Cairo, Egypt.*

A graphite–silver diethyldithiocarbamate electrode is described and satisfactorily used to monitor complexometric titration of multielements with NaDDC and argentimetric microdetermination of halides, thiols, and sulfonamides in organic compounds.

Microchem. J. **26**, 181–191 (1981).

Simultaneous Determination of Tin and Lead by Anodic Stripping Voltammetry in Aqueous–Alcoholic Medium. Application to the Direct Determination of These Elements in Canned Foods. P. DEBACKER, J. L. VANDENBALCK, G. J. PATRIARCHE, AND G. D. CHRISTIAN, *Institut de Pharmacie, Université Libre de Bruxelles, Campus Plaine C.P. 205/6, 1050 Bruxelles, Belgium.*

A method is described for which iron(III) in large quantities does not interfere, and mixtures of copper, lead, tin, and cadmium may be analyzed.

Microchem. J. **26**, 192–197 (1981).

Sensitive High-Density Lipoprotein Cholesterol Assay. J. D. ARTISS,* M. W. MCGOWAN,† AND B. ZAK,‡ **Department of Medical Biochemistry, St. Joseph's Hospital, London, Ontario, Canada, and, †Department of Biochemistry and ‡Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201.*

Precipitation of low-density and very-low-density lipoproteins is paired with a modified sensitized enzyme reagent system containing sodium 2-hydroxy-3,5-dichlorobenzenesulfonate for the determination of cholesterol by equilibrium reaction with the high-density lipoprotein fraction of serum.

Microchem. J. **26**, 198–209 (1981).

The Polarography of Oximes. Part II. 1,2-Acenaphthaquinone Monoxime. M. S. SETHI, P. S. RAGHAVAN, B. S. GARG, AND R. P. SINGH, *Department of Chemistry, University of Delhi, Delhi - 7, India,* AND MAHENDRA K. GADIA, *Atlantic Analytical Services Limited, P.O. Box 489, Springdale, Newfoundland, A0J 1T0, Canada.*

Polarography was carried out in buffers of constant ionic strength. The oxime group underwent diffusion-controlled reduction over the pH range 3.5–13.0.

Microchem. J. **26**, 210–216 (1981).

High Performance Liquid Chromatographic Determination of *n*-Butyl Glycidyl Ether. V. M. SADAGOPA RAMANUJAM, THOMAS H. CONNOR, AND MARVIN S. LEGATOR, *Division of Environmental Toxicology, Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, Texas 77550.*

A novel technique for the determination of *n*-butyl glycidyl ether at concentrations down to 1 ppb has been developed.

Microchem. J. **26**, 217–220 (1981).

The Use of Redox Reactions in Analysis of Dyes and Dye Intermediates. X. Polarographic and Constant-Potential Coulometric Determination of 4,4′-Dihydroxyazobenzene and 4-Nitro-4′-hydroxyazobenzene. J. BAREK, A. BERKA, AND V. BOREK, *Department of Analytical Chemistry, Charles University, 128 40 Prague 2, Czechoslovakia.*

Conditions have been found for a polarographic and constant-potential coulometric determination of 4,4′-dihydroxyazobenzene and 4-nitro-4′-hydroxyazobenzene in buffered media.

Microchem. J. **26**, 221–227 (1981).

Solvent Extraction and Spectrophotometric Determination of Iron(II) with 2,2′-Dipyridyl-2-quinolylhydrazone. MAKOTO OTOMO, SEIICHIRO ANO, AND HIROYUKI KAKO, *Department of Synthetic Chemistry, Nagoya Institute of Technology, Showa-ku, Nagoya 466, Japan.*

Iron(II) reacts with the reagent at pH 3.4–4.5 to form a water-insoluble 1:2 complex, which can be extracted with many kinds of organic solvents.

Microchem. J. **26**, 228–235 (1981).

Computerized Technique in Organic Microelemental Analysis. Part IV. Automated Determination of Chlorine, Bromine, and Iodine in Organic Compounds. E. KOZLOWSKI, *Institute of Inorganic Chemistry and Technology, Polytechnic Gdansk, Gdansk, Poland* AND G. M. MACIAK, *Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.*

Automated potentiometric titration with $AgClO_4$ to a preset endpoint potential, using a combination silver microelectrode, serves as the endpoint detection of the titration of halides.

Microchem. J. **26**, 236–241 (1981).

Application of the Vanadium(V)–Xylenol Orange Reagent to the Assay of Serum Glucose. CHIYO MATSUBARA, KAZUO ISHII, AND KIYOKO TAKAMURA, *Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.*

The reagent exhibits a characteristic absorption maximum at 582 nm and the presence of glucose together with glucose oxidase leads to a significant decrease in the absorbance of the reagent.

Microchem. J. **26**, 242–249 (1981).

Comparison of a Graphite Tube Micromethod for the Determination of Serum Iron and Total Iron-Binding Capacity with Spectrophotometric Techniques. PAUL BAILY, HELEN B. RÖLLIN, AND TERENCE A. KILROE-SMITH, *National Centre for Occupational Health, Department of Health, Welfare, and Pensions, P.O. Box 4788, Johannesburg 2000, South Africa.*

A rapid micromethod by flameless atomic absorption spectrometry is presented for the determination of total iron in serum or plasma. A clear distinction is drawn between "total iron in serum" and "serum iron."

Microchem. J. **26**, 250–261 (1981).

Spectrophotometric Study of the Reaction of Titanium with Bromopyrogallol Red in the Presence of Cetylpyridinium Bromide. Z. HAUSENBLASOVÁ, I. NĚMCOVÁ, AND V. SUK, *Department of Analytical Chemistry, Faculty of Natural Sciences, Charles University, Albertov 2030, 128 40 Prague 2, Czechoslovakia.*

Optimal conditions were found for the reaction which formed the basis for a new determination of titanium in the concentration range of 0.05–0.5 μg/ml.

Microchem. J. **26**, 262–268 (1981).

A Highly Effective One-Minute Thin-Layer Chromatographic Separation of Unconjugated Tri- and Dihydroxy Bile Acids. C. S. HO AND K. G. BLASS, *Department of Chemistry, University of Regina, Regina, Saskatchewan S4S 0A2, Canada.*

A new solvent system is used consisting of isooctane, diisopropyl ether, methanol, acetic acid, and formamide. A sensitive fluorescent visualization technique is employed.

Microchem. J. **26**, 269–279 (1981).

BRIEFS

Study and Application of the Complex of Sn(II) with Glycerol in Volumetric Analysis. J. Doležal, M. Roušal, and J. Zima, *Department of Analytical Chemistry, Faculty of Natural Sciences, Charles University, Albertov 2030, Prague 2, Czechoslovakia.*

The preparation of standard Sn(II) solutions in glycerol and ethanol media was studied and the most suitable conditions were found for their standardization using dichromate and potassium hexacyanoferrate.

Microchem. J. **26**, 280–287 (1981).

A Highly Sensitive Spectrophotometric Determination of Gallium with Chromal Blue G in the Presence of Cetyltrimethylammonium Chloride. Katsuya Uesugi and Mitsuo Miyawaki, *Laboratory of Chemistry, Himeji Institute of Technology, Shosha, Himeji, Hyogo, 671-22, Japan.*

A new spectrophotometric method for the determination of gallium with chromal blue G in the presence of cetyltrimethyl-ammonium chloride is described.

Microchem. J. **26**, 288–293 (1981).

Separation of Monofunctional Isomeric Naphthalene Derivatives by Means of Liquid Chromatography. Józef Sliwiok and Janusz Szulik, *Institute of Chemistry, Silesian University, 9, Szkolna Street, 40-006 Katowice, Poland.*

The work was undertaken to establish principal regularities enabling liquid chromatographic identification of selected naphthaline derivatives.

Microchem. J. **26**, 294–297 (1981).

MICROCHEMICAL JOURNAL 26, 149–154 (1981)

The Application of Thin-Layer and Gas Chromatography to Separation and Identification of Carbofuran and Its Metabolites

LEONARD OGIERMAN

Institute of Plant Protection, Department of Analysis and Quality Control of Pesticides, 44153 Sosnicowice, Poland

Received April 23, 1980

INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl-*N*-methylcarbamate) is one of the *N*-methylcarbamate pesticides, and with its formulations and residue forms as well as with toxic metabolites gas chromatographic methods of separation and detection seem to be the most suitable ones (*2, 5, 6, 9, 10*). To lower the barrier of detectability and to improve the possibility of determination, carbofuran is usually converted to its perfluoro derivatives and analyzed by means of GLC (*5, 6, 9, 10*). It enables a selective electron-capture (*5, 6, 10*) or electrolytic conductivity detection (*2, 9*). One study has reported on the application of the HPLC technique to determine the carbofuran residue and its metabolites (*6*) and also of gas–liquid chromatography—mass fragmentography (*1*).

In this work an effort was undertaken to separate carbofuran and its selected metabolites by means of TLC and GLC and to establish some linear correlations of the retention data, which would enable qualitative identification of these substances with an incomplete stock of standard compounds.

EXPERIMENTAL

Separation of carbofuran and its metabolites was performed using precoated TLC glass plates covered with silica gel 60 F_{254} and having the concentrating zone (E. Merck, Darmstadt, GFR). Plates were activated at 110°C for 30 min immediately before use. On the above-mentioned plates, 5 mm^3 of the 5 mg/cm^3 carbofuran and its metabolites in acetone was developed.

The following mobile phases were applied (volume ratios): (a) *n*-hexane–benzene–acetone (4:1:1), (b) carbon tetrachloride–ethyl ether–acetone (4:1:1). Visualization of the separated substances was accomplished with a 5% solution of ammonium molybdate in 30% sulfuric acid or uv light at 254 nm. The results of this separation are given in Table 1 and Fig. 1.

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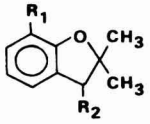
The separation performed on a thin layer was confirmed by the results received by means of gas chromatography. The Perkin–Elmer 900 chromatograph with flame ionization detector was used equipped with the glass columns (6 ft × 3 mm i.d.).

Stationary phases: (a) 3% OV-17 on Gas-Chrom Q, 80–100 mesh, (b) 3% EGSP-Z on Gas-Chrom Q, 80–100 mesh.

Analyses were performed with the detector temperature at 200°C and the injection chamber temperature at 220°C; hydrogen flow rate, 40 cm^3/min; air flow rate, 380 cm^3/min, and nitrogen carrier flow rate, 35 cm^3/min. The isothermal separation was performed at 160°C. The results of separations of carbofuran and its derivatives at two stationary phases (a) and (b) are given in Figs. 2 and 3.

TABLE 1
CHEMICAL CONSTITUTION AND THE R_f VALUES OF CARBOFURAN AND ITS METABOLITES

Compound	Common or short name	Structural formula	R_f values for the mobile phase	
			(a)	(b)
				
2,3-Dihydro-2,2-dimethyl-3-hydroxybenzofuran-7-yl-*N*-methylcarbamate	3-Hydroxycarbofuran	R_1: $-OCONHCH_3$ R_2: $-OH$	0.06	0.24
2,3-Dihydro-2,2-dimethyl-3-ketobenzofuran-7-yl-*N*-methylcarbamate	3-Ketocarbofuran	R_1: $-OCONHCH_3$ R_2: $=O$	0.15	0.46
2,3-Dihydro-2,2-dimethylbenzofuran--7-yl-*N*-methylcarbamate	Carbofuran	R_1: $-OCONHCH_3$ R_2: $-H$	0.18	0.51
2,3-Dihydro-2,2-dimethyl-7-yl-hydroxybenzofuran	Hydroxybenzofuran	R_1: $-OH$ R_2: $-H$	0.26	0.62
2,3-Dihydro-2,2-dimethyl-3-ketobenzofuran-7-yl methyl ether	3-Ketometoxybenzofuran	R_1: $-OCH_3$ R_2: $=O$	0.35	0.71
2,3-Dihydro-2,2-dimethylbenzofuran--7-yl methyl ether	Metoxybenzofuran	R_1: $-OCH_3$ R_2: $-H$	0.46	0.76

RESULTS AND DISCUSSION

The results of TLC separation of the examined substances are given in form of the R_f values in Table 1. The results given as the R_M value dependences for both mobile phases (a) and (b) also show a linear correlation. The linear correlation of the $R_M{}^a = f(R_M{}^b)$ function presented in Fig. 1 was found to be independent of the mobile phase systems.

The aforementioned fact gives evidence of the regularity with which substances belonging to the same group of chemical compounds, but differing with respect of the functional group order, can be characterized by the constant value of the R_M change in the same two mobile phases (*8*). The qualitative separation performed on a thin layer was confirmed by the results received by means of gas chromatography. The results of the isothermal GLC separation at two stationary phases OV-17 and EGSP-Z are given in Fig. 2.

Chromatograms shown in Fig. 2 suggest that independently from a stationary phase polarity, which used to be described by McReynolds constants (*7*), one obtains no separation of carbofuran from hydroxybenzofuran applying OV-17 (its McReynolds constant $\sum \Delta I = 844$) and EGSP-Z ($\sum \Delta I = 2278$) and the previously described analytical conditions. It should be mentioned that hydroxybenzofuran is one of the transition products in metabolism of carbofuran (*3*, *4*). These facts do not eliminate a possibility that when examining carbofuran residues and preserving the standard GLC analysis conditions there is a chance of getting too high

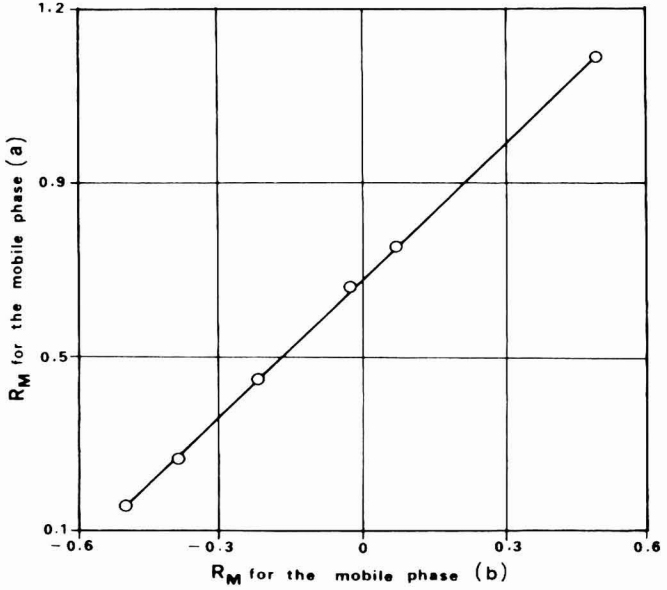


FIG. 1. The R_M values for carbofuran and its metabolites in two mobile phases.

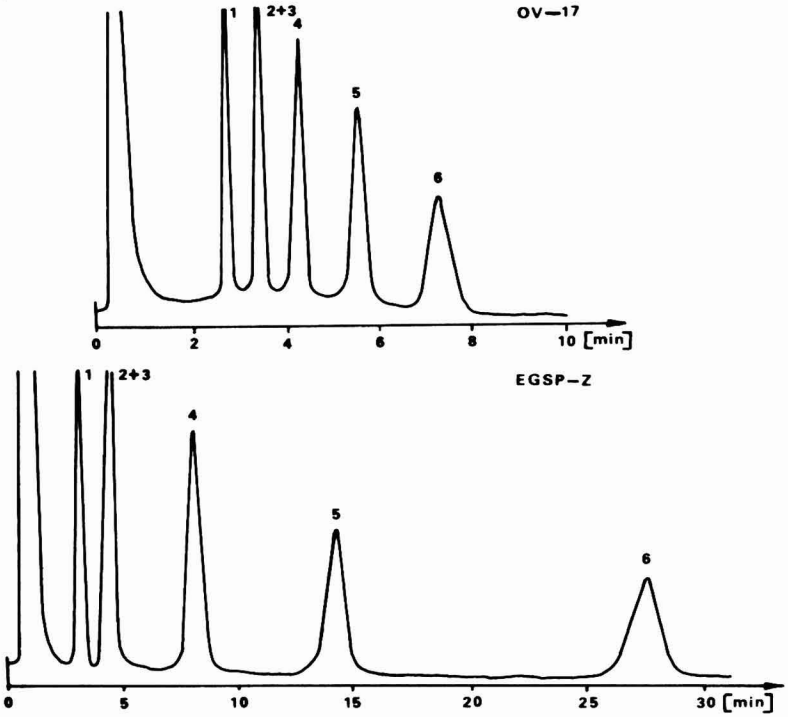


FIG. 2. Chromatograms of the separated substances. 1, Methoxybenzofuran; 2 and 3, hydroxybenzofuran and carbofuran; 4, 3-ketometoxybenzofuran; 5, 3-ketocarbofuran; 6, 3-hydroxycarbofuran.

results of determination of the discussed pesticide. Separation of carbofuran from hydroxybenzofuran is available by means of TLC both with the nonpolar mobile phase (a) and with the polar one (b). The ΔR_f values are in those cases 0.08 and 0.11, respectively.

Presentation of the retention data in the form of a dependency with the retention time logarithms determined for two stationary phases of a different polarity leads to the well-known linear correlation shown in Fig. 3. Both the linear correlation of the R_M coefficient values in TLC (Fig. 1) and of the log t_R coefficients in GLC (Fig. 3) can be useful for qualitative identification of carbofuran and its metabolites when lacking certain standard compounds. This observation might help those who study mechanisms of carbamate pesticide metabolism and take into assumption full identification of the reaction products.

SUMMARY

Conditions of chromatographical separation and identification by means of TLC and GLC were established for carbofuran and its selected metabolites, and some linear correlations

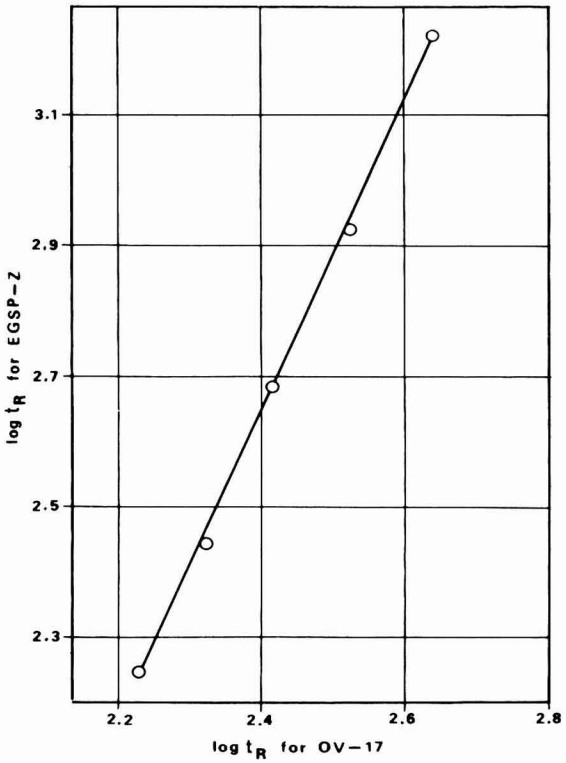


FIG. 3. Dependence of the log t_R for the carbofuran and its metabolites in two stationary phases.

were found for the R_M and log t_R coefficient. It was stressed that additivity of chromatographic coefficients in the case of the examined substances could help with their qualitative identification when a complete set of standard compounds is not available.

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MICROCHEMICAL JOURNAL 26, 155–163 (1981)

Analytical Applications of *N*-Phenylthiosemicarbazones

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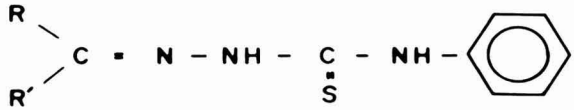
Received July 15, 1980

INTRODUCTION

Thiosemicarbazones have been widely used as analytical reagents in spectrophotometry, gravimetry, fluorimetry and also as visual indicators and in potentiometric studies. Their analytical applications have been recently reviewed (*23*) and also the structure of their metal chelates (*8*).

Phenylthiosemicarbazones are a class of compounds similar to thiosemicarbazones; the most common compounds studied are 4-phenylthiosemicarbazones named *N*-phenylthiosemicarbazones, whose general structure is



although in a few cases other phenyl derivatives have been studied. In the last years, several papers have dealt with the use of *N*-phenylthiosemicarbazones as analytical reagents. The aim of this review is to summarize these analytical applications and to confront their properties with the corresponding thiosemicarbazones.

PREPARATION AND CHEMICAL PROPERTIES

4-Phenylthiosemicarbazones are prepared by condensing 4-phenyl-3-thiosemicarbazide with an aldehyde or ketone, both in ethanolic solutions; in the case of certain di-phenylthiosemicarbazones the synthesis is a little difficult and requires addition of hydrochloric acid and precipitation as clorhidrate.

Phenylthiosemicarbazones act as weak acids, due to the dissociation of the thiol group. In general, ionization constants are less than that of the corresponding thiosemicarbazones. Biphenylthiosemicarbazones show only a deprotonation step, since the distance between the dissociable protons is relatively long, so that ionization constants would be expected to lie close together, and are not distinguishable by spectrophotometry (*16*).

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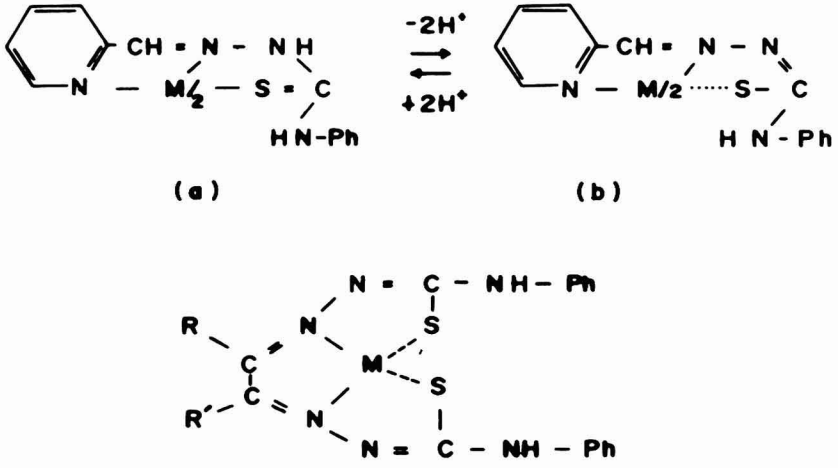
The behavior of phenylthiosemicarbazones as chelating agents is similar to the simple monothiosemicarbazones. Steric hindrance effects of the phenyl group have been not described. Both types of compounds act as bidentate ligands by bonding through the sulfur atom and the first hydrazino nitrogen; if the groups R_1 or R_2 in (I) show an additional coordinating atom (e.g., the nitrogen heteroatom of a pyridine ring, or an oxime group) they can act therefore as tridentate ligands; complexes formed may be charged (IIa) or uncharged (IIb). On the other hand, bi-phenylthiosemicarbazones appear to be tetradentate ligands with a convenient steric arrangement of its donor groups, and contain a conjugated system of π-electrons connected with donor system (III).



Solubility of phenylthiosemicarbazones in ethanol or ethanol–water mixtures is less than that of the corresponding thiosemicarbazones; however, their solubilities in dimethylformamide or dimethylformamide–water mixtures are much greater, as well as in chloroform and benzene. Extraction procedures of their chelates are necessary in diverse analytical uses; instead, solubility of simple thiosemicarbazones in organic solvents of little dielectric constant is small.

ANALYTICAL APPLICATIONS

The diverse mono- and bi-phenylthiosemicarbazones which have been used as spectrophotometric analytical reagents are summarized in Table 1.

Mono-phenylthiosemicarbazones

4-Phenylthiosemicarbazones of biacetylmonoxime ($PBTOH_2$) have been studied and compared with those of the thiosemicarbazone of biacetylmonoxime; the phenyl group produces an auxochromic effect on

the complexes formed; molar absorptivities are greater in all cases, and the absorption spectra show batochromic shifts. Extraction of metal chelates was feasible into chloroform (copper and nickel) and also into amyl alcohol (used in the extraction of copper, nickel, iron(II), cobalt(II), and manganese(III)). A selective spectrophotometric procedure for the determination of manganese(III) has been established; the red complex (λ_{max} = 550 nm) is formed by atmospheric oxidation above pH 9 (ammonium buffer); with increasing pH, the reaction rate increases. This complex can be extracted into chloroform, and perturbation of iron(III) could be eliminated by adding sodium tartrate. It was possible to determine 2–12 ppm of manganese in the presence of up to 100 ppm of iron (*10*).

Extraction of manganese(III) complex is carried out also in methyl isobutyl ketone; these extraction procedures have been used in the determination of manganese by atomic absorption spectrophotometry. In this extraction, a ternary complex (*24*) 1:2:1 Mn–$PBTOH_2$–tetrabutylammonium is formed previously.

$PBTOH_2$ have been used also in the photometric determination of copper(II) and bismuth(III). A spectrophotometric determination of both ions in mixtures has also been developed; copper is determined alone by using sodium tartrate as masking agent for bismuth; then, in the absence of tartrate, the sum of both ions is determined, so that the concentration of bismuth is obtained by the difference (*9*).

Picolinaldehyde 4-phenyl-3-thiosemicarbazone (PAPT) is a convenient reagent for the spectrophotometric determination of cobalt, nickel, and iron(II) and (III). Experimental results show an appreciable increase of sensitivity, twice at least, compared with picolinaldehyde thiosemicarbazone. A procedure for the selective determination of cobalt(II) in the presence of iron has been developed; determination is made in a strongly acid medium because it removes numerous interferences; iron(III) is reduced to iron(II) by the addition of ascorbic acid; this procedures has been applied to the determination of cobalt in steel (*13*). Moreover, determination of nickel and cobalt in mixtures has been studied, and a method has been established for the determination of both elements in industrial catalysts used in the petroleum industry (*12*). More recently, the iron–PAPT system has been investigated; iron(II) forms a green complex (λ_{max} = 625 nm) with PAPT at certain pH values, and iron(III) forms a yellow complex (λ_{max} = 390 nm) with a high absorptivity. The green complex is formed immediately in homogeneous solutions, but is oxidized in a few minutes by atmospheric oxygen to the yellow iron(III) complex. Ascorbic acid prevents the oxidation. This green iron(II) complex can be extracted into benzene; a photometric procedure has been established, and applied to the estimation of iron in industrial wastewater (*14*).

The reaction between iron(II) and PAPT with formation of the green 1:2 metal–ligand complex is similar when other thiosemicarbazones and

TABLE 1
SPECTROPHOTOMETRIC DATA OF THE COMPLEXES OF *N*-PHENYLTHIOSEMICARBAZONES

Reagent	Ion	λ_{max} (nm)	ϵ(liter mole^{-1} cm^{-1} $\times 10^{-4}$)	pH range	M:L	References
2-Acetylpyridine 4-phenyltsc[a]	Fe(II)	650	0.68	5.6–9.8	1:2	*20*
	Fe(III)	395	2.40	3.4–7.8	1:2	*20*
2-Benzoylpyridine 4-phenyltsc	Fe(II)	670	1.14	4.8–12.5	1:2	*18*
	Fe(III)	395	2.77	5.0–7.0	1:2	*18*
Biacetyl-bis(4-phenyltsc)	Cu(II)	485	1.27	1.8–11.9	1:1	*1*
	Zn(II)	440	2.16	4.5–9.0	1:1	*2*
	Pd(II)	428	2.53	0–10.0	1:1	*15*
Biacetylmonoxime 4-phenyltsc	Mn(III)	550	0.36	>10.0	1:2	*10*
	Co(II)	345	1.77	3.8–8.0	1:2	*10*
	Ni(II)	375	1.71	5.2–10.0	1:2	*10*
	Cu(II)	360	1.27	8.5–9.7	1:1	*9*
	Bi(III)	385	1.70	3.2–5.0	1:2	*9*
Bipyridylglyoxal-bis (4-phenyltsc)	Zn(II)	390	4.32	6.5–9.5	1:1	*17*
	Cd(II)	385	4.61	6.5–10.5	1:1	*17*
	Cu(II)	380	3.40	8.2–9.7	1:1	*17*
	Fe(II)	640	0.79	5.5	1:2	*16*
	Co(II)	390	3.28	5.3	2:3	*16*
	Ni(II)	370	3.37	6.3	2:3	*16*
	Pb(II)	390	2.86	9.5	1:1	*16*

Di-2-pyridylketone 4-phenyltsc	Fe(II)	665	1.41	3.0–12.0	1:2	19
	Fe(III)	400	3.23	5.0–7.2	1:2	19
Picolinaldehyde 4-phenyltsc	Co(II)	390	2.99	4.4–6.9	1:2	13
	Ni(II)	385	3.23	5.0–7.0	1:2	12
	Fe(II)	625	0.64	4.7–8.0	1:2	14
	Fe(III)	390	3.56	4.7–8.0	1:2	14
Salicilaldehyde 4-phenyltsc	Cu(II)	395		4.0–7.0	1:1	22
	Ni(II)	380		4.5–8.2	1:1	22
	Co(II)	390		4.0–6.0	1:2	22
	V(IV)	405		5.0–8.0	1:1	22
2(3′-Sulfobenzoyl)-pyridine 4-phenyltsc	Fe(II)	630	0.93	4.5–12.0	1:2	4
	Fe(III)	405	3.95	5.0–8.0	1:2	4
	Co(II)	405	2.90	4.0–8.5	1:2	5
	Ni(II)	390	3.44	5.0–8.5	1:2	5
	Cu(II)	400	2.12	5.0–9.5	1:1	5
	Zn(II)	410	3.69	6.0–9.0	1:2	5
	Cd(II)	400	3.20	5.0–7.0	1:2	5
	Hg(II)	375	1.85	5.0–8.5	1:2	5

[a] Phenylthiosemicarbazone.

phenylthiosemicarbazones of α-*N*-heterociclic aldehydes and ketones are used. The grouping $-N=\overset{|}{C}-\overset{|}{C}=N=C-S^-$ (thioazomethine ferroine group) contained in these compounds may be considered as a new functional group for iron(II). This grouping is not specific, since octahedral complexes are formed with other metal ions, both these complexes are yellow; the wavelength of maximum absorption of the iron(II) complexes allows the determination of iron in the presence of diverse metal ions.

Other diverse 4-phenylthiosemicarbazones containing the thioazomethine ferroine grouping have been studied: 2-acetylpyridine 4-phenylthiosemicarbazone (APPT) (*20*), 2-benzolypyridine 4-phenylthiosemicarbazone (BPPT) (*18*), and di-2-pyridyl ketone 4-phenyl-3-thiosemicarbazone (DPPT) (*19*). Sensitivity of the photometric determination of iron increases in this order; DPPT is more sensitive with the other reagents with the same functional group; moreover, the presence of two pyridine rings in the molecule produces an auxochromic effect on the absorption spectra of the complexes formed. Extraction into benzene make possible a selective procedure for determination of iron, in the presence of fairly large amounts of aluminum, chromium, cerium, lanthanum, tin, zinc, molybdenum, and tungsten.

Attempts to increase the solubility of phenylthiosemicarbazones have been made; 2(3′-sulfobenzoyl)pyridine phenylthiosemicarbazone (S-BPT) has been synthesized, and the reactions with iron(II), cobalt(II), nickel, and copper(II) have been studied (*4*, *5*). This compound shows a greater solubility than that of other phenylthiosemicarbazones, although the reagent must be used in dimethylformamide–water medium.

Only a phenylthiosemicarbazone is used as gravimetric reagents: 2-furaldehyde 4-phenylthiosemicarbazone. The reagent is used in 0.2% ethanolic solution in the determination of palladium. The orange complex is precipitated at pH 3–5, washed with 2:5 ethanol–water solution, and dried at 80–90°C. The gravimetric factor is 0.1784 (*11*).

Complexation of some phenylthiosemicarbazones has been studied potentiometrically by Bhatt *et al.* (*6*, *7*). Stability constants in water–dioxan media of Cu(II), Ni(II), Co(II), and VO^{2+} complexes with salicilaldehyde 4-phenylthiosemicarbazone and 2-hydroxy-1-naphthaldehyde 4-phenylthiosemicarbazone have been studied. A spectrophotometric study of the metal chelates formed between Cu(II), Ni(II), Co(II), and VO^{2+} with salicilaldehyde 4-phenyl-thiosemicarbazone has been made (*22*).

Biphenylthiosemicarbazones

Biphenylthiosemicarbazones for some α-diketones (biacetyl and glyoxal) were studied for the first time by Niederschulte and Ballschmitter

(*21*) and Ballschmitter (*3*). The principal application reported by these workers for this type of reagent has been the separation of complex chelate mixtures by thin-layer chromatography on aluminum oxide or by liquid chromatography; nevertheless, formation of highly colored metal chelates have also been reported.

Biacetyl-bis(4-phenyl-3-thiosemicarbazone) (BBPT) behaves as an interesting analytical reagent. The reaction between copper (II) and BBPT has been studied by spectrophotometry. The reddish orange 1:1 copper–BBPT complex (485 nm) is formed at pH 1.8–11.9, in a solution containing 60% v/v of dimethylformamide. The great stability of this complex makes possible the selective determination of copper, in the presence of iron(II), cobalt, nickel, lead, bismuth, antimony, tin, vanadium, mercury, and other metal ions, by using diverse masking agents: EDTA, fluoride, tartrate, and thiosulfate. The method has been applied to the determination of copper in industrial effluents from a sulfuric acid plant (pyrite process) (*1*). A method for the simultaneous photometric determination of zinc and copper has been developed, based on the different wavelength of absorption of the BBPT chelates. For both cations (440 and 480 nm), the results are evaluated by solving a system of two simultaneous equations. With this procedure it is possible to determine 0.15 ppm of zinc in the presence of 3.0 ppm of copper (*2*).

BBPT have been used in the photometric estimation of palladium. The green 1:1 complex, which is formed in acid and alkaline medium, is used for the determination of palladium, by means of extraction into chloroform (*15*).

Another reagent investigated is bipyridylglyoxal-bis(4-phenyl-2-thiosemicarbazone) (BGPT). The analytical possibilities of BGPT are superior to those of bipyridylglyoxal dithiosemicarbazone, owing to the higher molar absorptivities of the chelates and to the shift of the absorption peaks toward longer wavelengths; moreover, the BGPT chelates can be extracted more easily into benzene, toluene, and chloroform. Physicochemical properties, spectral characteristics, absorptivity, and the stoichiometric ratio of metal chelates formed have been described (*16*). The reagent acts as a tetradentate ligand with convenient steric arrangement of its donor groups in the complexes formed with Zn(II), Cd(II), and Cu(II) (1:1 stoichiometric ratio) in a similar manner to other vic-dithiosemicarbazones. In the case of the complexes of Fe(II), Co(II), and Ni(II) the stoichiometric ratios (1:2 and 2:3 metal ion–BGPT) indicate that each half of the molecule behaves independently; these complexes are similar to the complexes of picolinaldehyde 4-phenylthiosemicarbazone. Diverse spectrophotometric procedures for the determination of Zn(II), Cd(II), and Cu(II) with BGPT have been proposed, as well as the determination of mixtures of Zn(II), Cu(II), and Cd(II)–Cu(II).

SUMMARY

A review of the analytical uses of *N*-phenylthiosemicarbazones is given. Phenylthiosemicarbazones have been found in spectrophotometric and gravimetric procedures.

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Gas Chromatography Carbonate Microassay of Calcified Tissue

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INTRODUCTION

The mineral phase of calcified tissues contains carbonate ions (*3*); the concentration varies from sample and determines its properties: solubility, crystallinity, etc. An exact assay of the carbonates is necessary if the variation in their concentration is to be correlated with modifications of the properties of calcified tissues.

However, analysis of the carbonates present in the mineral phase is not at all straightforward, first because it is closely bound to the organic phase so it is extremely difficult to separate one from the other without disturbing them and second because the biological samples available are of very low mass (a few milligrams).

The methods which are currently used for this analysis—manometric (*4, 8, 11, 14, 17–19*), gravimetric (*2, 13*), chemical (*6, 7*), spectrometric (*12, 15*), and chromatographic (*5, 9, 10*)—require quantities of material of the order of tens of milligrams, so are unsuitable for carbonate assay in calcified tissues.

In the present paper we report a new technique aimed at carbonate microassay in biological materials based on the gas phase chromatography of the traces of carbon dioxide formed when calcified tissue undergoes acid attack. The apparatus described comports a reaction chamber where the carbonate decomposition takes place. The gasses which are released, including CO_2, are introduced by pressure difference into a circuit where they are prepared before being injected into a gas chromatograph through a gas valve. After calibration, samples of a few milligrams of calcified tissue are assayed for CO_2 release.

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MATERIALS AND METHODS

Reagents

All the chemicals and reagents used in this work were "pro-analysi" grade. *Perchloric*, 10 *M* (d = 1.67), *hydrochloric*, 12 *M* (d = 1.19), and *phosphoric*, 15 *M* (d = 1.70) acids were used at 10, 25, and 50% v/v dilutions.

Calcium carbonate standard. This was assayed gravimetrically by the method of Bonel (*2*); the carbonate content was found to be 59.43 ± 1.19%.

Barium carbonate and cow periostic tissue. The samples were assayed in this study by various methods (volumetry (*1*), gravimetry (*2*), and gas chromatography). The periostic tissue was ground by hand, then mechanically under liquid nitrogen.

Chromatographic adsorbents. Molecular sieve 13X (80–100 mesh) and Porapak N (80–100 mesh) were used.

Apparatus

Circuit of preparation and introduction of the gas samples. The circuit, developed by the authors, used for the analysis of the gasseous samples is shown in Fig. 1. This set-up allows the gas under investigation to be directed, when required, toward a thermal conductibility chromatographic detector and also a hydrogen flame ionization detector.

The circuit is composed of a group of fast shutting isolation taps (Edwards SC 5, England) (No. 1–9) and needle valves (Edwards OS ID) (No. 10–13) which, through fine setting, allow either a measured amount of gas into the system or the regulation of a pressure, and to this circuit is linked a series of peripheral elements:

The reaction chamber (R).

An input for standard calibration gasses contained in cartriges (C) (l'Air Liquide France).

A differential micromannometer (M) (Thommen 19 A 1, Switzerland) with a measurement range from 0 to 100 mbar (±0.05 mbar).

A pressure gauge (G) with digital display (Sogev Membranovac SD, France) working over two pressure ranges: 0 to 100 Torr and 100 to 1000 Torr.

Two gas valves (Chrompak, The Netherlands) V_1 and V_2 linked to two chromatographs on which different detectors can be used; the only type employed in this study was a thermal conductivity detector.

A vacuum pump (P) (Sogev Trivac D 2A) which can be used to empty all or part of the circuit.

The chamber (R) in which CO_2 release through acid attack takes place is a small Pyrex glass tube (outside diameter 16 mm, length 50 mm) which is

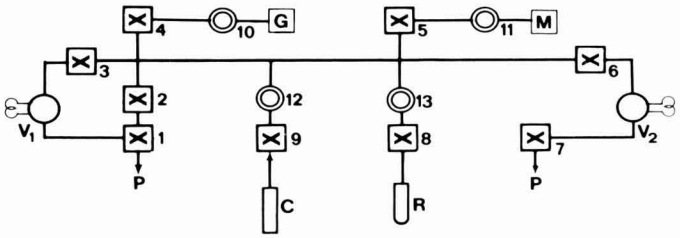

FIG. 1. Circuit used for the preparation and analysis of the gas samples.

joined to the general circuit by a vacuum joint (Edwards). The sample to be analyzed is weighed in a very small Pyrex spoon which is then hung on a small hook on the inside wall of the reactor. The reaction chamber and the spoon are shown in Fig. 2.

The different parts of the circuit are linked with a 2-mm-internal diameter stainless-steel tube.

Gas chromatography. A Hewlett-Packard 5750 model (Orsay, France) with a double column and a thermal conductivity detector linked to an electronic integrator (Hewlett-Packard 3380) was employed.

The carrier gas is helium at 24 ml min^{-1}. The gas injection valve is not heated. Analysis was carried out with two stainless-steel columns (3 m length and 22 mm internal diameter) filled with 13X molecular sieve and Porapak N heated to 180 and 25°C, respectively. The temperature of the detector is 200°C. The filament current intensity was 175 mA. The 13X sieve is regularly conditioned at 300°C in a stream of helium.

Balance. The weighings were carried out on a γ 21 N type microbalance (Setaram, France) which is sensitive to 10^{-6} g.

Procedure

After having placed 0.5 ml acid in the reactor, and when the spoon, containing a known amount of sample is placed on the hook, the reactor is connected to the injection circuit. The acid solution is degassed under

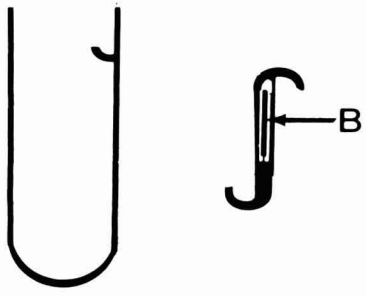

FIG. 2. Reaction chamber and spoon (B, magnetic bar).

vacuum over several freeze–thaw cycles. The reactor is then closed off by shytting valve No. 8 and the reactants heated to 35°C. A magnet on the outer wall of the reactor is used to unhook the spoon and lower it into the solution. The reaction:

$$CO_3^{2-} + 2H^+ \rightarrow CO_2 + H_2O$$

takes place in just a few seconds but the time allowed is kept at 1 min for absolute certainty that the reaction has finished. The CO_2 which is generated diffuses through the reaction chamber and then, after valve No. 8 is opened, through the whole circuit and in particular the chromatographic loop. Throughout the reaction the temperature is maintained constant; the vacuum is measured with an electronic gauge (G) and the total pressure of the released gasses by the differential micromannometer (M). The gasses reach pressure stability in the circuit after 15 min. The gaseous mixture contained in the loop is then injected into the chromatograph by rotation of the gas valve. At the end of every analysis the whole circuit is blown through with helium for about 10 min.

The chromatographic columns are kept under a constant light flow of helium (5 ml min^{-1}); between each series of experiments this has the effect of completely purging them thus eliminating any stabilization problems when the detector is switched on.

RESULTS AND DISCUSSION

Influence of the Type of Acid

In order to determine the best possible reaction conditions for the carbonate samples, three acids were tested at different concentrations. Experiments using calcium carbonate standards led to the results presented in Table 1.

The strongest signal given by the chromatographic detector for the same sample mass (1 mg) was obtained with 25% v/v hydrochloric acid.

TABLE 1
INFLUENCE OF THE TYPE OF ACID

Acids	Dilution[a] (v/v)			
	1/0 100%	1/1 50%	1/3 25%	1/9 10%
Perchloric (d = 1.67)	1,745	89,668	—	—
Phosphoric (d = 1.70)	1,386	73,907	265,282	153,480
Hydrochloric (d = 1.19)	19,763	139,820	430,425	283,350

[a] The dilution is expressed as the ratio of acid to water. The results (of CO_2 release) are given in the form of integration units.

It should be noted that for all the acids tested, the signal obtained, i.e., the CO_2 released, reached a maximum at average dilutions.

The acid which we decided to use in the trials is 25% v/v phosphoric acid since perchloric acid gave a very exothermic reaction with the dehydrated organic phase of the calcified tissues and hydrochloric acid led to the corrosion of parts of the sampling circuit.

Influence of the Organic Phase

Before considering the calibration using carbonate standards it was thought necessary to check, under the conditions used, if the organic phase of the calcified tissues to be assayed for carbonates did not become oxidized to CO_2, thus rendering the quantitative results inaccurate.

Table 2 presents the results of experiments carried out on fresh samples of calf muscle tissue not containing carbonate, with a strongly oxidizing acid (5 *M* perchloric acid). The tests, which were carried out at increasing temperatures and reaction times, show the absence of CO_2 formation. It is only above 80°C that traces of the gas start to be detectable.

The risk of inaccuracies in carbonate assay arising from oxidation of the organic phase can therefore be ignored at our working temperature of 35°C. Similar conclusions were drawn by Van Hall and Stenger (*16*) during a study concerning the oxidation of a certain number of organic substances. Samples subjected to attack by concentrated phosphoric acid at 100°C for several minutes showed no signs of oxidation.

Calibration

Figure 3 gives a typical example of the chromatogram of gasses released during the reaction.

The peak at 0.24 min after injection corresponds to a disturbance in the pressure of the carrier gas brought about on injection of the sample—the pressure in the loop being only a few Torr. Air and CO_2 have retention times of 1.07 and 5.77 min, respectively.

Calibration was carried out with $CaCO_3$ at 59.43% carbonate by weight. The results are summarized in Table 3.

TABLE 2
ORGANIC PHASE ACID OXIDATION

Temperature (°C)	Reaction time (min)		
	10	20	60
25	0	0	0
40	0	0	0
80	0	Traces of CO_2	Traces of CO_2

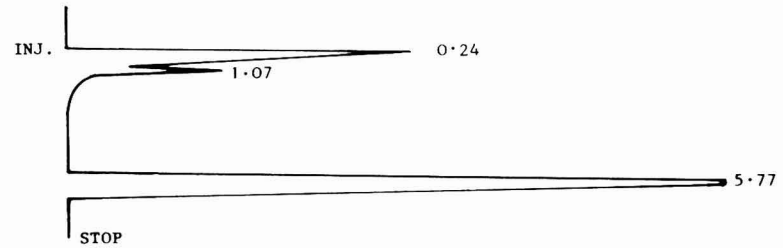

FIG. 3. Chromatogram of the generated CO_2. Column: stainless steel (3 m × 2 mm i.d.), filled with Porapak N (80–100 mesh) heated to 25°C. Carrier gas: helium 24 cm^3 min^{-1}. Injector: nonheated. Thermal conductibility detector: temperature 200°C. Filament current: 175 mA.

Column 4 shows the good repeatibility of the measurements since the variation coefficient (standard deviation divided by the mean) remains lower than 4.5%.

The curve in Fig. 4 shows that over the range 0.4 to 1.0 mg carbonate, the chromatographic response is linear.

Note that in order to establish the calibration curve and assay the CO_2 it is not necessary to know the internal volume of the circuit or the injection loop on the condition that it is always the same apparatus which is used and that the experimental conditions are kept strictly the same. With the calibration curve a direct relationship is obtained between the mass of reference carbonate placed in the reactor for chemical attack and the corresponding signal of the chromatographic detector. The carbonate content of the unknown sample is therefore easily found from the calibration curve.

Checking the Method. Application to Carbonate Assay in Calcified Tissues

The analytical technique described above was compared with more classical methods: gravimetry (*2*) and volumetry (*1*).

TABLE 3
MASS OF CARBONATE AND CORRESPONDING INTEGRATION SIGNAL

Mass of carbonate (mg)	Arithmetical mean[a] (integration units)	Standard deviation	Variation coefficient (%)
1.01	371,999	5,565	1.50
0.81	287,654	1,743	0.61
0.61	195,234	6,489	3.31
0.41	113,360	5,066	4.50
0.10	32,417	613	1.89

[a] Of five analyses.

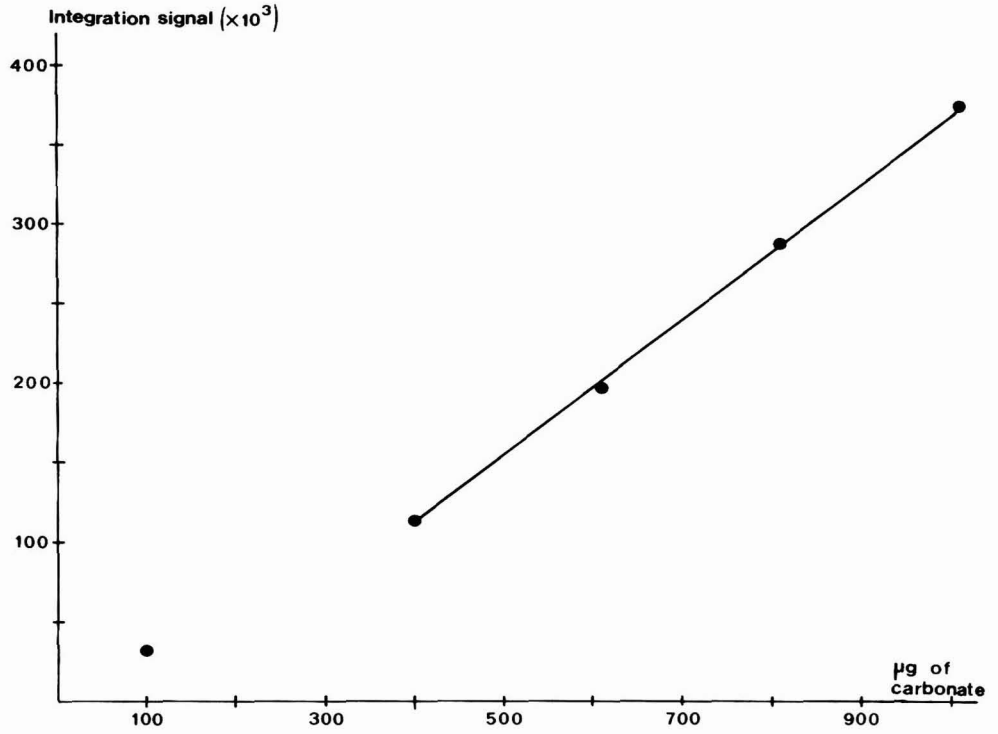

FIG. 4. Standard curve for the determination of carbonate.

TABLE 4
COMPARATIVE ASSAY OF $BaCO_3$ BY VOLUMETRY AND GAS CHROMATOGRAPHY

Volumetry			Gas chromatography		
Mass of $BaCO_3$ assayed (mg)	Experimental (%)		Mass of $BaCO_3$ assayed (mg)	Experimental (%)	
	CO_2	CO_3^-		CO_2	CO_3^-
200	22.65	30.89	1.53	23.10	31.50
200	21.74	29.65	1.00	22.80	31.09
200	20.93	28.54	1.50	22.37	30.50
200	22.83	31.13	1.50	22.06	30.08
200	21.15	28.84			
Mean	21.86	29.81		22.58	30.79
Standard deviation	0.77	1.04		0.40	0.54
Coefficient of variation (%)	3.5	3.5		1.80	1.80

TABLE 5

COMPARATIVE ASSAY OF THE CARBONATE CONTAINED IN SAMPLES OF PERIOSTIC BONE BY GRAVIMETRY, VOLUMETRY, AND GAS CHROMATOGRAPHY

Gravimetry			Volumetry			Gas chromatography		
Mass of tissue assayed (mg)	Experimental (%)		Mass of tissue assayed (mg)	Experimental (%)		Mass of tissue assayed (mg)	Experimental (%)	
	CO_2	CO_3^-		CO_2	CO_3^-		CO_2	CO_3^-
700	3.40	4.64	1000	3.33	4.55	3.60	3.37	4.60
750	3.45	4.70	1000	3.50	4.77	4.02	3.44	4.69
700	3.42	4.66	1000	3.44	4.70	5.07	3.43	4.68
700	3.38	4.61	1000	3.56	4.85	8.00	3.30	4.50
Mean	3.41	4.65		3.46	4.72		3.39	4.62
Standard deviation	0.03	0.04		0.08	0.11		0.06	0.08
Coefficient of variation (%)	1			2.3			1.80	

The comparative assay, using two different carbonate sample types, barium carbonate and periostic cow bone, gave the results summarized in Tables 4 and 5.

The results obtained by chromatography are in perfect agreement with those of the gravimetric and volumetric analyses. Only the chromatographic determination however can be carried out on just a few milligrams of sample with reproducible results: the coefficient of variation is around than 2% for the two samples assayed above.

SUMMARY

The aim of the study is to assay the carbonates held in the mineral phase of calcified tissues.

An apparatus is presented for the manipulation of the CO_2 released during acid attack of biological and mineral carbonate samples before injection into a gas chromatograph. The gasses are assayed for CO_2 by means of a calibration curve, previously established under the same experimental conditions using carbonate standards.

This analytical technique allows very small quantities of sample to be assayed: a few hundred micrograms in the case of mineral carbonates and a few milligrams for biological material. In spite of the low quantities involved the quantitative results obtained are of high accuracy.

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The Effect of 2,2′-Bipyridyl on the Stability of Cu(II) Complexes with Carboxylic Acids

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INTRODUCTION

The purpose of the present work was the comparison of stabilities for simple Cu(II) complexes with methoxyacetic, phenylacetic, and cyclohexylacetic acid with those for composite complexes in which one of the ligands is 2,2′-bipyridyl and another one is each of the above mentioned ligands. This specific subject has not been taken up in the literature although Griesser *et al.* (*1*) have also studied composite Cu(II) complexes whose coordination sphere contained other acids than those investigated here.

From among the simple Cu(II) complexes with methoxyacetic, phenylacetic, and cyclohexylacetic acid investigated by the author, only the methoxyacetic acid complexes were described. Sandell (*5*) provided as many as four values of successive stability constants for copper(II) methoxyacetate which does not seem to be very probable on account of bidentate properties of the carboxylic ion. Two constants for both Cu(II) methoxyacetate and Cu(II) phenylacetate equal to 2.01, 3.34 and 1.61, 2.40, respectively, are quoted by Sillen and Martell (*7*) but the results of their determinations obtained by Carson and Rossotti have not been published. No data on the stability of Cu(II) cyclohexylacetate have also been found.

This subject seemed to be interesting not only because of the absence of any reference data. Carboxylic acids investigated here and their derivatives are used, among others, as suitable extractive agents, as plant growth regulators and, therefore, studies of their complex-forming properties may be applied for analytical purposes. On the other hand, the Cu(II) complexes consisting of 2,2′-bipyridyl and carboxylic acid are interesting for biochemical reasons (*6*).

MATERIALS AND METHODS

Reagents and apparatus. The following acids were used as ligands: methoxyacetic CH_3OCH_2COOH E. Merck, G R, phenylacetic $C_6H_5CH_2COOH$, and cyclohexylacetic $C_6H_{11}CH_2COOH$, Fluka AG. Concentrations of

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acids were determined by titration with standard sodium hydroxide solution. The 2,2′-bipyridyl solution was prepared from the reagent made by POCh—Polish Chemical Reagents. $Cu(ClO_4)_2$ was prepared from copper carbonate and perchloric acid where $HClO_4$ concentration was determined potentiometrically and then neutralized. Constant ionic strength of $\mu = 2$ was maintained by introducing $NaClO_4$ (E. Merck, GR). All solutions were prepared from redistilled water. Absorbance was measured in a VSU-2 spectrophotometer (Carl Zeiss, Jena) using 0.5, 1, and 2 cm glass cells. Hydrogen ion concentration was measured in a PHM-4 Radiometer pH meter in the glass–silver silver chloride electrode system.

Measuring procedure and determination of stability constants. Prior to determination of the stability constants for simple and composite complexes, the dissociation constants of the acids under investigation were determined. To this effect emf values were measured and then hydrogen ion concentration of the "half neutralization" acid was calculated. Stability constants of the simple CuL^+ and CuL_2 complexes where L^- is the carboxylic acid anion were determined by the spectrophotometric Bjerrum method of corresponding solutions (*2*). Absorbance measurements were carried out at the 800 nm wavelength for each system. This wavelength was found to exhibit the largest difference between the absorbances of metal and complex. In each of the three measuring series a constant Cu(II) ion concentration was maintained. These concentrations were 10, 20, and 40 m*M* for the system containing methoxyacetic acid and phenylacetic acid and 5, 10, and 20 m*M* for the system with cyclohexylacetic acid. Absorbance measurements were carried out on solutions with increasing ligand concentrations up to the maximum methoxyacetic and phenylacetic acid concentration of 150 m*M*. The highest cyclohexylacetic acid concentration was 40 m*M* because of its limited solubility. Free ligand concentration $[L^-]$ and the average ligand number $\bar{n}$ was calculated according to the Bjerrum method. Stability constants were determined graphically by the Olerup method (*4*). The first stability constant of the Cu(II) complex with 2,2′-bipyridyl was determined as described above. Since the maximum 2,2′-bipyridyl concentration was 20 m*M*, suitably lower Cu(II) concentrations of 2.5, 5, and 10 m*M* were used in particular measuring series. Stability constants of the composite $Cu/bip/L^+$ complexes were determined by the same experimental and calculation technique as those for the simple complexes. Absorbance measurements were carried out on solutions containing increasing concentrations of the acid under investigation and constant $Cu(bip)^{2+}$ ion concentration of 2.5, 5, and 10 m*M* in each series.

RESULTS AND DISCUSSION

Studies of the complex-forming properties of methoxyacetic, phenylacetic, and cyclohexylacetic acid with respect to the Cu(II) ions showed

that in each system only the mononuclear complexes are formed since the positions of points in the Olerup diagram (Figs. 1, 2, and 3) do not depend on metal concentration.

The highest values of the average ligand number $\bar{n}$ determined for these systems are 1.50, 1.65, and 1.85, respectively, which indicates possible formation of the following complexes: CuL^+ and CuL_2. Table 1 summarizes the stability constants of these complexes and the pK values of the respective acids. A comparison of these values, that is, basicity of acid and complex stability, shows that there is no close correlation be-

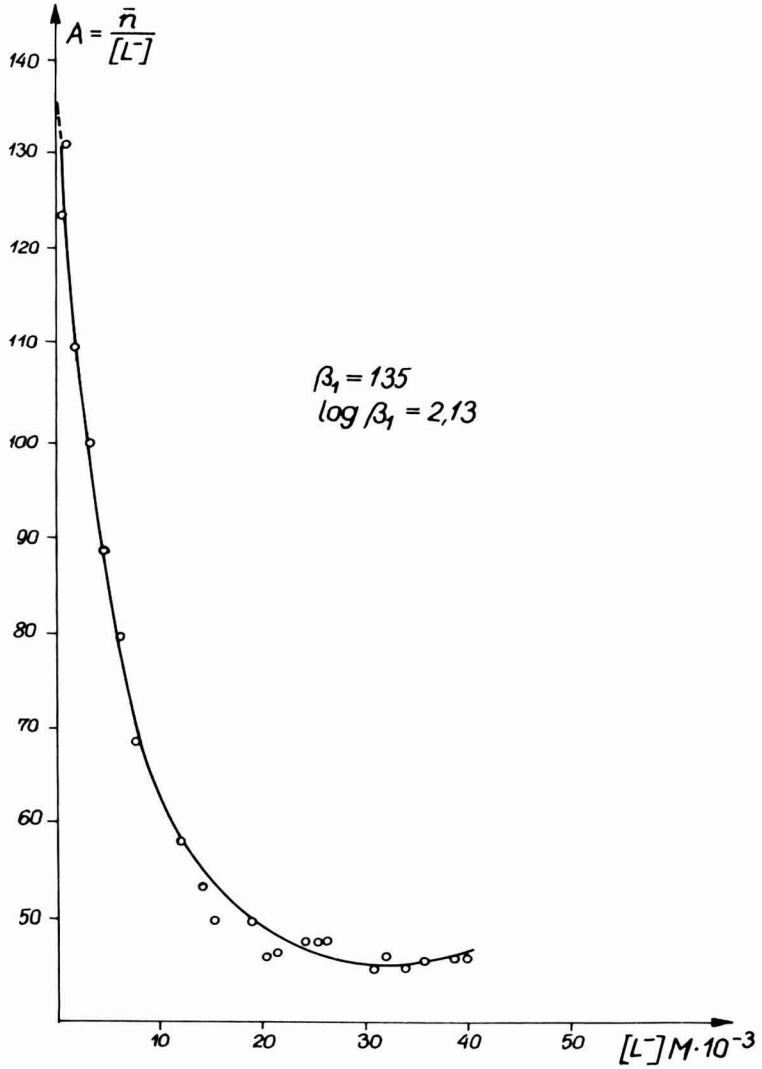

FIG. 1. Olerup diagram, system: Cu(II) methoxyacetic acid.

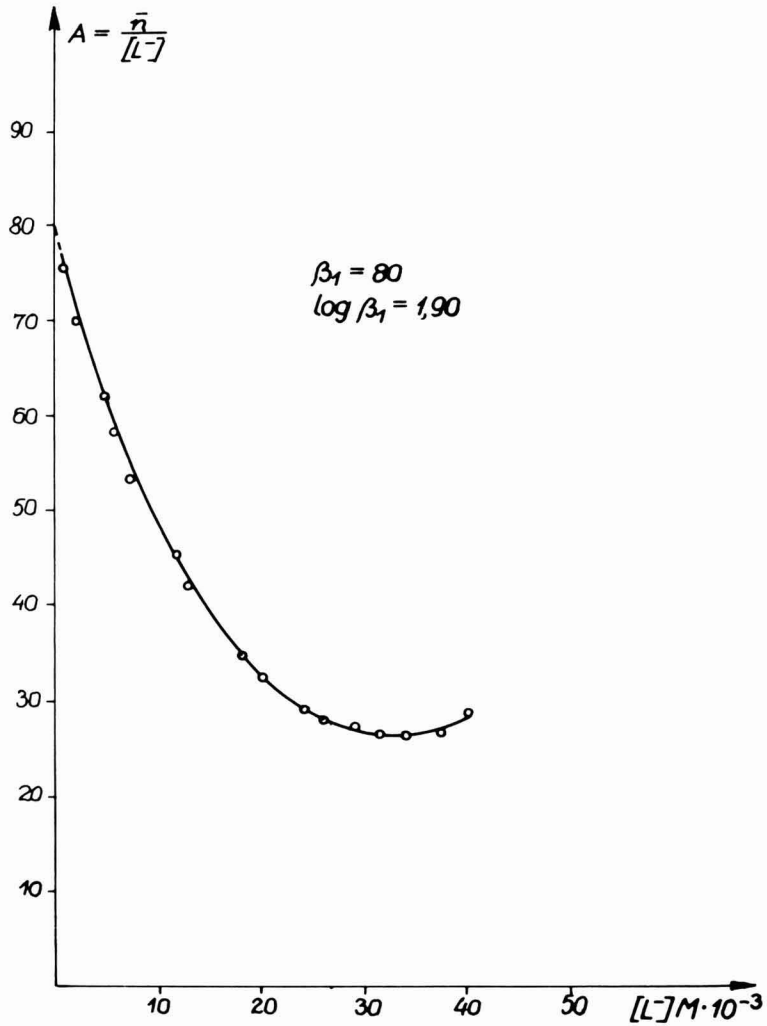

FIG. 2. Olerup diagram, system: Cu(II) phenylacetic acid.

tween them. Thus, methoxyacetic acid, the strongest acid among those under investigation, forms much more stable complexes than less dissociated phenylacetic acid. The following explanation of this anomaly is suggested: methoxyacetic acid binds the Cu(II) ions not only via the carboxylate ion as it happens for phenylacetic acid but also via the ether group CH_3-O-. On the other hand, a comparison of the stability of Cu(II) phenylacetate with that of Cu(II) cyclohexylacetate shows that the latter is much more stable. This relatively large difference in stabilities—exceeding one order—could not be explained as due merely to the difference in pK values of both these acids. It is quite probable that more

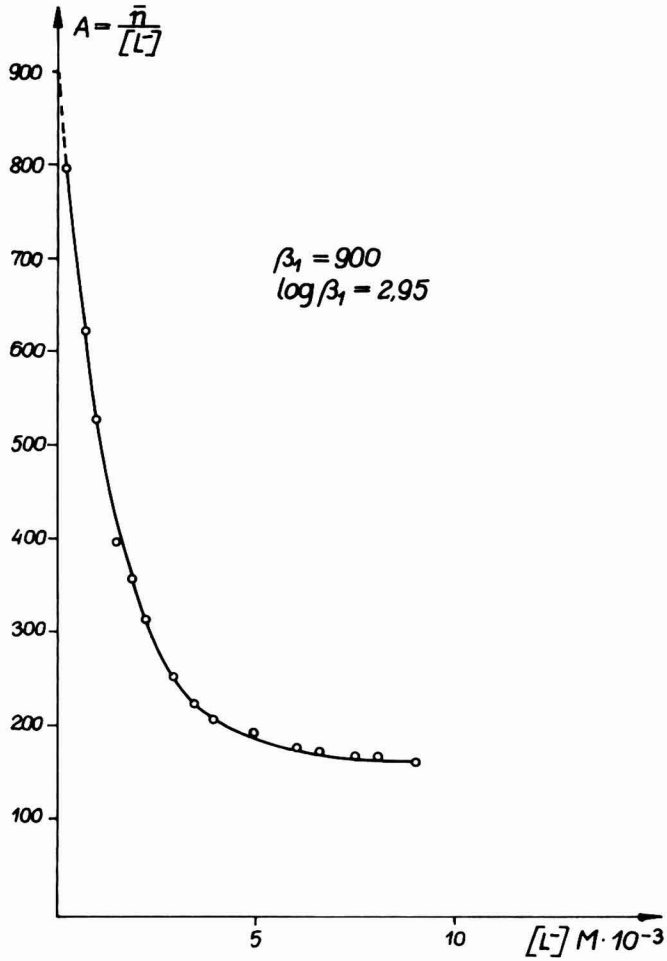

FIG. 3. Olerup diagram, system: Cu(II) cyclohexylacetic acid.

TABLE 1

STABILITY CONSTANTS OF SIMPLE Cu(II) COMPLEXES WITH CARBOXYLIC ACIDS: CuL^+ AND CuL_2 AND COMPOSITE COMPLEXES WITH 2,2′-BIPYRIDYL AND CARBOXYLIC ACIDS $Cu(bip)L^+$.

Acid	pK HL	CuL^+ log β_1	CuL_2 log β_2	$Cu(bip)L^+$ log β	$\Delta\log\beta$ log β-log β_1
Methoxyacetic	3.45	2.13	3.65	2.28	0.15
Phenylacetic	4.27	1.90	3.02	2.05	0.15
Cyclohexylacetic	4.63	2.95	5.30	3.12	0.17

[a] μ = 2 $NaClO_4$; T = 20°C.

favorable steric conditions of cyclohexylacetic acid determine higher stabilities of its complexes. Thus, the complex-forming properties of the acids under investigation may depend on several factors: basicity, steric conditions, formation of additional bonds (not only via the carboxylate group); their contribution to stability varies and is hardly determinable quantitatively.

The values of β_1 and β_2 summarized in Table 1 refer to simple complexes whose formation and dissociation are described by the following equations:

$$Cu^{2+} + L^- \rightleftharpoons CuL^+ \qquad \beta_1 = \frac{[CuL^+]}{[Cu^{2+}]\,[L^-]}$$

$$CuL^+ + L^- \rightleftharpoons CuL_2 \qquad \beta_2 = \frac{[CuL_2]}{[CuL^-]\,[L^-]}$$

where L^- is the anion of the acid under investigation.

The values of β provided in the subsequent column denote the stability constants of a composite complex formed in the reaction:

$$Cu(bip)^{2+} + L^- \rightleftharpoons Cu/bip/L^+ \; \beta = \frac{[Cu(bip)\,L^+]}{[Cu(bip)^{2+}]\,[L^-]}$$

On the other hand, $\Delta\log\beta$ is the difference between the stability constant for the composite and simple complex:

$$\Delta\log\beta = \log\beta - \log\beta_1.$$

This difference is positive for each system and is comprised between 0.15 and 0.17. This means that the acid anions under investigation form more stable complexes with the Cu(II) ions after 2,2′-bipyridyl is attached by the copper ions.

The first stability constant of the Cu(II) complex with 2,2′-biryridyl determined in this work is high and its logarithm is 6.65. The reason for the stability of the complexes with 2,2′-bipyridyl and its related ligands is certainly the synergic bond in which the electrons are transferred from σ orbitals of the N atoms to empty $d(eg)$ orbitals of the metal and from $d(t_{2g})$ orbitals of the metal to empty molecular π orbitals of the conjugated amine (*3*). On attaching 2,2′-bipyridyl as the first ligand by the Cu(II) ion, the π bond causes such a delocalization of the d electrons that the addition of the carboxylic acid anion as the second ligand is stronger than in a simple CuL^+ complex. Such a regularity was found to occur for each of the three acids under investigation and the determined values of $\Delta\log\beta$ are almost identical. This makes us believe that any increase in stability does

not depend substantially on the nature of the acid but is caused by the acceptor properties of 2,2′-bipyridyl.

SUMMARY

The stability constants β_1 and β_2 of simple Cu(II) complexes with methoxyacetic, phenylacetic, and cyclohexylacetic acid were determined spectrophotometrically and compared with the stability of composite complexes containing 2,2′-bipyridyl as the first ligand and the above mentioned acid as the second ligand. In each case the stability of the composite complex $Cu(bip)L^+$ was found to exceed that of the simple complex CuL^+ and the differences in the values of log β are comprised within 0.15 – 0.17.

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Graphite–Silver Diethyldithiocarbamate as a New Potentiometric Sensor for Titration of Some Metals, Halides, Thiols, and Sulfonamides[1]

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INTRODUCTION

Solid-state homogeneous, heterogeneous, and carbon paste electrodes for silver, based on the use of silver sulfide as an electroactive material, have been reported (*4*, *8*). Commercial availability of many of these electrode types has led to their applications in many fields (*4*, *7*) and has in turn resulted in a growing demand for new types of sensors of wider applicability. Construction of silver liquid membrane electrodes, consisting of silver chelates of diethyldithiophosphoric acid (*1*), 1-[2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl]-4-thioxo-5-methylthio-6-azauracil (*2*), and diketohydrindylidene diketohydrindamine (*3*) dissolved in organic solvents have also been described. However, the use of these electodes is limited due to the difficulties in preparing such complicated compounds and the high cost of commercial sensors.

This paper describes the preparation and applications of a new simple multipurpose electrode. It is based on the precipitation of silver diethyldithiocarbamate (AgDDC), as an electroactive material, within a graphic rod to give a sensitive sensor for both diethyldithiocarbamate (DDC) and silver ions. This electrode offers clear advantages over many of the available silver electrodes. Apart from the rather simple procedure for preparation, it presents a convenient approach to simultaneous determination of several elements without prior separation by direct titration with sodium diethyldithiocarbamate (NaDDC) and can be utilized for argentimetric microdetermination of many organic and inorganic compounds.

[1] Based on a paper presented at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, New Jersey, March 10–13, 1980.

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MATERIALS AND METHODS

Reagents

All the reagents used were of analytical grade unless otherwise stated and twice-distilled water was used throughout. The thiols, sulfonamides, and halogen compounds were of purity not less than 99% as confirmed by elemental analysis. A 0.01 *M* solution of sodium diethyldithiocarbamate (NaDDC) was prepared in 50% aqueous ethanol and standardized by potentiometric titration with standard 0.01 *M* silver nitrate using graphite–AgDDC electrode (*vide infra*). A 0.01 *M* solution of silver nitrate was prepared and standardized by titration with 0.01 *M* sodium chloride using graphite–AgDDC electrode. Stock solutions (0.01 *M*) of cadmium, cobalt, copper, lead, nickel, thorium, and zinc nitrates were prepared and standardized with EDTA using the standard visual titrimetric procedures (*14*).

Apparatus

All the potentiometric measurements were made with Orion Microprocessor Ionalyzer (Model 901) with a double junction reference electrode (Orion 90-02) in conjunction with graphite–silver diethyldithiocarbamate (AgDDC) electrode. Combined glass-calomel electrode (Orion 91-04) was used for pH adjustment.

Procedure

Preparation of graphite–AgDDC electrode. Cut a piece (2.5 cm long) of a spectral pure graphite rod (0.5 cm diameter) and place it in 20 × 1-cm clean test tube. Add 5 ml of a saturated solution of ethanolic sodium diethyldithiocarbamate. Keep the rod in the solution for 1 hr. Transfer the rod to another tube containing 5 ml of saturated aqueous solution of silver nitrate and leave for another 1 hr. Remove the rod, wash several times with twice-distilled water and 96% ethanol by placing the rod successively in beakers containing these solvents, and stir for at least 1 hr until no silver ions can be detected in the washing solutions (test with NaCl). Remove the rod and dry with a piece of filter paper. Wrap the rod from one end with a small piece of pure copper metal attached to a copper wire and insert in a plastic sleeve by which only 2 cm of the rod is exposed out of the sleeve. When the electrode is not in use, store in twice-distilled water, the electrode can be used for at least 8 months.

Microdetermination of metal ions. Transfer a 5-ml aliquot of the metal solution (containing 0.5–5 mg of Ag, Cu, Cd, Ni, Pb, Th, Co, Zn) to a 100-ml beaker and dilute with equal volume of 96% ethanol (the final solution contains 50–500 μg/ml). Insert the graphite–AgDDC indicator electrode in conjunction with a double junction reference electrode in the solution, connect to a pH meter, and potentiometrically titrate with stan-

dard 0.01 *M* NaDDC. Add the titrant slowly and stir the solution efficiently after each addition. (One equivalent of mono- and di- or tetravalent metal ions consumes 1 and 2 mol of NaDDC, respectively.)

Binary metallic mixtures containing silver (Ag–Ni, Ag–Pb, Ag–Cd, Ag–Cu, Ag–Th, Ag–Co, Ag–Zn) and tertiary mixtures (Ag–Cd–Zn, Ag–Pb–Zn, Ag–Ni–Zn) are titrated similarly. After the first inflection due to silver metal is attained (V_1), continue the titration to attain the second and third end points of the metals in the mixture (V_2 and V_3, respectively). Multiply the volume of NaDDC consumed at the first inflection (V_1) by the factor 1.33 to obtain the exact volume equivalent to silver (V_a). No correction is applied for mixtures of Ag–Co and Ag–Zn. Subtract (V_a) from (V_2) to obtain the exact volume equivalent to the second metal. The volume of NaDDC ($V_3 - V_2$) is equivalent to Zn in all the above mentioned tertiary mixtures.

Determination of Ag–Cu alloys. Weigh accurately 0.05–0.1 g of the alloy sample, transfer to a 25-ml beaker, and add 5 ml of 12 *M* nitric acid. Evaporate, on a sandbath, until dry and repeat four times or until complete dissolution of the alloy sample. Quantitatively transfer the content of the beaker, with twice-distilled water, to a 25-ml volumetric flask, complete to the mark with twice-distilled water, shake, and transfer a 5-ml aliquot to a 100-ml beaker. Add an equal volume of 96% ethanol, adjust the pH at 4–6, and follow up the above procedure.

Microdetermination of halogens in organic compounds. Weigh out 2–7 mg of the halogen compound into an L-shaped Whatman filter paper No. 42. Follow up the combustion procedure previously described (*5*) using the oxygen-flask combustion technique. After combustion, transfer the content of the flask to a 100-ml beaker and wash the flask several times, until the final volume of the solution in the beaker becomes 20 ml. Adjust the pH at 4–7 (using 0.5 *M* HNO_3). Insert the graphite–AgDDC electrode in conjunction with a double junction reference electrode and titrate with 0.01*M* silver nitrate solution.

Microdetermination of thiol and thiocarbonyl compounds. Weigh out 0.5–5 mg of the sample, transfer to a 100-ml beaker, dissolve in 10 ml 50% aqueous ethanol, and adjust the pH at 4–6. Titrate with 0.01 *M* silver nitrate solution as above. (One and two equivalents of silver are consumed per one equivalent of thiol and thiocarbonyl compound, respectively.)

Microdetermination of sulfonamides. Weigh out accurately 2–9 mg of the sample, transfer to a 100-ml beaker, and add 5 ml of twice-distilled water. Adjust the pH at 8 using 0.01 *M* sodium hydroxide and titrate with 0.01 *M* silver nitrate as above. (One equivalent of silver is consumed per mole of sulfonamide.)

For the determination of sulfonamides in pharmaceutical preparations,

weigh out the contents of five tablets of the sulfa drug in a small dish, mix the powder, and weigh a portion equivalent to one tablet. Dissolve in the least amount of water, adjust the pH at 8 (using 0.01 *M* NaOH), and transfer to a 100-ml measuring flask. Complete to the mark with twice-distilled water, shake, and titrate a 1.0-ml aliquot with silver nitrate as above.

RESULTS AND DISCUSSION

Graphite–AgDDC Electrode

Electrode performance. Impregnated graphite rods have been used in voltammetry as a substituent for nobel metal electrodes (*11*, *12*). In the present investigation AgDDC as an electroactive material is precipitated within a graphite rod which serves the dual purpose of supporting medium and conducting material. This electrode shows linear and fast response, in the range of 10^{-1} to 10^{-5} *M*, toward both silver and DDC ions with slopes of 50 and 65 mV per concentration decade, respectively. It can be used as indicator electrode for titrations involving silver and/or DDC ions. The life span of the electrode is at least 8 months.

Effect of pH and solvents. The effect of both pH and solvents on the electrode response was tested by titrating solutions of silver (50 μg/ml) with NaDDC at different pHs and in the presence of 50% aqueous solutions of some water-miscible organic solvents (e.g., dioxan, dimethyl sulfoxide, acetonitrile, methanol, ethanol, and isopropanol). Quantitative recovery (precision ± 0.3%) and maximum potential break at the equivalence point are obtainable at pH 4–7 in 50% aqueous ethanol.

Complexometric Titration with NaDDC

Microdetermination of metal ions. Sodium diethyldithiocarbamate (NaDDC) is a powerful ligand to chelate many metal ions (*6*). It is water soluble and stable in aqueous solutions of pH 4–7. Titration of divers of metal ions (e.g., Ag, Cu, Ni, Pb, Cd, Th, Co, Zn) with NaDDC at pH 4–7 using graphite–AgDDC indicator electrode shows inflection breaks (~50–350 mV) at points corresponding to stoichiometric 2:1 and 1:1 reactions for DDC:di- or tetravalent and DDC:monovalent metal ions, respectively. The results (Table 1) show good accuracy for metal concentrations down to 50 μg/ml. The average recovery is 97.9% and the mean standard deviation is ±0.9%.

Simultaneous microdetermination of several elements. Titration of some binary and tertiary metallic mixtures containing silver with NaDDC and graphite–AgDDC electrode shows two and three successive sharp inflections, respectively (Figs. 1 and 2), with sequence in harmony with

TABLE 1
MICRODETERMINATION OF SOME METAL IONS BY POTENTIOMETRIC TITRATION WITH NaDDC USING GRAPHITE–AgDDC ELECTRODE

Metal	Weight (μg/ml)		Recovery (%)
	Taken	Found	
Ag^{+}	107	106	99.1
	215	213	99.1
Cu^{2+}	63	62	98.4
	127	124	97.6
Ni^{2+}	66	65	98.5
	132	130	98.5
Pb^{2+}	217	215	99.1
	435	430	98.8
Cd^{2+}	123	120	97.6
	248	242	97.6
Zn^{2+}	73	70	95.6
	147	142	96.6
Co^{2+}	64	62	96.9
	129	125	96.9
Th^{4+}	80	78	97.5
	122	120	98.3

both the stability constants and solubility products of the metal–DDC complexes. However, the first and second inflections at all metal proportions appear at 75 and 125% of the expected values for silver and the second metal in sequence in the mixture, respectively. Thus, by multiplying the titer of silver by a factor of 1.33, the exact titer of silver is obtained which upon subtraction from the titer at the second inflection gives the exact titer of the second metal.

On the other hand, binary metallic mixtures of Ag–Co and Ag–Zn show stoichiometric equivalence points at the two inflections and need no correction. This may be due to the fact that the difference between the solubility products of AgDDC and $Co(DDC)_2$ or $Zn(DDC)_2$ is over several decades order of magnitude (*13*) permitting differential titration without overlapping. Synthetic binary and tertiary metallic mixtures in variable concentrations were titrated and the results are recorded in Tables 2 and 3. The mean average recovery calculated from the pooled data of all the metals is 98% and the mean standard deviation is ±1.2%.

Analysis of some silver-base alloys. Some silver-base alloys are satisfactorily analyzed by dissolving 50–100 mg sample in nitric acid, pH adjustment at 5–7 and potentiometric titration with NaDDC in 50% aqueous ethanol using a graphite–AgDDC electrode. The results obtained for silver and copper in sterling silver (92.5% Ag, 7.5% Cu), coin silver al-

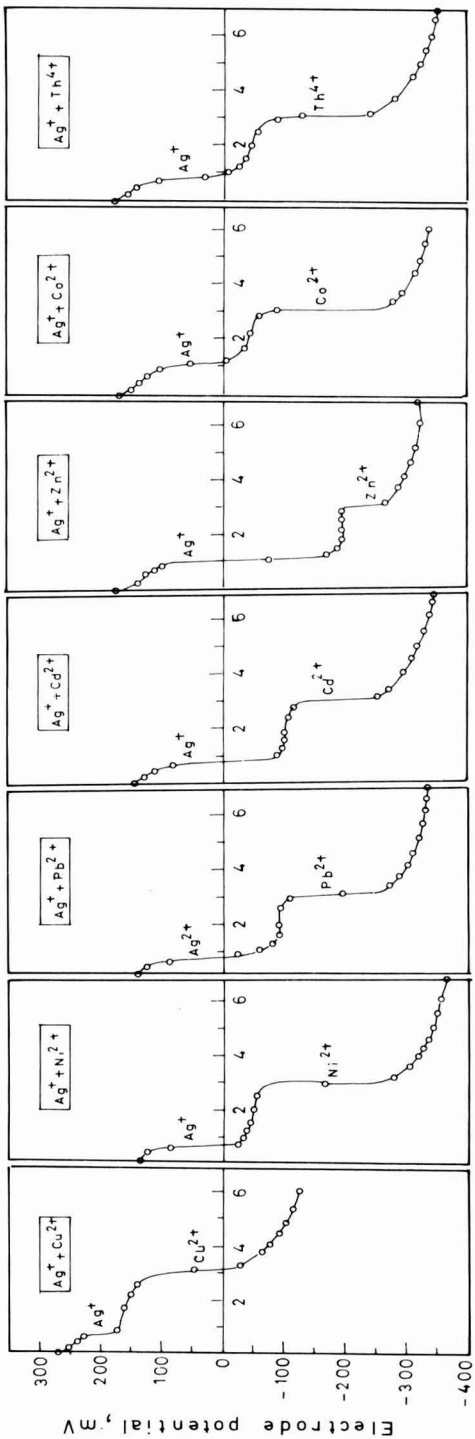

FIG. 1. Potentiometric titration of some binary metallic mixtures (1:1) with 50% ethanolic NaDDC at pH 4.5–6.5 using graphite–AgDDC electrode.

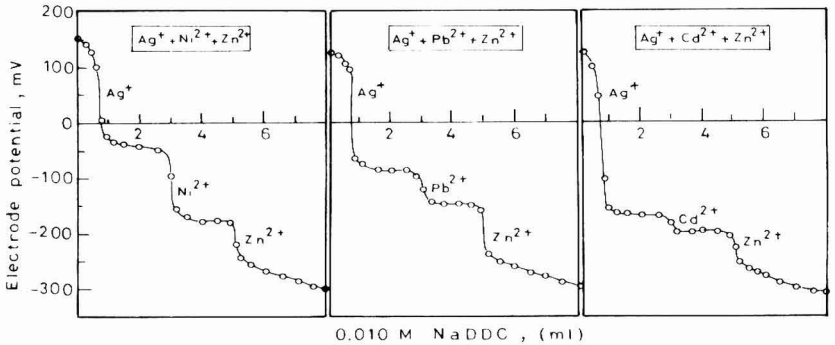

FIG. 2. Potentiometric titration of some tertiary metallic mixtures (1:1:1) with 50% ethanolic NaDDC at pH 4.5–6.5 using graphite–AgDDC electrode.

loy(I) (90% Ag, 10% Cu), and coin silver alloy(II) (72% Ag, 28% Cu) are in good agreement with the certified values.

Argentimetric Titration

Microdetermination of halogens. Halide ions (0.3–5 mg) are argentimetrically titrated at pH 4–6 in aqueous media using graphite–AgDDC electrode. The inflections at the equivalence points are not less than 150 mV for chloride, 200 mV for bromide, and 300 mV for iodide ions and the mean average recovery is 99.5%. Some organic chlorine and bromine compounds are similarly determined after combustion in an oxygen-filled

TABLE 2

SIMULTANEOUS MICRODETERMINATION OF SOME BINARY METALLIC MIXTURES BY POTENTIOMETRIC TITRATION WITH NaDDC USING GRAPHITE–AgDDC ELECTRODE

Mixture M_1-M_2	M_1			M_2		
	Taken (μg/ml)	Found (μg/ml)	Recovery (%)	Taken (μg/ml)	Found (μg/ml)	Recovery (%)
Ag–Ni	107	105	98.1	66	65	98.5
	215	212	98.6	66	66	100.0
Ag–Pb	215	214	99.5	217	215	99.1
	323	320	99.1	436	430	98.6
Ag–Cd	107	104	97.2	123	120	97.6
	215	211	98.1	123	121	98.4
Ag–Cu	107	107	100.0	66	64	97.0
	323	321	99.4	133	130	97.7
Ag–Th	107	106	99.1	243	240	98.8
	323	318	98.5	487	480	98.6
Ag–Co	107	104	97.2	64	62	96.9
	323	315	97.5	129	125	96.9
Ag–Zn	215	206	95.5	73	70	95.9
	323	309	95.5	147	148	100.7

TABLE 3

SIMULTANEOUS MICRODETERMINATION OF SOME TERTIARY METALLIC MIXTURES BY POTENTIOMETRIC TITRATION WITH NaDDC USING GRAPHITE–AgDDC ELECTRODE

Mixture $M_1-M_2-M_3$	M_1			M_2			M_3		
	Taken (μg/ml)	Found (μg/ml)	Recovery (%)	Taken (μg/ml)	Found (μg/ml)	Recovery (%)	Taken (μg/ml)	Found (μg/ml)	Recovery (%)
Ag–Cd–Zn	107	105	98.1	123	120	97.6	65	63	96.9
	215	210	97.7	123	123	100.0	130	127	97.7
	323	320	99.1	206	204	98.5	54	52	96.3
Ag–Pb–Zn	107	106	99.1	222	220	99.1	65	66	101.5
	215	214	99.5	222	218	98.2	130	130	100.0
	323	323	100.0	371	365	98.4	54	54	98.1
Ag–Ni–Zn	107	104	97.2	64	62	96.9	65	66	101.5
	215	212	98.6	64	63	98.4	130	128	98.5
	323	320	99.1	107	105	98.1	54	52	96.0

TABLE 4
MICRODETERMINATION OF SOME ORGANIC COMPOUNDS BY POTENTIOMETRIC TITRATION WITH SILVER NITRATE USING GRAPHITE–AgDDC ELECTRODE

Sample	Weight (mg)		Recovery (%)
	Taken	Found	
Halogen compounds			
p-Chlorobenzoic acid	2.52	2.47	98.0
	4.71	4.61	97.9
p-Chloro acetanilide	3.35	3.33	99.4
	6.21	6.12	98.6
Chloranil	4.11	4.04	98.3
	5.23	5.17	98.9
p-Bromo benzoic acid	3.12	3.10	99.4
	4.98	4.91	98.6
3-Bromo diphenyl	4.93	4.85	98.4
	6.08	6.02	99.0
Tetrabutyl ammonium bromide	5.12	4.99	97.5
	6.14	5.95	96.9
Thiols and thiocarbonyls			
2-Mercaptobenzothiazole	1.84	1.82	98.9
	3.68	3.67	99.7
o-Mercapto benzoic acid	1.64	1.61	98.2
	3.29	3.31	100.6
Thioacetamide	0.75	0.75	100.0
	1.50	1.48	98.7
Dithioxamide	0.60	0.59	98.3
	1.20	1.18	98.3
Sulfonamides			
Sulfamerazine	2.71	2.71	100.0
	5.52	5.42	98.4
Sulfamethoxy pyrazine	4.42	4.32	97.7
	5.80	5.71	98.4
Sulfathiazole	2.80	2.80	100.0
	5.62	5.51	98.0
Sulfadiimidine	2.81	2.81	100.0
	4.20	4.32	102.8

flask. Table 4 includes some representative results showing an average recovery of 98.4% and a mean standard deviation of ±0.6%. No effect is noticed due to the presence of $NaNO_2$ or H_2O_2 as absorbent in the oxygen-flask.

Microdetermination of thiols and thiocarbonyls. Argentimetric titration of thiols and thiocarbonyl compounds (0.5–5 mg) at pH 4–6 in 50% aqueous ethanol shows sharp inflections (~200 mV) at the equivalence points. One equivalent of silver is stoichiometrically consumed per mole of thiol group. The 1:2 molar reaction of thiocarbonyl compounds with

silver, however, is in a good agreement with the findings of other workers (*9, 10*). Table 4 presents results obtained for some available thio compounds; the average recovery is 99.1% and the mean standard deviation is ±0.8%.

Microdetermination of sulfonamides. Different sulfonamide samples (2–10 mg) are argentimetrically titrated at pH 8, to effect complete solution, since most of these compounds are insoluble in many organic solvents. The inflections at the equivalence point (1:1 molar reaction) are sharp and larger than those obtained by using the commercially available solid-state silver electrodes. The results recorded in Table 4, show an average recovery of 99% and a mean standard deviation of ±1.3%. the method was also applied to some pharmaceutical preparations (e.g., Bactrim tablet, Roche, Switzerland, Bayrena ampule, Bayer, Germany, and Mecozine tablet, Memphis, Egypt). The active ingredients of these drugs are sulfamethoxazole, sulfamethoxydiazine, and sulfadimethoxine, respectively. The results obtained show an average recovery of 98.5% of the nominal amounts and no interferences are caused by the excipients and diluents used in drug formulation.

SUMMARY

A new simple potentiometric sensor based on the precipitation of AgDDC within a graphite rod is described. The electrode is sensitive for both DDC and silver ions down to 10^{-5} *M* and can be used as an indicator electrode for complexometric titration of metallic ions with NaDDC and argentimetric titration of some organic and inorganic compounds. Accurate results are obtained for microdetermination of some metal ions singly or simultaneously (in binary and tertiary mixtures and alloys) and thiols, sulfonamides, and halides in organic compounds.

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MICROCHEMICAL JOURNAL 26, 192–197 (1981)

Simultaneous Determination of Tin and Lead by Anodic Stripping Voltammetry in Aqueous–Alcoholic Medium. Application to the Direct Determination of These Elements in Canned Foods

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INTRODUCTION

Many techniques are available for the determination of tin and lead but few allow the simultaneous determination of these two elements (*3*). Trace quantities may be determined by atomic absorption spectrometry (*1*, *4*) and milligram quantities by complexometric titration (*6*). Mixtures have also been analyzed by polarography (*7a*, *7b*). We have undertaken a study to determine the optimum conditions of solvent and supporting electrolyte for the simultaneous measurement of tin and lead by differential pulse anodic stripping voltammetry. This technique may be used for the direct determination of these elements in canned food.

MATERIALS AND METHODS

Polarographic measurements were carried out with a Bruker Universal DC polarograph E100 equipped with a three electrode system and an x-y recorder (Hewlett-Packard 7004 B). The working electrode was a hanging mercury drop electrode (HMDE) Metrohm E 410.

The reference electrode was a satured calomel electrode (SCE) and to avoid any contamination of the test solution, it was immersed into a "Purley" tube filled with alcoholic hydrochloric acid medium saturated by potassium chloride.

The auxilary electrode was a platinum foil electrode and a Metrohm cell was thermostated to 25.0 ± 0.1°C. Micropipettings were performed with a 5 μl Eppendorf pipet.

All reagents were analytical reagent quality. Stock tin(IV) solutions (1 g/liter) were prepared in 6 *M* HCl from $SnCl_4 \cdot 5H_2O$ to avoid hydrolysis.

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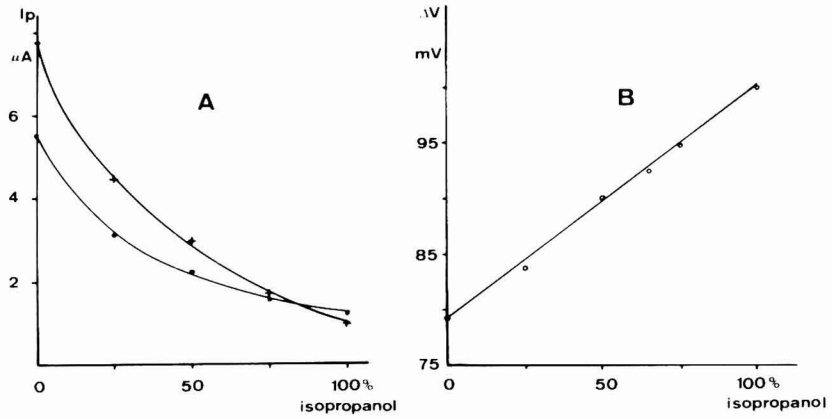

FIG. 1. Influence of the isopropanol percentage in solutions. (A) On peak heights of lead (●) and tin (X). (B) On ΔV.

Stock lead(II) solutions (1 g/liter) were prepared in 1 *M* HNO_3 from $Pb(NO_3)_2$. The initial test solution contained 15 μg each of tin and lead in 25 ml of 1 *M* HCl in methanol, prepared by dilution of the stock solutions. Anodic stripping voltammetry was carried out as follows: plating voltage, −1.00 V; plating time, 240 sec; resting time, 30 sec; pulse amplitude, 40 mV; voltage scan, 5 mV sec^{-1}; drop area, $1.986 \times 10^{-7} \cdot m^2$. Solutions were deaerated prior to electrolysis by bubbling for 10 min with nitrogen.

RESULTS AND DISCUSSION

Differential pulse anodic stripping voltammetry is among the most sensitive techniques for trace metal analysis (*2*). It is useful for simultaneous determination of several elements.

Effect of Solvent

We have observed that the addition of isopropanol to the initial test solution affects both the a.s.v. peak heights and the potential difference between the two peaks. The peak heights decrease with increasing isopropanol (Fig. 1A), while the resolution between peaks increases (Fig. 1B). Hence there is a compromise between these two effects, and we have arrived at an optimum compromise mixture of 50% isopropanol–50% methanol containing 1 *M* hydrochloric acid. The hydrochloric acid content influences the relative peak height (Fig. 2). A concentration of 1 *M* HCl results in maximum sensitivity for both elements.

The minimum concentration of water in the solvent–electrolyte is 5%, coming from the hydrochloric acid. The presence of water has a pronounced effect on the peak separation. Figure 3 illustrates the influence of water on the relative peak potentials.

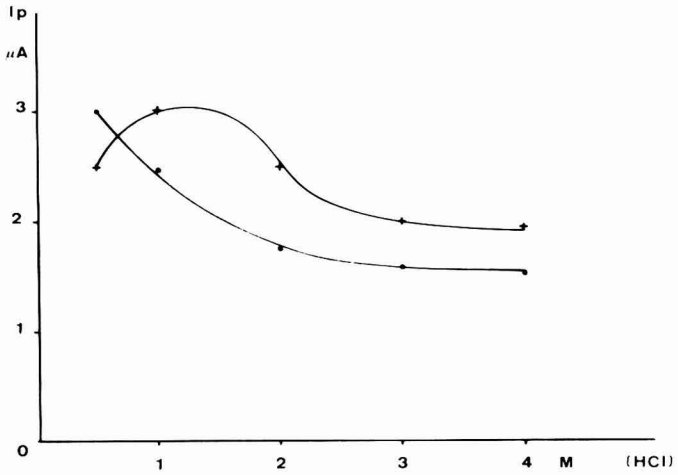

FIG. 2. Influence of the hydrochloric acid concentration on peak heights of lead (●) and tin (+). Solvent: methanol–isopropanol–water (38–38–24).

Effect of Electrolysis Potential

Figure 4 illustrates the influence of the plating potential on the relative peak heights under the above solution conditions. The peak heights of the two cations present a plateau in the plot between −1.00 and −0.85 vs SCE.

Simultaneous Measurement of Tin and Lead

Calibration curves for either tin or lead (40–400 ppb) were unchanged

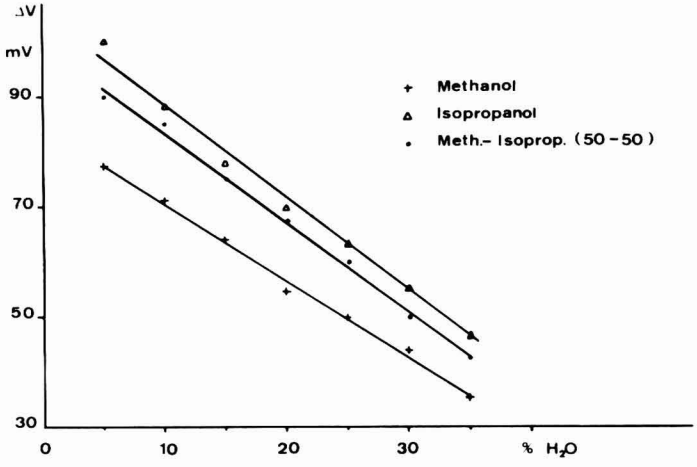

FIG. 3. Influence of water percentage on ΔV between tin and lead peaks.

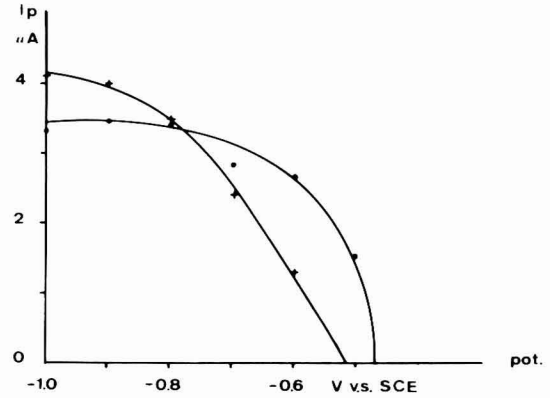

FIG. 4. Effect of electrolysis potential on the peak heights of lead (●) and tin (+).

when the other element was present at up to 400 ppb. Measurements of mixtures of copper, lead, tin, and cadmium were possible. Figure 5 illustrates a.s.v. recordings for a mixture of the four elements with varying concentrations of tin and lead. A large excess of iron(III) exhibits no influence on the peak. Either tin(II) or tin(IV) results in the same peak height. Table 1 summarizes results for the measurement of mixtures of copper, lead, tin, and cadmium in a solution that contains 100 ppb of each element, using the method of standard additions. Recoveries were 95 to 102%.

Application to Analyses of Juices

We have observed that the classical method of destruction of organic matter by acid digestion results in loss of tin in organic media. Hence, we

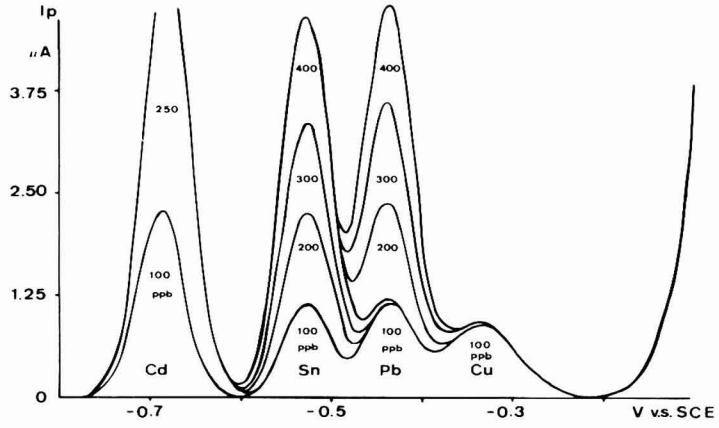

FIG. 5. Anodic stripping voltammograms of various concentrations of tin and lead in the presence of cadmium and copper ions.

TABLE 1
DETERMINATION OF TIN, LEAD, CADMIUM, AND COPPER USING THE METHOD OF STANDARD ADDITION[a]

Cd (added) (ppb)	I_p (μA)	Sn (added) (ppb)	I_p (μA)
0	2.27	0	1.16
+ 50	3.41	+100	2.31
+100	4.63	+200	3.46
+150	5.90	+300	4.67
Recovery: 95 ppb		Recovery: 100 ppb	
Pb (added) (ppb)	**I_p (μA)**	**Cu (added) (ppb)**	**I_p (μA)**
0	1.19	0	0.96
+100	2.43	+100	1.87
+200	3.67	+200	2.91
+300	4.91	+300	4.01
Recovery: 96 ppb		Recovery: 102 ppb	

[a] Initial elements concentrations: 100 ppb.

have investigated the possible direct determination of the above trace metals in fruit juices by differential pulse a.s.v. All juice samples were prepared by diluting 5 μl to 25 ml with the supporting electrolyte.

Lead and tin added to the juices at levels of 1 μg per 5 μl of juice were recovered with 95 to 98% efficiency compared to standards prepared in the supporting electrolyte. A number of juice samples were analyzed using the method of standard additions. No lead was detected in any of the samples (detection 10 ppm in the sample or 2 ppb in the analysis solution). Lead can be detected in the presence of 30-fold excess of tin. Tin was readily measurable in all samples. The detection is the same as for lead, and tin can be accurately measured in the presence of 12-fold excess lead and can be detected in the presence of 30-fold excess lead. The results of the analyses (5 replicates per samples) were as follows: grape fruit juice (fresh can): 129 ppm Sn (old can): 421 ppm Sn. vegetable juice: 108 ppm Sn.

These results are in agreement with a study made by Garcia-Olmedo *et al.* (*5*) and demonstrate the gradual dissolution of tin from the can upon standing.

Conclusion

The method described here for the determination of tin and lead in juices should be applicable to the systematic investigation of foods stored in cans. The evidence of gradual dissolution of tin with time should be

investigated in detail for other samples and related to possible human health effects.

SUMMARY

Tin and lead may be determined in mixture in a solution of 50% methanol–50% isopropanol containing 1 *M* hydrochloric acid. The solvent–electrolyte composition affects both the relative a.s.v. peak heights and peak resolution. Iron(III) in large quantities does not interfere, and mixtures of copper, lead, tin, and cadmium may be analyzed. Juice samples can be analyzed without digestion, by simple 1:5000 dilution (5 μl to 25 ml) with the above solvent electrolyte. It was demonstrated that tin gradually dissolves from cans containing the juice.

ACKNOWLEDGMENTS

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Sensitive High-Density Lipoprotein Cholesterol Assay[1]

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INTRODUCTION

One of the important and critical needs in the determination of high-density lipoprotein cholesterol (HDL-C) is the ability to measure accurately the relatively low concentrations of cholesterol that one is apt to encounter when the other lipoproteins are removed. It would seem to be apparent that if the total cholesterol (TC) is determined spectrophotometrically by an adequate colorimetric reaction that has a low molar absorptivity, then the removal of most of the cholesterol by a precipitation reaction may not create an environment that is the best for quantification. As recently shown (*11*), normal HDL-C specimens are in the concentration range of 500 ± 140 mg/liter (1.259 ± 0.363 m*M*) while the normal range for serum TC is 1890 ± 400 mg/liter (4.90 ± 1.036 m*M*). However, abnormal specimens may have HDL-C well below that concentration range. In using a version of the currently popular Gibbs reaction in a Trinder-type modification involving oxidative coupling of phenol (Ph) and 4-aminoantipyrene (*16*), the absorbance for 1000 mg/liter when using the recommended 1:100 addition of sample to reagent was approximately 0.14–0.16 *A* units/1000 mg/liter. This is adequate for the determination of serum TC where the average value may be approximately 2000 mg/liter (5.18 m*M*) or more. When the values encountered are in the range of 350–650 mg/liter (0.907–1.684 m*M*) and there is a need to be accurate to within ±50 mg/liter (*17*), the problem in measurement is intensified. The absorbance for 50/1000, a few milliabsorbance units, is obviously small enough to make its measurement difficult to be reliably precise at either end of the normal range.

The present investigation will describe a modified reaction using sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) in place of phenol. This compound was synthesized by us in our laboratories as

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previously described (*2*). The auxochromic effect of the chloro substituents will magnify the absorbances obtained with no basic change in the reaction condition, thereby allowing a more reproducible signal from the equilibrium reaction measured.

Several factors, as outlined below, should be considered when using such a modification. The competing interference of bilirubin in the final step should be studied spectrophotometrically because its concentration ratio to HDL-C will be much larger than it would be to TC. Perhaps HDL-C should not be determined in a serum from a juandiced patient. However, these requests are sometimes made of the clinical laboratory. The concentration of protein that can be tolerated with such low concentrations of cholesterol must be studied in order to be able to use an optimum aliquot of serum. The effect of removing low-density and very-low-density lipoproteins from severely lipemic serum as a water displacement effect should also be considered here inasmuch as such a loss of volume could be considerable (*1*), causing a problem in the determination of the ratio of HDL-C to TC as a potential risk factor. The effect of the reagents needed to separate HDL from low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) on the final sequence of reactions used to quantify HDL-C should be studied inasmuch as negative interference from the precipitating chemicals has been described recently (*7*).

MATERIALS AND METHODS

Reagents

Sodium-2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) was prepared as previously described (*2*) and was added to a dry cholesterol reagent prepared for us by Abbott Laboratories, Diagnostics Division (South Pasadena, Calif.). The latter contained per liter of reconstituted reagent solution, cholesterol oxidase, EC 1.1.3.6, 167 IU/liter; cholesterol ester hydrolase, EC 3.1.1.13, 117 IU/liter; horseradish peroxidase, EC 1.11.1.7, 27667 IU/liter; sodium cholate 3 m*M*; 4-aminoantipyrine (4AAP), 0.8 m*M*; Na_2HPO_4, 50 m*M*; NaH_2PO_4, 50 m*M*; and Carbowax-6000, 0.2 m*M*. To this mixture were added sufficient quantities of 4AAP and HDCBS so that their final reagent mixture concentrations were 2.4 and 9 m*M*, respectively. When reconstituted in water, 40 mg of the final mixture/ml of solution had a measured pH of 6.70 ± 0.20.

Magnesium chloride, 2m. The contaminated solution used to dissolve the enzyme reagent instead of water contained 9×10^{-4} *M* Mg^{2+} and 9×10^{-3} g/liter of dextran sulfate.

Dextran sulfate, 20 g/liter. We used the Kostner technique and reagents (*9*) for precipitation of LDL and VLDL as the source of preparation with

the understanding that there is some controversy over their use (*7*). But we carried out the reaction at 37°C rather than at ambient temperature and the reaction temperature is considered to be the cause of the discrepancies (*7*). Other reagents for precipitating these lipoproteins shown to be compatible with Trinder reactions could be substituted. It is our purpose to go on and determine the other lipids of the HDL fraction and we have found the $MgCl_2$–dextran precipitation to be most compatible with our phospholipid and triglyceride reactions, a study that is presently being carried out in our laboratories.

Procedure

Some alternative technology is feasible as the procedure for the preparation of the HDL-C containing serum fraction. It is possible to prepare dried quantities of reagent in centrifuge tubes so that no corrective mathematics is necessary owing to dilution of the sample by the precipitating chemical or, alternatively, concentrated reagents can be added in a small volume to the sample thereby minimizing the correction required. The latter approach is more convenient when reagents are prepared rather than purchased. Therefore, 50 μl of each reagent (or 100 μl of the mixed reagent) was added to 1.0 ml of serum and mixed. After 10 min, the serum was centrifuged to remove LDL and VLDL leaving only the HDL in the supernatant fluid. Then 0.005 or 0.01 ml of the treated serum was added to either 0.5 or 1.0 ml of the enzyme reagent solution. After 15 min at 37°C the absorbance was determined against a reagent blank at 510 nm and corrected for the volume dilution by the reagent. If the specimen contains bilirubin, it is important that a serum blank not be subtracted because this would have the effect of lowering the value inasmuch as bilirubin and its color are not static (*7, 8*). This phenomenon will be treated further under Results and Discussion.

Total cholesterol was determined for the calculation of a cardiovascular risk factor (*6*) by pipetting 0.005 ml of serum into 1.0 ml of reagent solution containing 4AAP–HDCBS as the equilibrium reagent, mixing well, holding the sample 10–15 min at 37°C, then measuring the endpoint absorbance at 510 nm against a reagent blank.

RESULTS AND DISCUSSION

The primary reason for undertaking this investigation was to minimize the problem of determining the cholesterol concentration of the HDL-containing fraction within very small concentration limits. The ability to do this represents an important advantage for the 4AAP–HDCBS substitution for 4AAP–Ph as the hydrogen donor in the peroxidase-coupled reaction step involving the generated hydrogen peroxide. The sensitivity factor is shown graphically in the comparative calibration

slopes of Fig. 1 where a decided increase in the molar absorptivity with a ratio of 4.4/1 is demonstrated. In this case the ±50 mg/liter cholesterol for the 4AAP–Ph reaction would result in an absorbance of 0.0077 whereas 50 mg/liter for the HDCBS-containing system would result in an absorbance of 0.034. The ratio of the 4AAP–HDCBS to the phenol equilibrium reaction generated the factor above. The advantage becomes obvious for adding an auxochrome and providing the needed sensitivity for a reaction which has previously resulted in a small, perhaps too small, delta absorbance signal for a concentration of cholesterol capable of generating only a few milliabsorbance units with what is the presently accepted quantification reagent system. Obviously, the larger one can make a reproducibly accurate signal, the better chance one is provided with for distinguishing small signal differences.

On the assumption that the operating range for HDL-C determination would be below 1000 mg/liter with most samples, averaging about half of that value or less, a precision study was carried out to determine the kind of results one could expect to obtain under ideal conditions. The conditions used for the study were the replicate determination of standards in the range of 250 to 1000 mg/liter with assay of the standards accomplished by both the 4AAP–Ph (*16*) and 4AAP–HDCBS (*3*) equilibrium reactions. This was obviously an ideal circumstance for obtaining precise results inasmuch as it was carried out without the addition of LDL–VLDL precipitating reagents (*9*) or the use of ultracentrifugation (*14*) or electrophoretic separation (*4*, *5*). The findings are shown in Table

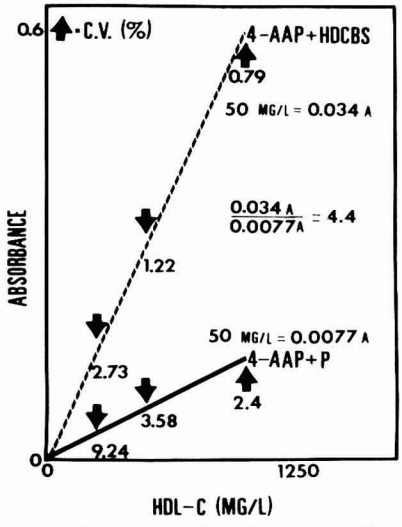

FIG. 1. The comparative slope characteristics of the reactions involving 4AAP + phenol versus 4AAP + HDCBS.

1. It can be seen that the coefficient of variation (C.V.) of each concentration is better for the 4AAP–HDCBS reaction in going from low to high concentrations of cholesterol and that the 4AAP–Ph reaction is the lesser of the two in terms of the precision to be expected under these circumstances. If one substituted serum for standards with any of its potential interfering compounds along with the addition of the precipitating agents with their attendant dilution of the absorbances to be attained, then one might expect to see some increase in C.V. for the reactions. In considering the ratio of sensitivities shown in the figure which favors the 4AAP–HDCBS reagent by a very large factor, the chances for attaining better precision obviously reside with the 4AAP–HDCBS system since it takes approximately a 5× scale expansion for the 4AAP–Ph reagent to make its slope appear to be the same as the slope of the more sensitive

TABLE 1
REPRODUCIBILITY STUDY FOR PHENOL VERSUS
2-HYDROXY-3,5-DICHLOROBENZENESULFONATE SYSTEMS

	Phenol			HDCBS		
	250 mg/l	500 mg/l	1000 mg/l	150 mg/l	500 mg/l	1000 mg/l
	0.034	0.061	0.119	0.158	0.316	0.660
	0.033	0.063	0.119	0.173	0.309	0.664
	0.033	0.068	0.118	0.158	0.313	0.658
	0.036	0.060	0.119	0.165	0.309	0.656
	0.030	0.061	0.121	0.159	0.314	0.661
	0.041	0.061	0.122	0.160	0.308	0.665
	0.031	0.065	0.125	0.160	0.312	0.651
	0.032	0.060	0.119	0.161	0.309	0.651
	0.033	0.065	0.125	0.162	0.306	0.670
	0.032	0.063	0.120	0.160	0.304	0.662
	0.034	0.061	0.120	0.159	0.308	0.662
	0.032	0.060	0.120	0.161	0.311	0.658
	0.034	0.062	0.120	0.154	0.307	0.654
	0.033	0.065	0.116	0.162	0.308	0.649
	0.038	0.063	0.117	0.157	0.314	0.656
	0.039	0.064	0.123	0.169	0.309	0.657
	0.029	0.064	0.118	0.166	0.311	0.661
	0.030	0.063	0.116	0.162	0.304	0.658
	0.031	0.060	0.114	0.164	0.302	0.659
	0.032	0.060	—	0.157	0.304	—
N	20	20	19	20	20	19
$\bar{x}$	0.0333	0.0625	0.1195	0.161	0.3089	0.6585
SD	0.0031	0.0022	0.0029	0.0044	0.0038	0.0052
C.V. (%)	9.24	3.58	2.4	2.73	1.22	0.79
mg/l	250 ± 23.1	500 ± 17.9	1000 ± 24.0	250 ± 6.8	500 ± 6.1	1000 ± 7.9

reaction. The sensitivity ratio obtained here between the two reactions approaches a factor of 5 which is slightly better than that of the two calibration slopes shown in Fig. 1. Perhaps, when enzyme reagents get too old, they may lose some of their overall sensitivity. In our experience, it does not seem expedient to use them beyond their expiration date without critical evaluation.

In spite of the fact that it has been described that bilirubin can interfere with peroxidase-coupled reactions sequenced to peroxide-generating reactions of oxidases, some manufacturers' instructions persist in suggesting a static sample blank to correct for a presumptive static background of irrelevant absorption even though the bilirubin interference is obviously dynamic (*10*) and the presumption is specious. It can be demonstrated that such a technique is in error and will result in values unrelated to the cholesterol content being determined. It can also be demonstrated for the 4AAP–Ph reaction that if uncorrected, the end absorbance will approximate that of the true cholesterol even though the intended indicator reaction is only measured in part (*10, 19*). This fortunate compensatory action results from the fact that within limits of bilirubin concentration, the competing hydrogen donor in the peroxidase step, bilirubin, has just enough remaining color to compensate for the loss of the oxidized 4AAP–Ph complex that should have been generated. Some additional considerations should be noted. If a different indicator system were used which does not overlap the bilirubin interference spectrum in a similar manner, then the results obtained may be uncompensated. Also, if a new indicator color reaction is used whose molar absorptivity greatly exceeds that of the 4AAP–Ph indicator reaction, then the results might be even lower owing to the lesser ability to compensate. However, if the new indicator color reaction has a reaction rate many times faster than that of bilirubin as a competitive reaction, then the interfering effect of bilirubin might be more static than dynamic and therefore more amenable to corrective action by simple sample blanking (*13*). In our own experience, this latter phenomenon has not yet been encountered in oxidase-coupled reactions.

The potential problem of bilirubin interference in the enzyme reagent system including the chlorinated–sulfonated phenol modification has been previously described for serum total cholesterol assays (*10*). However, when determining HDL-C, the concentrations at which such an interference could react is magnified in a relative way by the fact that low-density and very-low-density lipoproteins are removed prior to determining HDL-C and the latter protein may contain a quarter or less of the original total cholesterol. In view of the precision required in HDL-C assays, should a request like this be received on a specimen which con-

tains a high concentration of bilirubin, it might be difficult to carry out the determination without some prior specimen treatment to avoid what could be a severe interference.

To emphasize the effect of bilirubin, a repeat of a previous experiment was carried out (*3, 10*) in which the bilirubin concentration was at a high level whereas the cholesterol concentration was kept at the moderately high level that one might expect to encounter in an HDL-C determination. The results are shown in Fig. 2. The findings are predictably similar to those previously described (*3, 10*) in which bilirubin is shown to be a successful competitor with the suggested indicator reaction used in the peroxidase coupled step. Similar considerations can be shown for drugs which unlike bilirubin generate no compensating colors, making values obtained always lower than those theoretically expected (*8, 12*). All direct oxidase procedures using similar endpoint devices are prone to this weakness in measurement.

When speculating on what might occur in the face of severe lipemia, the addition of a chemical reagent to remove lipoprotein fractions of serum other than HDL could lead to a decrease in the volume of that serum, a decrease whose significance would depend on the level of the triglycerides. The presence of high concentrations of lipids as a water

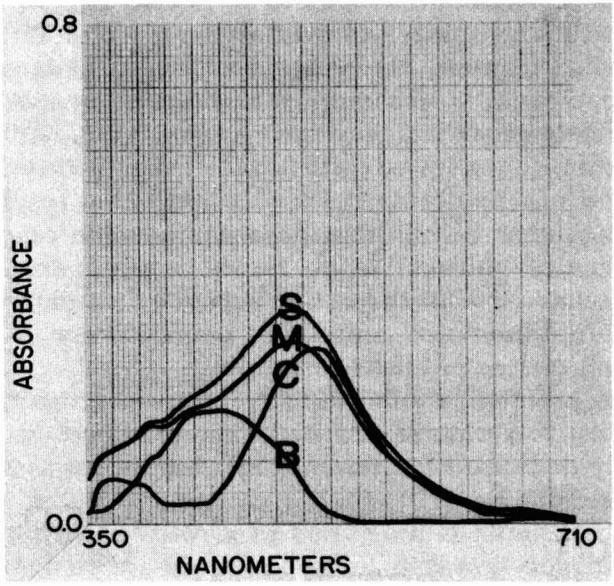

FIG. 2. Spectra are shown for bilirubin in albumin (B), cholesterol (C), the mixture of the two reacted simultaneously (M), and the summation of C and B by exhausting H_2O_2 before adding bilirubin (S).

displacement error (*1*, *15*) has resulted in artifactually low electrolyte values that are correctible by mathematical treatment in which the triglyceride concentration is considered to be the major contributor (*1*, *15*). However, in the circumstance of severe lipemia, because a water displacement phenomenon resulting in a change in volume may be caused by use of a chemical precipitation method, the HDL-C is obviously higher than it would have been could it have been measured without the shrinkage in volume. Conversely, the TC because it is determined on the original sample would have been calculated on the basis of the original volume. This difference in treatment of TC versus HDL-C could cause a distortion of the ratio obtained between them, and that distortion would vary as a function of how the triglycerides of serum varied in concentration (*15*). The question of what to do about such potential artifactual ratios in the study of risk factors awaits study and clarification, but obviously it should be considered when dealing with severely lipemic specimens.

The displacement of water by triglycerides as a potential volume exclusion error (*1*, *15*) has received little if any speculative or theoretical investigation other than with electrolytes. If a separation scheme to remove all lipoproteins except HDL were perfect and only the HDL remained behind, the question of the volume may have to be considered if triglyceride values are high. In the extreme case involving severely turbid serum where the triglyceride concentration may be represented by a significant volume of serum, the removal of that volume would mean that whatever remained in the clarified fraction might have to be corrected for the change in volume. One might speculate that if the volume change was ignored, any relationship of HDL-C to TC would be distorted. The ratio of HDL-C:TC must, in the final analysis, be based on a constant relationship of the volume characteristics of the serum aliquots used to carry out these two important determinations.

A hypothetical example of how this water displacement error could affect concentration is shown in Fig. 3. In this case the corrective equation was the same one used in a similar way when determining electrolytes in serum (*15*). It was applied here because little corrective effort has been devoted toward anything but electrolytes and therefore no corrective equation other than this one has been suggested. Each of the serums are arbitrarily established to originally contain the same concentration of HDL cholesterol shown as 1.0 before treatment but to vary in their triglyceride concentration by up to 15 g/dl. If removal of the turbidity-causing lipids is complete (*9*), as it might be, and if the water displacement error is calculable as it should be (*15*), the values found would be represented by this calculated line shown where the water displacement causes an approximate 2% error on the original sample for each g/dl of triglycerides

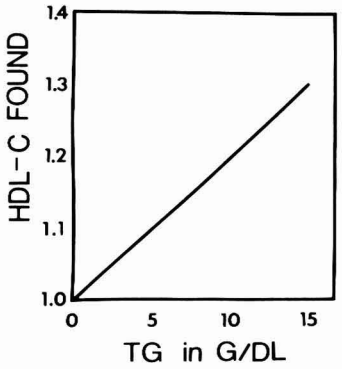

FIG. 3. Variation of HDL-C found in supernatant fluid after theoretically correcting for triglyceride concentrations in g/liter (*15*).

present (*15*). At the limit shown for this hypothesized and imaginary experiment, where the difference between treated specimens approaches 30%, the final volume of serum tested could obviously be concentrated with respect to the HDL and its lipids by a very large factor, undoubtedly resulting in a volume problem not unlike that encountered when sodium is determined in lipemic specimens by flame photometry (*18*). Such an error would artifactually elevate HDL-C values in treated specimens and compromise comparisons to untreated specimens as in electrophoresis were this dilution factor to be disregarded. Furthermore, in the calculation of a risk factor such as HDL-C:TC ratios, the TC would have been determined on the untreated specimen whereas the HDL-C would have been determined on the concentrated specimen resulting in incorrect ratios which could be misleading to both patient and physician if the volume change was ignored.

A simple experiment was then carried out to test whether the LDL and VLDL precipitating reagent, $MgCl_2$–dextran sulfate interfered with the determination of cholesterol in the HDL fraction remaining after these chemicals were added to the serum. The serum was itself diluted with saline to prepare a concentration of cholesterol approximating that of normal HDL concentration, roughly 500 mg/liter, and extending beyond that into the normal range of total cholesterol at roughly 2000 mg/liter. The enzyme reagent to be used for the assay of this serum was dissolved with a dilute solution of $MgCl_2$–dextran sulfate at a concentration of the latter which would be present when 10 μl of treated serum was added to 1.0 ml of the enzyme reagent. This contaminated enzyme reagent was then compared to the uncontaminated enzyme reagent prepared by solution with water. The reactions were monitored at room temperature and at 37°C in order to see if the $MgCl_2$–dextran sulfate interfered with the reaction as has recently been described (*6*). Spectra of the serum and its

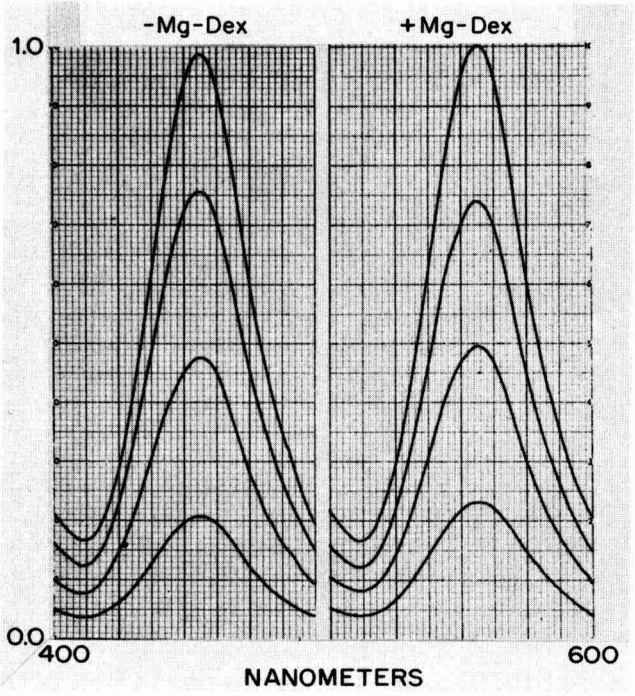

FIG. 4. Spectra of various concentrations of serum cholesterol with (right) and without (left) the inclusion of magnesium chloride–dextran sulfate precipitating reagent.

dilutions are shown for the reactions of both reagent solutions with several serum concentrations of cholesterol. These spectra were obtained at equilibrium at 37°C and the results are plotted in Fig. 4. It can be seen that within experimental error the reaction is similar for both reagent solutions.

The experiment in reactivities was continued by drawing rise curves for the cholesterol reactions with and without precipitating reagents. These curves were compared for the water-diluted reagent and the $MgCl_2$–dextran sulfate-contaminated reagent using an intermediate concentration of cholesterol while carrying out the reactions at both 25 and 37°C. The results indicated a significant difference between the two solutions at the two temperatures with a 10% lower value obtained for the test concentration at 25°C. On observing the two sets of rise curves one can see that the reaction at 25°C had not reached equilibrium at 15 min although it was approaching it, whereas the reaction at 37°C had definitely plateaued beyond 7 min. The rise curves are shown in Fig. 5.

The effect of protein concentration on the 4AAP–HDCBS reaction was demonstrated previously in a cerebrospinal fluid study on the determination of cholesterol (*3*). There, it was shown that interference only

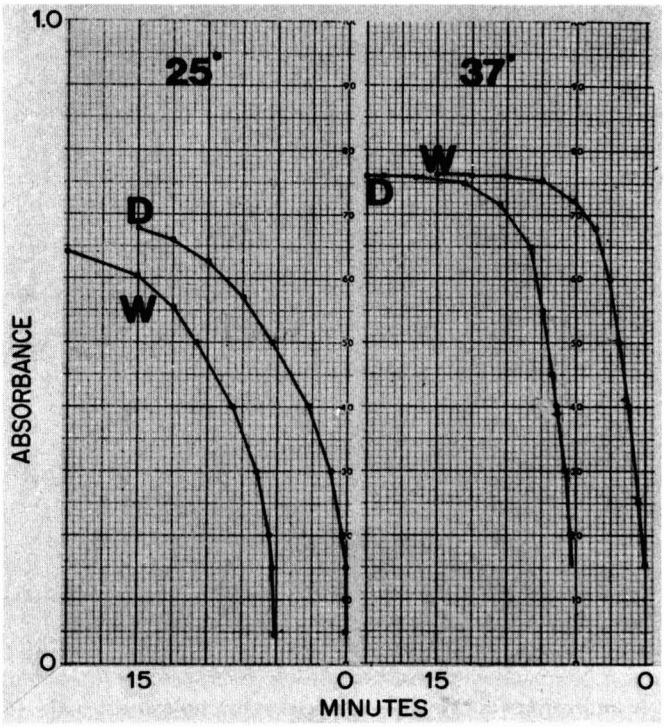

FIG. 5. Rise curves demonstrating effect of temperature on reaction of cholesterol in serum for aqueous (W) and magnesium chloride–dextran sulfate (D) presence at 25 and 37°C.

began when 2.5 g/liter of protein in the final dilution was exceeded, but the error was small, becoming 2% at 5 g/liter. This concentration of protein would correspond to a much higher concentration of protein than one could ever encounter in normal or most pathological serum specimens, for a pathologically high 100 g/liter serum, would be less than 1 g/liter when diluted to its final reaction mixture concentration. Therefore, more than twice the conventional sample could be tolerated before interference from this source would manifest itself.

SUMMARY

A procedure has been described in which a currently acceptable simple procedure for the precipitation of low-density and very-low-density lipoproteins is paired with a modified sensitized enzyme reagent system containing sodium 2-hydroxy-3,5-dichlorobenzenesulfonate for the determination of cholesterol by equilibrium reaction with the high density lipoprotein fraction of serum. The resulting increase in absorbances by using the auxochromic derivative of phenol is some four to five times that of the phenol system and therefore makes it possible to discriminate with more assurance within the critical ±50 mg/liter concentration difference. Factors such as comparative calibration slopes for the phenol reaction and its auxochrome substitute, the water displacement error, the presence of bilirubin, and the contamination by LDL–VLDL precipitating reagents are considered and discussed.

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The Polarography of Oximes

II. 1,2-Acenaphthaquinone Monoxime

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INTRODUCTION

The polarographic behavior of oximes has been studied by several authors (*1, 4–9, 13, 14, 18, 19*). In general, the reduction products of an oxime group at the dme was the corresponding amine. This process was shown to involve four electrons per molecule. It has been shown (*3, 5*) with α-ketoximes that, unlike simple oximes, these compounds are reduced to the corresponding amines even in a strongly basic medium. The present investigation deals with the polarographic behavior of 1,2-acenaphthaquinone monoxime, which is an α-ketoxime, on which no polarographic work is reported so far. This compound has been shown to be a good complexing and precipitating agent (*17*) and hence was chosen for the present study.

MATERIALS AND METHODS

1,2-Acenaphthaquinone monoxime was prepared from its parent quinone, as described by Sharwan Kumar (*17*). Stock solutions of the parent quinone and its oxime were prepared in purified ethanol. All other chemicals were of analytical reagent grade purity. HCl–KCl, HCl–sodium acetate, Na_2HPO_4–citric acid, and boric acid–NaOH buffers were used. The ionic strength was adjusted to 0.5 *M* with KCl. All experiments were carried out in 40% v/v alcoholic media. pH measurements were made with an Elico pH meter (Model PH821A). Nitrogen

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gas used for deoxygenation of the solutions was purified as described by Meites (*15*, p. 89) and was passed through a 40% v/v alcohol water mixture prior to its entry in the polarographic cell. Triton X-100 was used as a maximum suppressor. The temperature was maintained with an accuracy of ±0.5°C at 25°C. All potentials are referred to that of saturated calomel electrode (SCE). A manual dc polarographic setup was used. The capillary characteristics were: m = 2.35 mg/sec and t = 3.00 sec/drop in 0.5 M KCl (open circuit) at $h_{corr.}$ = 48.4 cm. The mercury used for the dme was first purified chemically and subsequently distilled under reduced pressure.

Millicoulometric analyses were carried out at pH 3.5, 7.5, 10.45, and 13.0. The diffusion coefficients were determined by using a McBain–Dawson diaphragm cell and applying the King–Cathcart equation (*11*).

RESULTS AND DISCUSSION

In order to identify the wave(s) due to the reduction of the oxime group and due to lack of the data on 1,2-acenaphthaquinone in the same buffer system and ionic strength, preliminary studies on 1,2-acenaphthaquinone and 1,2-acenaphthaquinone monoxime were carried out under identical conditions at 25°C in the pH range 3.5–13.0. It has already been established (*10*) that 1,2-acenaphthaquinone exhibits two one-electron waves: the first corresponds to the formation of semiquinone radical anion and the second results from the reduction of this radical. In the present study two waves each were observed with both the quinone and the oxime, up to pH 7.5, beyond which the second wave disappeared. Further, the height of the first wave of 1,2-acenaphthaquinone monoxime was four times the height of the corresponding wave of 1,2-acenaphthaquinone. This indicated that the number of electrons involved in the reduction of 1,2-acenaphthaquinone monoxime was four, which has been confirmed by other methods described later. A number of workers (*1*, *5*, *6*, *8*, *9*, *14*, *18*) have reported a 4e reduction for oximes culminating in the formation of the corresponding amines. In most cases it has been observed that the wave heights of oximes decreased in strongly basic media, which has been attributed to the lesser tendency of the anion to be reduced at the electrode (*2*, *18*) or to the slow rate of recombination of the oxime anion (at pH > pK_a) with H^+ prior to its reduction (*20*). This is discussed later.

Having established the identity of the wave due to the oxime group, a detailed investigation was carried out to examine the nature of the wave. The tests of i_d/C, $i_d/h^{1/2}$, negative shifts of $E_{1/2}$ with concentration, slopes of $\log i/(i_d\text{-}i)$ vs E plots, and the temperature coefficients of $E_{1/2}$ and i_d were carried out at pH 3.50, 7.40, 10.45, and 13.00. All these tests showed that the oxime group underwent diffusion-controlled irreversible reduction.

The diffusion coefficient D of the oxime was determined under conditions similar to those for polarographic test solutions with regard to composition and pH. The values of D obtained were 2.92, 2.90, 2.88, and 3.02 $\times$ 10^{-6} cm^2/sec at pH 3.50, 7.40, 10.50, and 13.00, respectively. These values of D were determined by using a McBain–Dawson cell and applying the King–Cathcart equation (*11*). It can be seen that D does change much with pH and a mean value of D was taken for further calculations. Since the wave is diffusion controlled, the value of D can be incorporated into the Ilkovic equation to obtain the value of the number of electrons (n) involved in the reduction process. A value of $n = 3.98 \simeq 4$ was obtained at pH 3.50 and the n values approximated to 4 at other pH's as well. This implies that the reduction of the oxime group goes up to the amine stage.

Millicoulometric studies at the above-mentioned pH's also yielded a value of n close to 4. Controlled-potential electrolysis (cpe), using a mercury pool cathode, was also carried out at pH 3.50 and 7.50 at a potential on the plateau of the second wave. The resulting solutions showed a positive test for ammonia, thereby indicating the fission of the C–N bond.

The wave heights of the first wave of 1,2-acenaphthaquinone monoxime were found to be almost equal to those of 9,10-phenanthraquinone monoxime (*16*) under similar conditions. It has already been shown that 9,10-phenanthraquinone monoxime undergoes a 4*e* reduction (*16*). Hence, it is reasonable to infer that 1,2-acenaphthaquinone monoxime also undergoes a 4*e* reduction to form the corresponding amine. The $E_{1/2}$ values of the first wave showed a cathodic shift with pH. This showed that H^+ are involved in the potential-determining step. The pK_a of the oxime has been found to be 9.30 $\pm$ 0.02 in water (*17*). It can be justifiably assumed that the oxime anion is the predominant species in the bulk of the solution at pH $>$ 10.0. The existence of a wave at pH $>$ 10.0 whose height remains unaltered indicates that either the oxime anion is being reduced directly or the recombination of the anion with H^+, at the electrode surface, is fast. Since $E_{1/2}$ values showed a cathodic shift even beyond pH 10.0, it can be concluded that the anion is not reduced. The $E_{1/2}$ of the reduction of the anion is expected to be pH independent (*20*) at pH $>$ pK_a, i.e., the slope of the $E_{1/2}$–pH plot is expected to be zero in this region. The slope is, however, observed to be 142 mV/pH with 1,2-acenaphthaquinone monoxime in this region. Oximes are known to exhibit two waves at pH $\simeq$ pK_a, corresponding to separate reduction of the oxime and its monoanion to an α-aminoketone (*18*). Where there is no separation in current–voltage curves, logarithmic analysis can reveal the existence of two waves (*20*). In the present study only one wave was observed and the log plots were linear. This is additional evidence for the nonreducibility of the anion, and it could be concluded that the neutral oxime molecule is reduced since the height of the wave is maintained in the alkaline region too. Hence it is

reasonable to infer that the anion recombines with H^+, at the electrode surface, prior to being reduced and that the rate of recombination is fast as compared to the rate of the reduction of the oxime at the dme, i.e., at the electrode surface there is a rapid establishment of equilibrium between the anion and H^+ on the one hand and the neutral oxime on the other.

The $E_{1/2}$–pH plot showed two segments and the pH at the intersection was about 10.25, which is near the expected pK_a value of the oxime, on the basis of the pK_a reported (*17*) in aqueous medium. The plots of E vs $[\log i/(i_d - i) - 0.546 \log t]$ were linear at all pH values. αn_a values at various pH's were calculated from the slopes of these plots using relevant equations (*15*, pp. 242–248). These values of αn_a were introduced in the following equation to obtain the value of p, the number of H^+ involved in the rate determining step (*15*, pp. 242–248):

$$dE_{1/2}/d(\text{pH}) = 0.061\, p/\alpha n_a.$$

The value of p was found to be 0.86 and 0.98, respectively, in the pH range 3.50–9.20 and 10.5–13.0. These values of p are close to 1, showing that the rate-determining step involved one proton. The values of kinetic parameters αn_a and $-\log k^\circ_{f,h}$ have been calculated by Koutecky's procedure (*12*) and are given in Table 1. The values of αn_a thus calculated are

TABLE 1

POLAROGRAPHIC CHARACTERISTICS OF 1,2-ACENAPHTHAQUINONE MONOXIME [a] IN BUFFERS CONTAINING 40% ETHANOL AT 25°C

pH	$-E^{h}_{1/2}$ (vs SCE)	i_d (μA)	Slope of log plot[c] (mV)	αn_a		$-\log K^\circ_{f,h}$ (NHE)
				From slope of log plot	Koutecky's method	
3.50	0.324	5.15	83	0.653	0.657	3.71
4.40	0.380	5.15	82	0.660	0.666	4.48
5.50	0.445	5.20	85	0.637	0.620	4.84
6.50	0.480	5.20	85	0.637	0.620	5.20
7.45	0.545	5.20	86	0.630	0.619	5.99
8.40	0.576	5.10	86	0.630	0.619	6.29
9.30	0.622	5.20	86	0.630	0.607	6.47
10.50	0.697	5.20	110	0.492	0.486	6.77
11.45	0.790	5.15	110	0.492	0.483	7.19
12.40	0.948	5.10	118	0.459	0.442	8.08
13.00	1.061	5.20	120	0.451	0.441	8.78

[a] Concentration of oxime = 0.6 mM; ionic strength 0.5 M.

[b] From the intercept of the log plot.

[c] E vs $[\log i/(i_d - i) - 0.546 \log t]$.

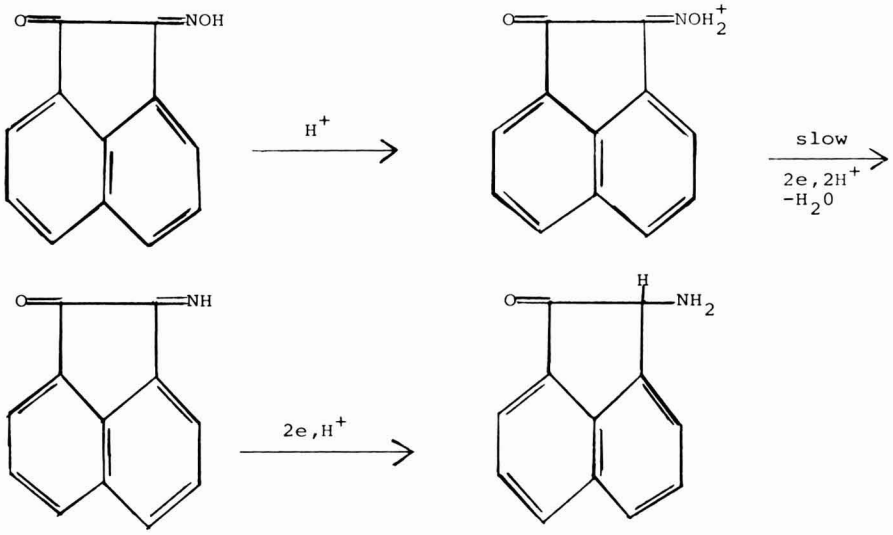
close to those calculated from the log plots. The value of $-\log k^{\circ}_{f,h}$ increased successively with pH showing thereby that the reduction was rendered more and more irreversible as the pH increased.

The second wave in the case of the oxime up to pH 7.50 which is half the height of the first wave shows that two electrons are involved in the second stage of reduction. This wave could be attributed to the reduction of the amine, with the liberation of ammonia to form acenaphthenone. A similar behavior has been reported in the reduction of 2-oxyimino-1,2-dihydropyrrolizin-1-one (*3*) and some α-aminoketones (*4*).

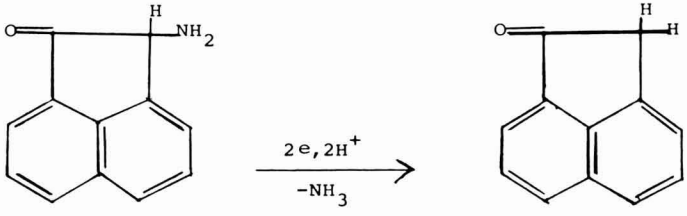
On the basis of the forgoing discussion the following mechanisms are proposed.

I. $pH < pK_a$

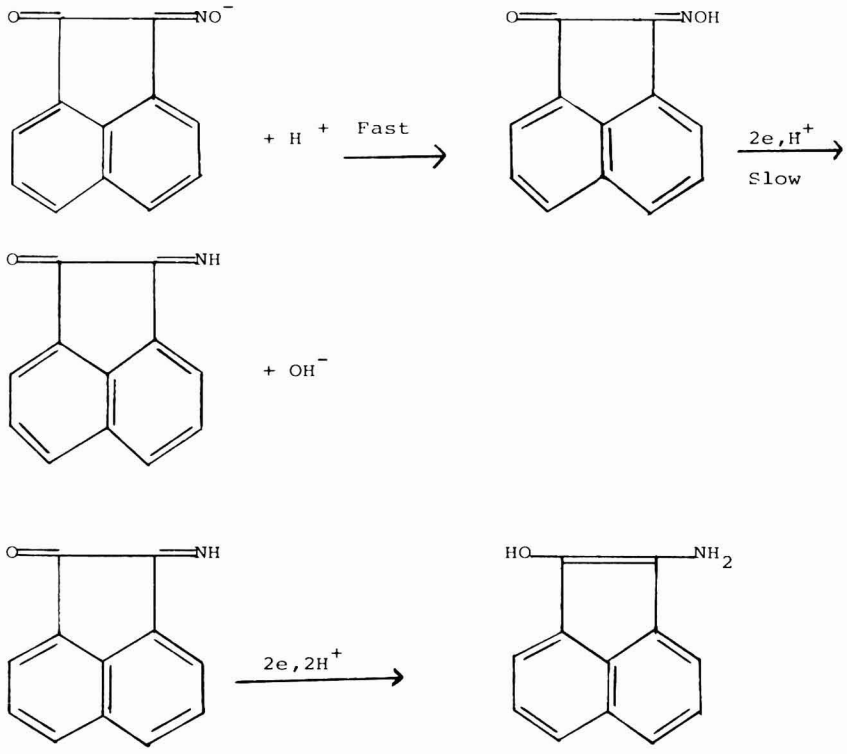
(i) First wave



(ii) Second wave



II. $pH > pK_a$



SUMMARY

Polarography of 1,2-acenaphthaquinone monoxime has been carried out in buffers (pH 3.5–13.0) of constant ionic strength 0.5 *M* and 40% alcohol v/v at 25 ± 0.5°C. The oxime group underwent diffusion-controlled reduction (4*e*) over the whole pH range. The number of electrons involved in the reduction was found by coulometric method as well as by incorporating the values of diffusion coefficients, obtained by using a McBain–Dawson cell, into the Ilkovič equation. Koutecky's method has been used to compute the kinetic parameters (αn_a and $-\log K^{\circ}_{f,h}$) for the reduction of the oxime group and a reduction mechanism is proposed.

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High Performance Liquid Chromatographic Determination of *n*-Butyl Glycidyl Ether

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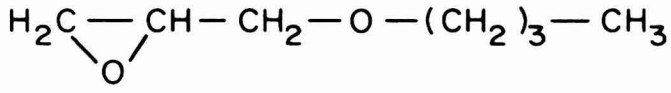
INTRODUCTION

n-Butyl glycidyl ether (*n*-BGE; 1-butoxy-2,3-epoxypropane) is an important member of the group of compounds known as glycidyl ethers, which are commonly used as reactive diluents in epoxy resins (*4*). Even though no studies have yet investigated the carcinogenic effects of long-term inhalation of *n*-BGE at low concentrations in humans or animals, this



n-BGE

compound has been implicated as a mammalian mutagen (*1*, *5*, *6*) and causes skin and eye irritation and sensitization (*4*). Hence the National Institute for Occupational Safety and Health (NIOSH) recommends that the limit for workers' exposure to *n*-BGE be set at the lower limit of detectability permitted by the NIOSH-recommended gas chromatographic (GC) method (*3*), 30 mg/m^3 (4.4 ppm), as a ceiling concentration. This NIOSH-validated GC analysis seems to be the most sensitive analytical method for the quantitative determination of *n*-BGE when compared to other presently available methods in the literature (*2*). Since this NIOSH-validated GC analysis is not sufficiently sensitive to detect levels of *n*-BGE less than 4 ppm, we have recently developed a very sensitive high-performance liquid chromatographic (HPLC) procedure for the determination of 1- to 10-ppb levels of this compound. To our knowledge, no report of any HPLC determination of *n*-BGE has appeared in the literature. The HPLC method described in this article is simple and accurate, and allows the rapid determination of *n*-BGE at concentrations down to 1 ppb (1 μg/liter).

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MATERIALS AND METHODS

A Varian Model 5000 analytical liquid chromatograph (Varian Associates, Inc., Walnut Creek, Calif.) connected with a Varichrom variable wavelength dual-beam uv-visible spectrophotometric detector (Varian Associates, Inc., Walnut Creek, Calif.) was used for the analysis. The chromatograms were recorded using a Varian Model 9176 strip chart recorder (Varian Associates, Inc., Walnut Creek, Calif.).

The chromatographic analyses were carried out with a stainless-steel micro CH-10 reversed-phase column (30 cm × 4 mm i.d.; Varian Associates, Inc., Walnut Creek, Calif.).

Materials. The sample of *n*-BGE was supplied by the Dow Chemical Co., Freeport, Texas. The acetonitrile used in this investigation was of liquid chromatographic grade (distilled in glass; Burdick & Jackson, Muskegan, Mich.). The water (distilled) was filtered through Millipore filters prior to use.

Chromatographic analysis. Different solutions containing varying amounts of *n*-BGE (1–10 μg/liter) were prepared in acetonitrile–water 90:10 (v/v) solvent mixture by appropriate dilution. The analysis was carried out by reversed phase HPLC, injecting 10 μl volumes of each diluted sample and by eluting with acetonitrile–water 90:10 (v/v) solvent mixture at a flow rate of 1 ml/min. The effluent was monitored at 245 nm (λ_{max} of *n*-BGE in this solvent composition) with a sensitivity of 0.02.

RESULTS AND DISCUSSION

Figure 1 shows the typical chromatogram for the sample of *n*-BGE in acetonitrile–water 90:10 (v/v) solvent mixture. The retention time for *n*-BGE under the present chromatographic conditions is 3 min 40 sec. The

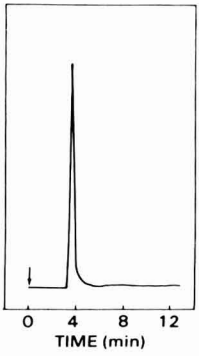

FIG. 1. High-performance liquid chromatogram of *n*-butyl glycidyl ether. Column: micro CH-10 reversed phase (30 cm × 4 mm i.d.; Varian Associates, Inc.); mobile phase: acetonitrile–water 90:10 (v/v); flow rate: 1 ml/min; temperature: ambient; detection: uv at 245 nm; sensitivity = 0.02.

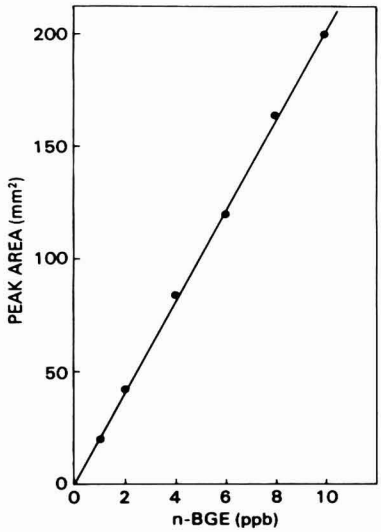

FIG. 2. Calibration graph (plot of peak area versus ppb) for *n*-butyl glycidyl ether.

calibration graph (plot of peak area versus ppb) for *n*-BGE is linear in the range of 1–10 ppb or μg/liter (Fig. 2). The peak area of 20 mm² for 1 ppb of *n*-BGE at 0.02 sensitivity indicates that this HPLC technique is highly sensitive for its determination.

The present HPLC method has great potentials for the accurate and rapid determination of *n*-BGE in biological samples such as blood, urine, bile, etc.

SUMMARY

A novel high-performance liquid chromatographic (HPLC) technique for the determination of *n*-butyl glycidyl ether (*n*-BGE) at concentrations down to 1 ppb (1 μg/liter) has been developed. This HPLC procedure is simple, highly sensitive, and rapid within the limits described.

ACKNOWLEDGMENTS

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MICROCHEMICAL JOURNAL 26, 221–227 (1981)

The Use of Redox Reactions in Analysis of Dyes and Dye Intermediates

X. Polarographic and Constant-Potential Coulometric Determination of 4,4′-Dihydroxyazobenzene and 4-Nitro-4′-hydroxyazobenzene[1]

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INTRODUCTION

In one of the previous papers (*1*) we studied a reductometric and polarographic determination of *N,N*-dimethyl-4-amino-4′-hydroxyazobenzene. As a continuation of this study, the present paper describes possibilities of analytical use of polarographic and coulometric reduction of 4,4′-dihydroxyazobenzene and 4-nitro-4′-hydroxyazobenzene in buffered media.

EXPERIMENTAL

Reagents. A 0.005 *M* solution of 4,4′-dihydroxyazobenzene was prepared by dissolving 0.2678 g of pure substance (Research Institute of Organic Syntheses, Pardubice, Czechoslovakia) in 125 ml methanol and diluting to 250 ml with distilled water. A 0.005 *M* solution of 4-nitro-4′-hydroxyazobenzene was prepared by dissolving 0.3040 g of pure substance (Research Institute of Organic Syntheses, Pardubice, Czechoslovakia) in 125 ml methanol and diluting to 250 ml with distilled water. The purity of both substances was checked by thin-layer chromatography on a Silufol UV 254 plate (Kavalier, Sázava, Czechoslovakia) with eluent systems chloroform–methanol (95:5) and methanol–ethyl acetate (1:4).

Britton-Robinson buffers with an ionic strength of 0.15 and containing 15% methanol were prepared according to Ref. (*2*). The solution pH was measured using a PHM 52 pH meter (Radiometer, Copenhagen, Denmark).

Apparatus. Polarographic measurements were carried out in a Kalousek

[1] Part IX: The Reductometric, Polarographic, and Coulometric Determination of 4-Nitrotoluene-2-sulfonic Acid. *Microchem. J.* (in press).

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cell with a dropping mercury indicator and a saturated calomel reference electrode, on an LP 60 polarograph with an EZ 2 line recorder (Laboratorní Přístroje, Prague, Czechoslovakia). The depolarizer solutions were added using an Agla microburette (Burroughs Wellcome, London, England).

Coulometric measurements were performed on an OH-404 universal coulometric analyzer (Radelkis, Budapest, Hungary), with a three-electrode circuit (mercury pool working electrode, SCE reference electrode, and a Pt foil counter electrode) in a compartment separated from the working space by a frit.

PROCEDURES AND RESULTS

Dependence of the Half-Wave Potential on the pH

To 10 ml of a Britton-Robinson buffer of a required pH ($I = 0.15$), 0.10 ml of a 5×10^{-3} M solution of a test substance and 1 drop of 0.5% gelatine were added, the solution was deaerated by 5 min passage of nitrogen and the polarographic curve was recorded.

It has been found that 4,4′-dihydroxyazobenzene yields a single well-developed wave at pH 2 to 11, whose position and height are pH dependent. 4-Nitro-4′-hydroxyazobenzene gives two well-developed waves at pH 2 to 9, the second wave being about twice as high as the first and the position and the height of both waves depending on pH. The half-wave potentials at various pH values are given in Table 1 and plotted in Fig. 1.

Dependence of the Limiting Current on the pH

The dependence of the limiting current on the pH, obtained from the above polarographic curves, is given in Table 2 and Fig. 2.

Dependence of the Limiting Current on the Height of the Mercury Reservoir

This dependence was obtained in Britton-Robinson buffers of pH 2.85

TABLE 1

DEPENDENCE OF THE HALF-WAVE POTENTIAL OF 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE ON THE pH[a]

pH	2.50	3.40	4.00	5.00	6.00	7.50	9.00	11.50
$E_{1/2}$(mV)[b]	−110	−200	−260	−345	−430	−535	−615	−850
$E_{1/2}$(mV)[c]	−45	−50	−60	−175	−275	−380	−455	−520
$E_{1/2}$(mV)[d]	−320	−310	−355	−400	−455	−600	−700	−800

[a] Depolarizer concentration, 5×10^{-5} M; reservoir height, 36 cm, SCE.

[b] 4,4′-Dihydroxyazobenzene.

[c] 4-Nitro-4′-hydroxyazobenzene, first wave.

[d] 4-Nitro-4′-hydroxyazobenzene, second wave.

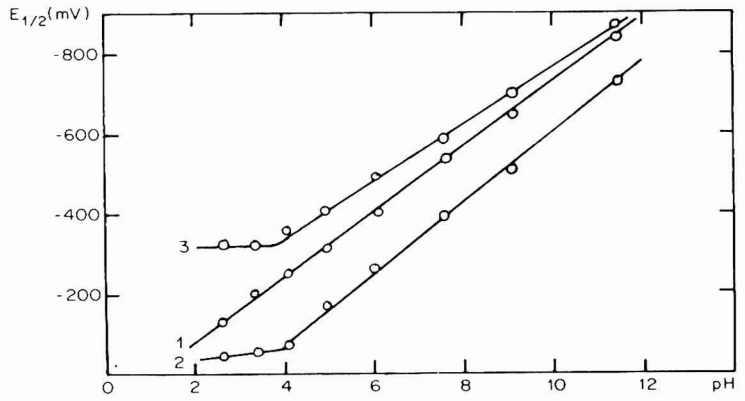

FIG. 1. Dependence of the half-wave potential of 4,4′-dihydroxyazobenzene (1) and the first (2) and second (3) wave of 4-nitro-4′-hydroxyazobenzene on the pH. The depolarizer concentration is 5×10^{-5} *M*; the reservoir height is 36 cm.

and 9.25 for 4,4′-dihydroxyazobenzene and 3.35 and 9.0 for 4-nitro-4′-hydroxyazobenzene, at a depolarizer concentration of 5×10^{-5} *M*. It is given in Table 3 and plotted in Fig. 3.

Dependence of the Limiting Current on Temperature

This dependence was measured in the same solutions as the dependence on the height of the mercury reservoir and the temperature coefficients found, $\Delta i/\Delta t$, are given in Table 4.

Dependence of the Limiting Current on the Depolarizer Concentration

For determination of 4,4′-dihydroxyazobenzene the optimal solution has a pH of 4 or 9, for determination of 4-nitro-4′-hydroxyazobenzene a pH of 3.5 or 9; best-developed waves were obtained in these media. The concentration dependence of the limiting current was measured in these solutions as follows: To 10 ml of a Britton-Robinson buffer of a required pH were added 1 drop of 0.5% gelatin and 10 to 500 μl of 0.005 *M* depolarizer from a microburette. The solution was deaerated by 5 min pas-

TABLE 2

DEPENDENCE OF THE LIMITING CURRENT OF 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE ON THE pH[a]

pH	2.50	3.40	4.00	5.00	6.00	7.50	9.00	11.50
$i \times 10^3$ (μA)[b]	6.46	5.75	4.67	4.30	3.95	2.77	3.25	2.15
$i \times 10^3$ (μA)[c]	6.68	6.48	6.48	5.16	5.26	4.40	3.96	3.50
$i \times 10^3$ (μA)[d]	11.88	11.52	10.80	10.80	10.80	10.44	10.06	9.62

[a] For conditions see Table 1.
[b] 4,4′-Dihydroxyazobenzene.
[c] 4-Nitro-4′-hydroxyazobenzene, first wave.
[d] 4-Nitro-4′-hydroxyazobenzene, second wave.

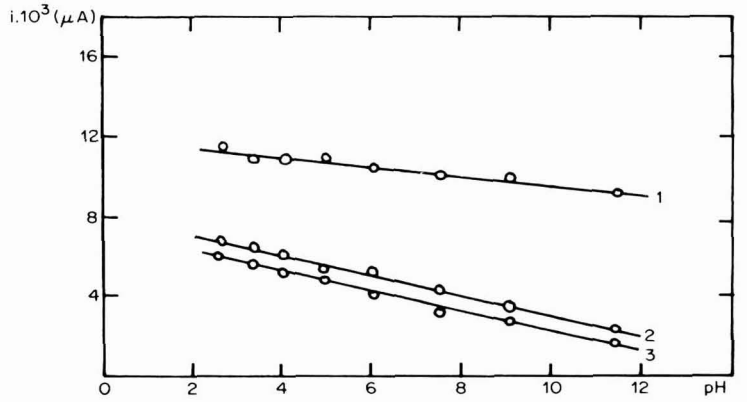

FIG. 2. Dependence of the limiting current of 4,4′-dihydroxyazobenzene (3) and the first (1) and second (2) wave of 4-nitro-4′-hydroxyazobenzene on the pH. The depolarizer concentration is 5×10^{-5} *M*; the reservoir height is 36 cm.

sage of nitrogen after each addition of the depolarizer and the polarographic curves were recorded. The concentration dependence of the limiting current is given in Table 5.

Constant-Potential Coulometric Determination of the Number of Exchanged Electrons

In the coulometer vessel was added 30 ml of a buffer solution of a required pH and a potential corresponding to the limiting current of the test substance was applied to the mercury pool. The residual current was measured and 1.00 to 3.00 ml of 0.05 *M* solution of the test substance was added to the buffer solution. After the current decrease to the original residual value (10-times the half-time), the charge corresponding to the passage of the residual current was subtracted from the charge deter-

TABLE 3

DEPENDENCE OF THE LIMITING CURRENT OF 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE ON THE SQUARE ROOT OF THE HEIGHT OF MERCURY RESERVOIR[a]

$h^{1/2}$ ($cm^{1/2}$)		5	6	7	8
$i \times 10^3$ (μA)[b]	pH 2.85	17.3	22.0	27.6	33.5
	pH 9.25	14.0	15.1	15.8	17.3
$i \times 10^3$ (μA)[c]	pH 3.35	2.26	3.06	3.80	4.32
	pH 9.00	2.88	3.60	4.32	5.04
$i \times 10^3$ (μA)[d]	pH 3.35	8.28	10.44	10.94	12.60
	pH 9.00	11.88	15.12	17.28	20.16

[a] Depolarizer concentration, 5×10^{-5} *M*.
[b] 4,4′-Dihydroxyazobenzene.
[c] 4-Nitro-4′-hydroxyazobenzene, first wave.
[d] 4-Nitro-4′-hydroxyazobenzene, second wave.

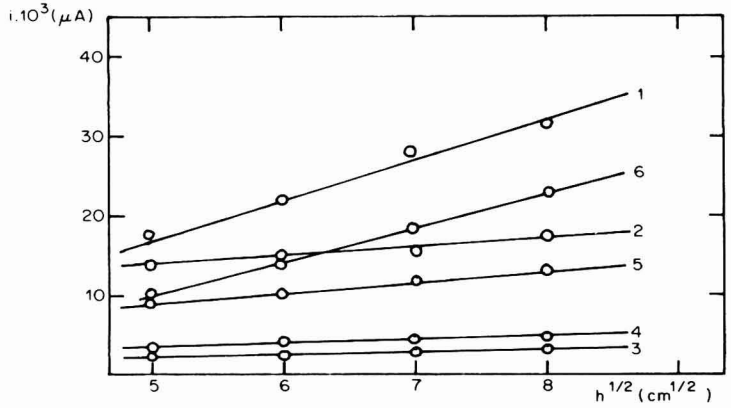

FIG. 3. Dependence of the limiting current of 4,4′-dihydroxyazobenzene at pH 2.85 (1) and 9.25 (2), of the first wave of 4-nitro-4′-hydroxyazobenzene at pH 3.35 (3) and 9.00 (4) and of the second wave of 4-nitro-4′-hydroxyazobenzene at pH 3.35 (5) and 9.00 (6) on the square root of the mercury reservoir height.

TABLE 4
TEMPERATURE COEFFICIENTS FOR 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE

pH	2.85[a]	9.25[a]	3.35[b]	3.35[c]	9.00[b]	9.00[c]
$\Delta i/\Delta t$ (%)	1.89	1.71	1.71	1.74	1.82	1.84

[a] 4,4′-Dihydroxyazobenzene.
[b] 4-Nitro-4′-hydroxyazobenzene, first wave.
[c] 4-Nitro-4′-hydroxyazobenzene, second wave.

TABLE 5
DEPENDENCE OF THE LIMITING CURRENT ON THE CONCENTRATION OF 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE[a]

$c \times 10^6$ (mol liter^{-1})		30	60	90	120	150
$i \times 10^3$ (μA)[b]	pH 4.25	7.5	17.1	24.14	32.7	40.7
	pH 9.25	4.8	11.3	16.6	21.6	27.1
	pH 3.40	6.6	12.1	18.5	24.1	30.7
$i \times 10^3$ (μA)[c]	pH 9.45	9.4	18.2	27.3	35.0	44.9

[a] Reservoir height, 36 cm.
[b] 4,4′-Dihydroxyazobenzene.
[c] 4-Nitro-4′-hydroxyazobenzene, first wave.

mined. The number of exchanged electrons was calculated from the Faraday law and is given in Table 6 as the average of three measurements. The values obtained were simultaneously treated as analytical results for integral numbers of exchanged electrons (i.e., 4 e^- for the azo group and 6 e^- for the nitro group, see Table 7).

TABLE 6
COULOMETRIC DETERMINATION OF THE NUMBER OF ELECTRONS EXCHANGED IN THE REDUCTION OF 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE AT A CONSTANT POTENTIAL

pH	4.25[a]	9.25[a]	3.40[b]	3.40[c]	3.40[d]	9.45[b]	9.45[c]	9.45[d]
E (mV/SCE)	−450	−900	−250	−650	−650	−750	−1150	−1150
Number of electrons	4.0	3.2	4.6	5.5	10.1	4.8	6.3	10.9

[a] Reduction of 4,4′-dihydroxyazobenzene.
[b] Reduction of 4-nitro-4′-hydroxyazobenzene at a potential of the first wave.
[c] Reduction of 4-nitro-4′-hydroxyazobenzene at a potential of the second wave, using the solution after reduction under *b*.
[d] Reduction of 4-nitro-4′-hydroxyazobenzene at a potential of the second wave.

TABLE 7
ACCURACY AND REPRODUCIBILITY OF THE DETERMINATION OF 4,4′-DIHYDROXYAZOBENZENE AND 4-NITRO-4′-HYDROXYAZOBENZENE BY CONSTANT-POTENTIAL COULOMETRY

4,4′-Dihydroxyazobenzene[a]			4-Nitro-4′-hydroxyazobenzene[b]		
Taken (mg)	Found (mg)	Standard deviation (mg)	Taken (mg)	Found (mg)	Standard deviation (mg)
1.071	1.073	0.007	1.126	1.129	0.009
2.142	2.157	0.02	2.432	2.441	0.015
3.213	3.225	0.03	3.648	3.662	0.021

[a] pH 4.25, potential −450 mV vs SCE.
[b] pH 3.4, potential −650 mV vs SCE.

DISCUSSION

Attention was first paid to polarographic reduction of 4,4′-dihydroxyazobenzene and 4-nitro-4′-hydroxyazobenzene. The pH dependence of the half-wave potentials and the limiting currents of the two substances indicate that a fast and mobile acid−base equilibrium precedes the electrode process. The dependence of the limiting currents on the mercury reservoir height, the temperature coefficients, and the linear concentration dependence in the range 3×10^{-5} to 1.5×10^{-4} mol liter^{-1} indicate that the limiting currents of all the waves are diffusion-controlled. Hence the above working procedure and experimental conditions can be considered as a recommended procedure for a polarographic determination of the substances in buffered media.

The coulometric determination of the number of exchanged electrons indicates that the first wave of 4-nitro-4′-hydroxyazobenzene corresponds to the reduction of the azo group (exchange of 4 e^- corresponds to the

reaction, $-N=N- + 4\ H^+ + 4\ e^- \rightarrow 2\ -NH_2$). The second wave, appearing at more negative potentials, corresponds to the reduction of nitro group (exchange of 6 e^- corresponds to the reaction, $-NO_2 + 6\ H^+ + 6\ e^- \rightarrow -NH_2 + 2\ H_2O$).

It can further be seen that in alkaline media the reactions do not exhibit a 100% current efficiency, apparently owing to subsequent reactions of the reduction intermediates. Therefore, a medium with pH 4.25 and a potential of −450 mV (SCE) can be recommended for a constant-potential coulometric determination of 4,4′-dihydroxyazobenzene.

In the reduction of 4-nitro-4′-hydroxyazobenzene at the potential of the reduction of azo group, the nitro group is partially reduced, giving rise to a positive error of determination. Reduction at more positive potentials disproportionately lengthens the determination time (from 75 to 250−300 min). For the determination of 4-nitro-4′-hydroxyazobenzene can thus be recommended a medium with pH 3.4 and a potential of −650 mV (SCE), corresponding to the limiting current of the second wave.

SUMMARY

Conditions have been found for a polarographic and constant-potential coulometric determination of 4,4′-dihydroxyazobenzene and 4-nitro-4′-hydroxyazobenzene in buffered media.

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Solvent Extraction and Spectrophotometric Determination of Iron(II) with 2,2′-Dipyridyl-2-quinolylhydrazone

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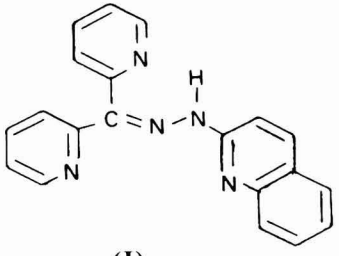
INTRODUCTION

Considerable attention has been directed in recent years to the preparation of new, nitrogen-containing heterocyclic hydrazones, as suitable reagents in spectrophotometric determination of metal ions; the extent of the contribution of the particular heterocyclic systems attached to either the aldehyde (or ketone) or the hydrazine groups to the overall characteristics of the metal chelates formed has also been discussed (*4–7, 11–13*).

As part of a study concerning the analytical possibilities of 2,2′-dipyridyl-2-quinolylhydrazone (I, abbreviated as DPQH) (*7, 14*), the results on the iron(II)–DPQH complex are described in this paper and a rapid and sensitive method for the extractive spectrophotometric determination of iron(II) with DPQH is proposed. The extraction mechanism for the iron(II) complex is also discussed.



(I)

EXPERIMENTAL

Reagents

DPQH. The ligand was prepared as previously described (*7*) by direct condensation of equimolar proportions of di-2-pyridylketone and 2-hy-

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drazinoquinoline. A 1×10^{-3} *M* DPQH in benzene was freshly prepared daily.

Iron(II) solution. Iron(II) standards were obtained by dilution of 0.01 *M* ammonium iron(II) sulfate acidified with sulfuric acid and standardized complexiметrically.

All inorganic and organic chemicals were of analytical grade. Special care was taken to avoid contamination by iron.

Apparatus

Absorbance measurements were made with a Nippon Bunko UVIDEC-1 digital double-beam spectrophotometer using matched 1.00-cm quartz cells. For pH measurements a Toa Dempa HM-6A pH meter was used with a combination electrode. All measurements were made at 25°C.

Recommended Procedure

To a suitable aliquot containing less than 33 μg of iron(II, III) in a 50-ml separatory funnel, add 1 ml each of 1% ascorbic acid, 1 *M* acetate buffer adjusted to pH 4.0, and 1 *M* sodium chloride solution. Dilute to 10 ml with doubly distilled water and equilibrate with exactly 10 ml of 1×10^{-3} *M* DPQH in benzene for 10–15 min. Measure the absorbance of the benzene extract at 644 nm (or 504 nm for less than 14 μg iron in the absence of interfering ions) against the reagent blank. Calculate the amount of iron present from a previously prepared calibration curve.

RESULTS AND DISCUSSION

Characteristics of the Iron(II) Complex

The iron(II) complex formed with the reagent is sparingly soluble in water, but readily soluble in various organic solvents such as partially halogenated hydrocarbons, acetate esters, ketones, and lower aliphatic alcohols. Benzene, toluene, and chlorobenzene proved to give the highest absorbance at the wavelengths of maximum absorption. Under the optimal conditions of the reagent concentration, pH, and shaking period, iron(II) can be quantitatively extracted from aqueous solution with one 10-ml portion of DPQH in benzene over the concentration range studied. The color thus obtained remains unchanged for at least several hours. Figure 1 illustrates the visible absorption spectrum of the iron(II) complex extracted into benzene along with that of the reagent blank. The absorption spectrum is characteristic for iron(II), three absorption maxima being located at 473, 504, and 644 nm. Iron(III) did not react with DPQH and should be reduced by ascorbic acid.

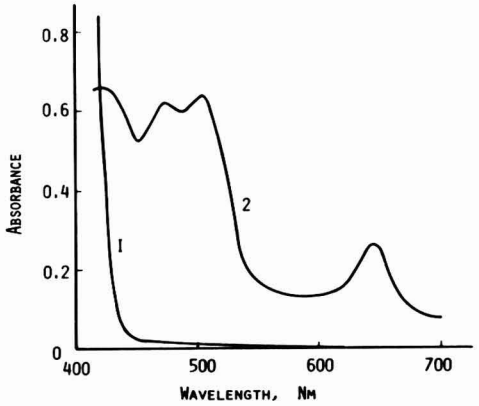

FIG. 1. Absorption spectra obtained under the experimental conditions given in the recommended procedure. Concentrations of iron(II) and DPQH are 2×10^{-5} and 1×10^{-3} *M*, respectively. (1) Reagent blank; (2) Fe(II)–DPQH complex.

Effects of Experimental Conditions

A pH study was carried out over the pH range 2.0–6.0. The data are plotted in Fig. 2, indicating that maximal absorbance is obtained between pH 3.4 and 4.5. In more acidic or more basic solutions, the absorbance decreases because of incomplete complex formation and of slow extraction of the complex, respectively. (When the iron(II) complex was extracted into benzene after completion of the complex formation in a 12.5% (v/v) aqueous ethanol, a maximal and constant absorbance was obtained between pH 4.0 and 9.6.)

The absorbance of the organic phase was studied as a function of the

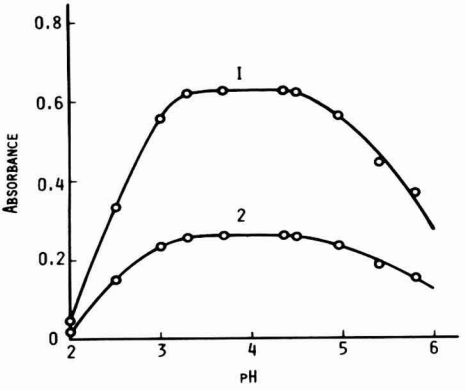

FIG. 2. The pH dependence of the iron(II)–DPQH complex formation. Initial concentrations of iron(II) and DPQH are 2×10^{-5} and 1×10^{-3} *M*, respectively. (1) Measured at 504 nm; (2) at 644 nm.

initial concentration of DPQH in benzene. About 15-fold molar excess of the reagent was sufficient for complete extraction of iron(II) within 10–15 min. An excess of the reagent up to 100-fold molar did not produce any change in absorbance. The rate of complex formation somewhat increased with increasing reagent concentration.

Addition of 1 ml of 1 *M* sodium chloride was sufficient for preventing emulsification during the extraction. An aqueous to organic phase ratio of 0.5–5:1 did not affect the absorbance.

Conformance to Beer's Law

The analytical species of interest obeys Beer's law over the range studied up to about 6×10^{-5} *M* (33 μg) iron(II) in the organic phase. The

TABLE 1
COMPARISON OF SENSITIVITY FOR IRON(II) WITH SOME OTHER REAGENTS OF FERROIN TYPE

Reagent	λ_{max} (nm)	ϵ_{max} ($\times 10^{-4}$)	Extractant	Reference
DPQH	473	3.06	Benzene	Present work
	504	3.14		
	644	1.30		
Pyridine-2-aldehyde-2-quinolylhydrazone	461	2.69	Benzene	*8*
	491	2.72		
	623	0.64		
2-Acetylpyridine-2-quinolylhydrazone	460[a]	2.86[a]	Benzene	*8*
	494[a]	2.63[a]		
	640[a]	0.70[a]		
2-Benzoylpyridine-2-quinolylhydrazone	466	3.10	Benzene	*8*
	502	2.90		
	645	1.06		
2,2′-Dipyridyl-2-pyridylhydrazone	538	1.50		*1*
2,2′-Dipyridyl-2-thiazolylhydrazone	600	1.23		*13*
1,10-Phenanthroline	510	1.11		*10*
4,7-Diphenyl-1,10-phenanthroline	533	2.24		*10*
2,2′-Bipyridine	522	0.87		*10*
4,4′-Diphenyl-2,2′-bipyridine	552	2.11		*10*
Tris(2′-pyridyl)-1,3,5-triazine	594	2.26		*10*
4,7-Di-*p*-benzeneazoanilino-1,10-phenanthroline	520	4.38	Isopentanol	*9*

[a] Unstable complex was formed.

molar absorptivity was calculated as $1.30 \times 10^4\ M^{-1}\ cm^{-1}$ at 644 nm; it was $3.14 \times 10^4\ M^{-1}\ cm^{-1}$ at 504 nm and less than $2.5 \times 10^{-5}\ M$ (14 μg) of iron(II) can be determined at this wavelength.

Table 1 presents a comparison of the spectral properties of the iron(II) complexes with several heterocyclic hydrazones containing the $-N=C-C=N-NH-C=N-$ chromophoric group and some of promising or widely used ferroin-type reagents. The present reagent, DPQH, is found to be one of the most sensitive reagent of those tested for iron(II).

Effect of Diverse Ions

The possible interference of various ions was examined by introducing them into a solution containing 11.2 μg of iron(II). The absorbance measurements were made at 644 nm and interference was regarded as significant when it produced a difference of more than $\pm 3\%$ in absorbance from that found with iron(II) ions alone. The results are summarized in Table 2. The present method is found to be relatively interference free. The influence of silver(I), bismuth(III), copper(II), mercury(II), tungsten(VI), and vanadium(V) could be overcome by addition of suitable masking agents as noted in Table 2. Cobalt(II) and nickel(II), however, interfered when their concentration exceeded five times that of iron(II). EDTA must be absent.

Composition of the Complex

The stoichiometry of the extracted species was established by Job's method of continuous variations. The results showed a definite 1:2 ratio of iron to DPQH in the complex (Fig. 3), indicating that DPQH acts as a tridentate ligand.

TABLE 2
EFFECT OF FOREIGN IONS ON DETERMINATION OF 11.2 μg OF IRON(II)

Tolerance limit ([Ion]/[Fe(II)])	Ion
$\geq$10,000	Br^-, Cl^-, NO_3^-, SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, tartrate
$\leq$5,000	PO_4^{3-}, citrate, thiourea
$\leq$1,000	SCN^-
$\geq$100	Ag^{+a}, Cd^{2+}, Hg^{2+b}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, Al^{3+}, Pt^{4-}, Mo^{6+}
$\leq$100	Cu^{2+c}, Bi^{3+d}, Ir^{3+}, Rh^{3+}, Os^{8+}, $C_2O_4^{2-}$
$\leq$50	Au^{3+}, Ga^{3+}, In^{3+}, Ru^{3+}, V^{5+d}, U^{6+}, W^{6+d}
$\leq$10	Pd^{2+a}
$\leq$5	Co^{2+d}, Ni^{2+}
0	EDTA

[a] One milliliter of 0.4 *M* thiourea was added.
[b] Two milliliters of 1 *M* sodium hydrogen sulfite was added.
[c] One milliliter of 1 *M* sodium thiosulfate was added.
[d] Four milliliters of 0.5 *M* tartaric acid adjusted to pH 4.0 was added.

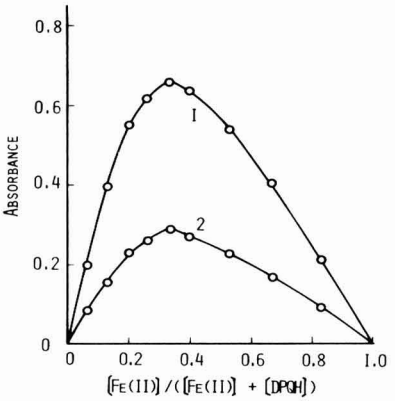

FIG. 3. Continuous variations plot for the iron(II)–DPQH system. Total concentration of iron(II) and DPQH is 1.2×10^{-4} *M*. (1) Measured at 504 nm; (2) at 644 nm.

Possible Extraction Mechanism

It is generally known that the cationic bis complexes of divalent transition metals with nitrogen-containing heterocyclic hydrazones (as tridentate ligands) can easily be converted by the loss of two protons to highly colored uncharged bis complexes, soluble in many organic solvents but almost insoluble in water. In 40% (v/v) aqueous ethanol, iron(II) reacts with DPQH to form colored complexes with maximal absorbance at about 470 and 555 nm (red complex) below pH 2.8, at about 467 and 595 nm (brownish green complex) between pH 3.2 and 4.5, and at about 482 and 625 nm (green complex) above pH 6. Mole ratio and continuous variations studies clearly showed that all the three complexes have a metal–ligand ratio of 1:2, while an ion-exchange study using Amberlite IR-120A and IRA-400 suggested that the former two complexes are cationic and the last one uncharged. Any experimental evidence, however, could not be obtained for the formation of the cationic mono complex. This would imply that the stability constant of the cationic bis complex, $Fe(HL)_2^{2+}$, is greater than that of the lower complex, $Fe(HL)^{2+}$, as previously described by Lions and his co-workers (*2*, *3*). The red and the brownish green complexes are therefore assigned to the species, $Fe(HL)_2^{2+}$ and $FeHL_2^{+}$, respectively. Figure 4 shows the absorption spectra of the iron(II)–DPQH system in 40% (v/v) aqueous ethanol at varying pH values. Distinct isosbestic points are located at about 569 nm between pH 4.11 and 4.61 (Fig. 4A) and about at 613 nm between pH 4.64 and 6.88 (Fig. 4B), indicating that the deprotonation constants, pk_2' and pk_1', of $Fe(HL)_2^{2+}$ can be estimated by a spectrophotometric method previously described (*4*). The absorbance measurements obtained at 597 and 626 nm lead to values of $pk_2' = 4.3_2$ and $pk_1' = 5.7_7$ ($\mu = 0.1$), respectively.

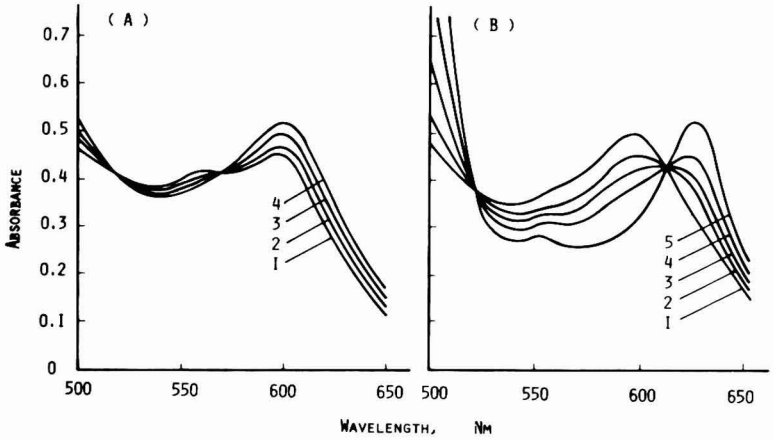

FIG. 4. Absorption spectra of the iron(II)–DPQH system is 40% (v/v) aqueous ethanol. Concentrations of iron(II) and DPQH are 6.03×10^{-5} and 1.21×10^{-5} *M*, respectively. (A) Absorption spectra between pH 4.11 and 4.61: (1) pH 4.11; (2) pH 4.20; (3) pH 4.39; (4) pH 4.61. (B) Absorption spectra between pH 4.64 and 6.88: (1) pH 4.64; (2) pH 5.07; (3) pH 5.50; (4) pH 5.85; (5) pH 6.88.

Dissociation of the imino-hydrogen in DPQH is extremely weak ($pk_1 > 14$) (*7*), so that the magnitude of the acid-strengthening effect of the coordinated iron(II) on the imino group amounts to as much as 10 pk units. It should be noted that the absorption spectrum of the green complex in 80% (v/v) aqueous ethanol exhibited three absorption maxima at 465 (big shoulder), 488, and 632 nm, the absorption pattern being consistent with that found in the complex extracted into water-immiscible organic solvents.

Consideration of the foregoing results and the deprotonation constants, pk_3 and pk_2, of DPQH in aqueous ethanol (*7*) leads to the conclusion that, if sufficient ligand is present to form the bis complex, the following equilibria would be involved in the extraction process of the iron(II) complex with an immiscible organic solvent.

$$(HL)_{org} \rightleftharpoons HL \text{ (partition)}$$
$$H_3L^{2+} \rightleftharpoons H_2L^{+} + H^{+} \text{ (deprotonation, } pk_3 = 2.4_8)$$
$$H_2L^{+} \rightleftharpoons HL + H^{+} \text{ (deprotonation, } pk_2 = 5.5_7)$$
$$Fe^{2+} + HL \rightleftharpoons Fe(HL)^{2+} \text{ (dipositively charged mono-complex formation)}$$
$$Fe(HL)^{2+} + HL \rightleftharpoons Fe(HL)_2^{2+} \text{ (dipositively charged bis-complex formation)}$$
$$Fe(HL)_2^{2+} \rightleftharpoons FeHL_2^{+} + H^{+} \text{ (deprotonation, } pk_2' = 4.3_2)$$
$$FeHL_2^{+} \rightleftharpoons FeL_2 + H^{+} \text{ (deprotonation, } pk_1' = 5.7_7)$$
$$FeL_2 \rightleftharpoons (FeL_2)_{org} \text{ (partition)}$$

SUMMARY

2,2′-Dipyridyl-2-quinolylhydrazone (DPQH) was used for the spectrophotometric determination of trace amount of iron(II) after the extraction process. Iron(II) reacts with DPQH at pH 3.4–4.5 to form a water-insoluble 1:2 complex, which can be extracted with many kinds of organic solvent. The extracted species with benzene has absorption maxima at 473, 504, and 644 nm and obeyed Beer's law over the range 0–14 μg of iron at 504 nm and 0–33 μg at 644 nm. The molar absorptivities at 504 and 644 nm are 3.14×10^4 and 1.30×10^4 M^{-1} cm^{-1}, respectively. DPQH is one of the most sensitive reagents for iron(II) and trace amount of iron(II) can be determined in the presence of fairly large amounts of other ions. Possible equilibria involved in the extraction process were also studied.

ACKNOWLEDGMENT

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MICROCHEMICAL JOURNAL 26, 236–241 (1981)

Computerized Technique in Organic Microelemental Analysis

Part IV: Automated Determination of Chlorine, Bromine, and Iodine in Organic Compounds[1]

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INTRODUCTION

In the course of automation and computerization of the LRL Microanalytical Laboratory (*3*) it became desirable to develop instrumentation and methodology for the automatic determination of elements other than CHN. Earlier we reported titration methods for the determination of sulfur (*5*) as well as fluorine (*4*) in the fully automated and computerized fashion, through the use of colorimetry for the endpoint detections. In spite of the convenience and precision of colorimetric endpoint detections of sulfur or fluorine, this method could not be applied to the argentometric halogen determination due to the formation of heavy precipitate of silver halides which interfered with instrumental colorimetric procedures. Argentometric determination is the only convenient and uniform method for determining all three halogens, individually or as the sum of halogens in organic compounds. Therefore, we designed a system based on a potentiometric titration of halides with silver salts in the presence of a silver electrode, to a preselected (*2*) endpoint potential. This classical determination of halides has been used in form of an automatic micromethod in conjunction with the mineralization of organic substances (*1*, *7*, *8*).

The titration of the three halides may be conducted to the same endpoint potential, or as described by Scheidl and Toome (*7*), to the particular equivalence point potential of Cl^-, Br^-, or I^-. The titration to the

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same endpoint potential permits determination of unknown halides or a mixture of halides. Since the kind of halogen in the compound submitted for analysis is not always known in advance, titration to the same potential is necessary in the automatic and computerized mode of analysis. In daily microanalytical practice most of the analyzed halogen-containing substances are chloro-organic compounds. For this reason the equivalence point potential of Cl^- was chosen as the preselected endpoint potential for argentometric titration of all three halides.

TECHNIQUE

Mineralization

Apparatus and technique for the mineralization of halogen-containing compounds and transfer of combustion products are identical to those described in our sulfur determination (*5*).

Transfer of the Combustion Products from the End of the Combustion Tube to the Titration Vessel

Combustion products of the halogen-containing compounds are, as is known, a mixture of corresponding acids and free halogens, and therefore require reduction of free halogens to hydrohalides. Traditionally this reduction is done very successfully by absorbing the combustion products in a solution containing $NaHSO_3$ (*6*). However, this reagent may cause contamination of the combustion tube with alkali metals from thermally decomposed sulfurous salts and may cause irreversible binding of halogens in the combustion tube. Therefore, the preferred wash solution is sulfurous acid which is prepared weekly by dissolving SO_2 in water.

In the course of our investigation, reductive solutions containing a mixture of water in isopropyl alcohol were successfully tested (*7*). The latter may cause an accidental explosion if alcohol vapors come in contact with oxygen at high temperature when a disturbance of the pressure equilibrium between oxygen and wash solution occurs in the combustion tube.

Endpoint Determination

The proposed potentiometric titration of halogens was first examined in a model experiment using potentiographic presentation of titration curves for Cl^-, Br^-, and I^- using the same conditions established for potentiostatic determinations. A solution was prepared by adding ca. 3 ml of 0.01 *N* KCl, KBr, or KI to 20 ml of acidified ($HClO_4$) 50% isopropanol and 3 ml of SO_2-containing wash solutions (see Reagents). This solution was titrated with 0.005 *N* $AgClO_4$ in 50% isopropyl alcohol using a Metrohm combination silver electrode EA-252 and Metrohm recording potentiograph E-436. From the graphical presentation (Fig. 1) of the titration curves

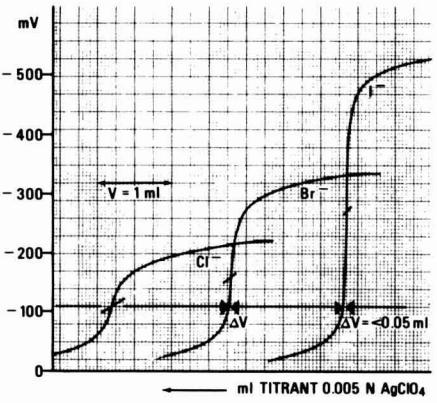

FIG. 1. Experimental evaluation of systematic errors ($\Delta\nu$) by titration of all three halides to the preset potential.

it is apparent that the equivalence point potential for Br^- and I^- are quite different from that of Cl^-. However, only a very small increase of the titrant volume ($\Delta\nu$) is observed (about 0.05 ml of 0.005 *N* $AgClO_4$) when the potentiostatic titrations of Br^- and I^- are carried to the equivalence point potential of Cl^-. This volume of titrant represents a systematic positive error equal to +0.02 mg Br or +0.03 mg I, which can be incorporated in the empirical calculation factor of the titrant. This mode of error compensation is applicable to a limited range of amounts of the determined halogen (Br or I).

The given potential values, as apparent from the graphic presentation of the titration curves, are characteristic for a particular specimen and condition of silver electrode and titration medium, and should be checked periodically.

REAGENTS AND EQUIPMENT

Reagents

Purified-grade oxygen gas without additional treatment.

Wash solution: 3% solution (by weight) of SO_2 in water prepared freshly once a week.

Absorption solution: 50% isopropyl alcohol (reagent grade) acidified with 10 ml $HClO_4$ (70%) per 1 liter solution.

Titration solution: approximately 0.005 *N* $AgClO_4$ in 50% isopropyl alcohol. The calculation factor of the titrant must be determined experimentally by combustion of Cl^-, Br^-, or I^- containing microanalytical standard substances. For Br and I the sample weight should be adjusted to consume about 5 ml of titrant (50% of the burette volume).

TABLE 1
INTERFERENCE OF PHOSPHORUS IN THE CHLORINE DETERMINATION

Atom. ratio Cl:P	Percentage Cl	
	Found	Calculated
1:1	22.97	22.64
2:1	22.82	
1:2	22.89	

Equipment

The equipment for the halogen determination is set up as described in the previous publication dealing with sulfur determinations (*5*). The final determination of halides is conducted potentiometrically, utilizing the following equipment:

Impulsomat: Metrohm model E-473.
pH meter: Metrohm model E-512.
Combination silver micro electrode: Metrohm model EA-252.
Dosimat: 10 ml electric burette, Metrohm model E-415.

EXPERIMENTAL

The automatic procedure of the analysis is conducted as described in the sulfur determination (*5*). It was established that the method functions blank-free.

Sample size 1–8 mg should be adjusted according to the amount of halogen expected in the substance. If more than 10 ml of titrant is used, the computer will disregard the calculation. (This precautionary factor incorporated in the software excludes the possibility of obtaining calculation on an uncompleted automatic titration, rejection volume of titrant if it is larger than 10.00 ml.)

Among the various elements present in organic compounds, only phosphorus may cause small interference in the determination. To establish the magnitude of this interference, *p*-chloro-benzoic acid was combusted in the presence of various amounts of triphenylphosphine, with the results shown in Table 1.

TABLE 2
STATISTICAL EVALUATION OF ANALYTICAL RESULTS

		Cl^-	Br^-	I^-
Mean error	$\Delta\bar{X}$	0.01	0.00	−0.02
Standard deviation	S	0.05	0.14	0.18

Since the found values of Cl^- determined in the presence of phosphorus are within the +0.3% limit of the theory and do not depend on the atomic ratio of phosphorus to halogen, phorphorus-containing substances can be analyzed.

RESULTS AND DISCUSSION

During the last 6 years we performed thousands of analyses of Cl, Br, and I on various organic and organometallic compounds using the described procedure. Organometallic compounds were combusted in the presence of vanadium pentoxide (V_2O_5) as an additive to the analyzed substance. Volatile compounds were analyzed in sealed capsules and liquids in quartz capillaries. The combustion tube was periodically removed, inspected, and washed with HF, particularly after a series of analyses of metallo- or phosphoroorganic compounds. Based on daily instrument control analyses over a period of 2–4 weeks, the accuracy and precision of the results are computed and presented in Table 2.

Samples of Cl-benzoic acid, Br-benzoic acid, and I-benzoic acid were used as standard substances. From the data summarized in Table 2 one can conclude that the obtained results of halogen determination are accurate and precise. This conclusion, based on obtained results of control analyses, confirms at the same time the reliability of the developed procedure during a prolonged period of time.

SUMMARY

The automatic and computerized rapid microanalytical determination of chlorine, bromine, and iodine in organic and organometallic compounds is described. The method consists of combustion of the compound in a large diameter empty tube connected to a titration vessel. The combustion products are transferred from the end of the combustion tube to the titration vessel by means of an automatic reductive SO_2-containing wash. Automatic potentiometric titration with $AgClO_4$ to the preset endpoint potential, using a combination silver microelectrode, serves as the endpoint detection of the titration of halides. The entire analysis, including automatic preparation of the equipment for the next determination, is programmed in a 6-min cycle. The method is blank-free; the results are accurate. Standard deviations amount to: 0.05, 0.14, and 0.18% absolute for Cl, Br, and I, respectively.

The analyzer is interfaced to a real-time, time-sharing computer along with electronic microbalances and other analyzers, as part of the microanalytical laboratory computer service. The results of the analyses are reported on CR-terminals and are stored on magnetic disk for further processing.

ACKNOWLEDGMENTS

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Application of the Vanadium(V)–Xylenol Orange Reagent to the Assay of Serum Glucose

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INTRODUCTION

In our previous paper, the use of the mixture of vanadium(V) and xylenol orange was recommended as a new colorimetric reagent (abbreviated as V–XO reagent) in the determination of traces of hydrogen peroxide (*5*). The absorption spectrum of the V–XO reagent exhibits the characteristic peak at 582 nm due to the formation of the vanadium(V)–xylenol orange binary complex. The addition of hydrogen peroxide to the V–XO reagent led to a significant decrease in the absorbance and its magnitude (denoted as ΔA) was proportional to the concentration of hydrogen peroxide. The reagent was found to be useful for the trace analysis of hydrogen peroxide with high sensitivity, and the data were little affected by the presence of some reducing substances. These findings suggest the applicability of the reagent to the colorimetric determination of traces of hydrogen peroxide in biological fluids.

In usual clinical tests for D-glucose in serum, the colorimetric methods using the glucose oxidase–peroxidase system and a chromophore such as 4-aminoantipyrine-phenol are used. However, various reducing substances interfere with the coloration of such chromophore in the usual tests because of incomplete selectivity of peroxidase (*1*, *3*, *7*).

The paper is concerned with the extention of our earlier study of the V–XO reagent to the assay of glucose in serum using glucose oxidase alone. No peroxidase is needed in the present method.

MATERIALS AND METHOD

Reagents. All the chemicals were reagent grade and were used without further purification.

Stock vanadium(V) solution (1.00 m*M*): 0.0910 g of vanadium pentoxide was dissolved in 4 ml of 0.5 *M* sodium hydroxide and then diluted with

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water to 1000 ml. The final solution was used as a stock solution of vanadium(V).

Stock XO solution (1.00 m*M*): Stock solution of XO was prepared by dissolving 0.7166 g of sodium salt of XO in 1000 ml of water.

V–XO reagent: The V–XO reagent was prepared by mixing 10 ml of each stock solution of vanadium(V) and XO, and diluting with water to 100 ml.

Standard glucose solution: Standard glucose solution was prepared by dissolving 90.0 mg of glucose in 100 ml of water. The solution can be kept for a week in a refrigerator without any detectable change in the concentration.

Stock hydrogen peroxide solution (100 m*M*): 2.60 ml of 30% hydrogen peroxide solution was diluted with water to 500 ml; the solution was standardized by titration with potassium permanganate.

Glucose oxidase solution (10 U/ml): Glucose oxidase (15.3 U/mg) was purchased from Sigma Chemical Co. Ltd. The solution was prepared by dissolving 66 mg of glucose oxidase in 100 ml of acetate buffer (pH 5.6). The solution can be kept for a week in a refrigerator without any detectable change in the activity.

Apparatus. Visible absorption spectra were recorded on a Hitachi double-beam spectrophotometer, Model 200-10, with 10-mm quartz cells. The pH of each solution was obtained by measurement with a Toshiba-Beckman pH meter, Model SS-2.

Procedure of determination of glucose. The absorption measurements for the assay of glucose in serum were made in the following way: 10 μl of serum, 0.5 ml of the V–XO reagent, and 1.0 ml of the glucose oxidase solution were mixed and the solution was incubated for 5 min at 35°C. After addition of 3.5 ml of acetate buffer (pH 4.0) to the solution, the absorbance at 582 nm (A_s) was measured. For the control assay, the solution containing the same components as above except the standard glucose solution instead of serum was prepared and the absorbance at 582 nm (A_c) was measured. The absorbance corresponding to the reagent blank (A_b) was obtained at the same wavelength. All the measurements were made with reference to water.

Since the values given by $\Delta A_s = A_b - A_s$ and $\Delta A_c = A_b - A_c$ were proportional to the concentration of glucose in serum and the standard glucose solution, respectively, the glucose content in serum can be expressed as,

$$\frac{\Delta A_s}{\Delta A_c} \times 90.0 = \text{glucose in serum (mg/100 ml)}.$$

RESULTS

Absorption Spectra

In Fig. 1, the absorption spectrum of the V–XO reagent is shown as curve a. A sharp peak having a maximum at 582 nm is given in the pH range 3 to 5. On the addition of hydrogen peroxide to the V–XO reagent, the absorption peak was lowered significantly (curves b and c), and the magnitude of the decrease in absorbance (ΔA) was proportional to the concentration of added hydrogen peroxide. The value of ΔA per 1 M hydrogen peroxide is found to be 2.5×10^4. This value is relatively large compared with the molar absorptivity of the ternary complexes observed in the Ti(IV)–XO–H_2O_2 system by Nordschow (*6*) and also by the present authors (*4*).

In the previous paper, applicability of the V–XO reagent to the determination of traces of hydrogen peroxide was proved, and the condition that [XO]/[V(V)] = 0.5 ~ 1.2 in the pH range 3.5 ~ 4.5 was found to be optimum (*5*). Since an extension of the applicability of the V–XO reagent to the determination of glucose in clinical samples seems promising, the following experiments were made in order to decide the appropriate conditions in the combined use of the V–XO reagent and glucose oxidase.

Optimum Conditions for the Determination of Glucose

The pH value of 5.6 is found to be optimum for the enzymic reaction of glucose oxidase, whereas the optimum pH is about 4.0 for obtaining ΔA through the reaction between the V–XO reagent and hydrogen peroxide. Then the solution was first adjusted to pH 5.6 for the incubation with glucose oxidase, and afterward readjusted to pH 4.0 for the measurement of absorbance.

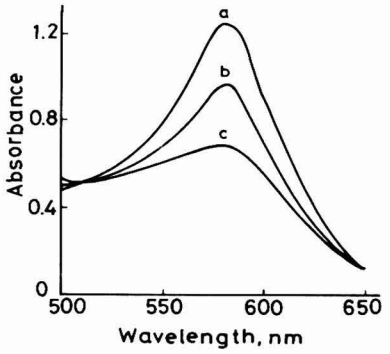

FIG. 1. Absorption spectra of the V–XO reagent in the absence (a) and in the presence of 1.0×10^{-5} (b) and 2.0×10^{-5} (c) MH_2O_2 at pH 4.

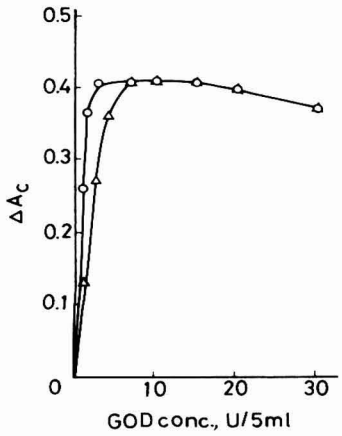

FIG. 2. Effect of glucose oxidase (GOD) concentration on ΔA_c obtained after a 3-min (△) and a 20-min (○) period incubation at 35°C. Concentration of glucose, 2×10^{-5} *M*.

A favorable temperature to complete the enzymic reaction of glucose oxidase is known to be about 35°C. Fortunately the reaction of the V−XO reagent with hydrogen peroxide attains equilibrium within 3 min at this temperature (*5*). Then the temperature was maintained at 35°C during the incubation.

The effect of glucose oxidase activity on the ΔA_c was examined to determine the optimum concentration of glucose oxidase, and the result are shown in Fig. 2. The constant value of ΔA_c was obtained in the ranges of 7 to 15 U and 4 to 15 U glucose oxidase upon incubating for 3 and 20 min, respectively, with the use of 20 μl of the standard glucose solution. The effect of incubation time on the ΔA_c was also examined with the use

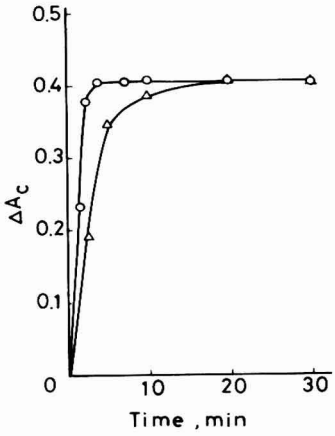

FIG. 3. Effect of incubation time on ΔA_c obtained for 4 U (△) and 7 U (○) GOD in 5 ml of solution I. Concentration of glucose, 2×10^{-5} *M*. Incubation was made at 35°C.

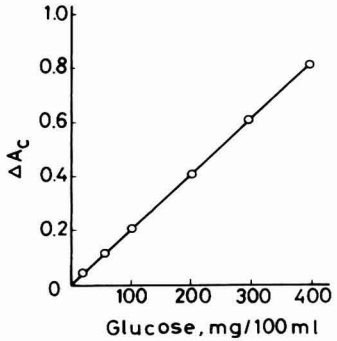

FIG. 4. Proportionality of ΔA_c to glucose concentration.

of 7 and 4 U glucose oxidase. In the former case the maximum value of ΔA_c was obtained within 3 min, but 20 min was needed in the latter (Fig. 3). The value of ΔA_c remained virtually unchanged on standing the solution at room temperature for 1 hr after readjusting the pH to 4. Based on these experimental results, the following procedure was decided: incubating for 5 min at pH 5.6 with 10 U glucose oxidase.

The ΔA_c obtained through the above procedure was plotted against the glucose concentration. A linear relationship exists between them as shown in Fig. 4, from which the method is found to be applicable to the determination of glucose in the concentration range of 5 to 400 mg in 100 ml sample. The data were reliable with a coefficient of variation below 2.8%.

Further experiments were made to examine the utility of the V–XO reagent in the determination of glucose in human sera. The recovery of

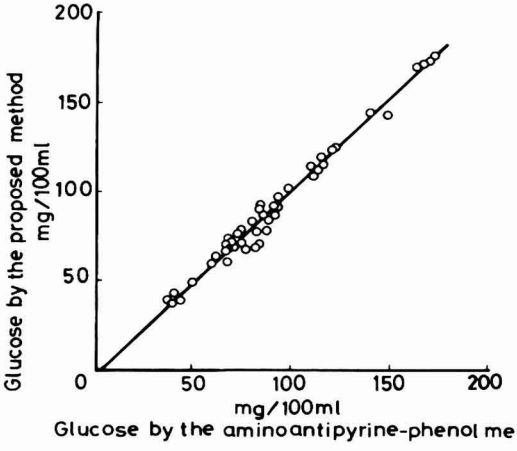

FIG. 5. Correlation of the results by the aminoantipyrine-phenol and the proposed methods.

glucose added to serum (100 mg/100 ml) was 98.6% with a coefficient of variation below 3.0% ($n = 20$). Glucose in 50 serum samples was assayed by the present method and the results are compared with those by the 4-aminoantipyrine-phenol method as shown in Fig. 5. The correlation coefficient was found to be 0.970 and $Y = 1.06X - 5.90$ was set up as the linear regression equation, indicating high correlation between these two methods.

Effects of Foreign Substances on the Determination of Glucose

Effects of some foreign substances which are usually contained in serum were examined, and the results are summarized in Tables 1 and 2.

As seen in Table 1, some inorganic ions such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, F^-, Cl^-, I^-, ClO_4^-, and NO_3^- scarcely affect the absorption measurement even when these are present in large excess compared with glucose. The presence of SO_4^{2-} and PO_4^{3-} causes only a minor error. On the other hand, Fe^{3+} and Cu^{2+} give rise to serious errors, even at low concentration, a rising from the complex formation between these metal ions and XO. However, as mentioned above, relatively large value of ΔA_c corresponding to 1 M glucose permits dilution of sample fluids, as a result of which these metal ions exert practically no effect in the solution (e.g., the final concentrations of Fe^{3+} and Cu^{2+} in the solution prepared from human serum is ca. 5×10^{-7} and 3×10^{-8} M, respectively).

All the organic compounds tested have virtually no effect on the determination of glucose, as seen in Table 2.

TABLE 1
EFFECT OF INORGANIC COMPOUNDS ON THE DETERMINATION OF GLUCOSE[a]

Inorganic compound	Concn (M)	Glucose found (%)
NaCl	1.0×10^{-3}	100
KCl	1.0×10^{-3}	100
$MgCl_2$	1.0×10^{-4}	100
$CaCl_2$	1.0×10^{-4}	100
$CuSO_4$	1.0×10^{-5}	160
$ZnCl_2$	1.0×10^{-4}	101
$FeCl_3$	1.0×10^{-5}	170
NaF	1.0×10^{-3}	101
KI	1.0×10^{-3}	100
$KClO_4$	1.0×10^{-4}	100
$NaNO_3$	1.0×10^{-4}	100
Na_2SO_4	1.0×10^{-4}	98
NO_3PO_4	1.0×10^{-4}	97

[a] Concn of glucose added: 1.0×10^{-5} M.

TABLE 2
EFFECT OF ORGANIC COMPOUNDS ON THE DETERMINATION OF GLUCOSE[a]

Organic compound	Concn (M)	Glucose found (%)
Acetic acid	1.0×10^{-3}	100
Ascorbic acid	2.0×10^{-4}	100
Citric acid	1.0×10^{-5}	100
Glycolic acid	1.0×10^{-5}	100
Glyoxylic acid	1.0×10^{-5}	100
Lactic acid	1.0×10^{-5}	100
Pyruvic acid	1.0×10^{-5}	101
Amino acids[b]	1.0×10^{-5}	100
Bilirubin	1.0×10^{-5}	100
Uric acid	1.0×10^{-6}	100
Urea	1.0×10^{-5}	100

[a] Concn of glucose added: 1.0×10^{-5} M.
[b] Amino acids used: Arg, His, Cys, Met, Asn, Asp, Gly, Ala, Thr, Ser, Orn, Cit, Trp, Tyr.

DISCUSSION

The results in the present paper made it possible to use the V−XO reagent in the assay for glucose in serum. The present method has several advantages over usual methods. One of them is that there is no need to use peroxidase, as a result of which no pretreatment of sample, such as deionization and deproteinization, is required in this method without leading to a lowering of selectivity. In general cases using the glucose oxidase−peroxidase enzyme system, the presence of reducing substances often results in serious errors. For example, ascorbic acid appreciably inhibits the color development of chromophores. Sharp (*7*) reported that the presence of 0.4 mM ascorbic acid apparently led to about 20% depression of glucose content obtained by the 2′,2-diazo-di(3-ethyl benzothiazoline-6 sulfonic acid) method. Uric acid is also liable to cause interference. Contrary to this, such substances exert no effect on the present experimental results as seen in Table 2.

A purple coloration of the V−XO reagent will provide further advantage because it prevents interference of bilirubin contained in biological samples.

The sensitivity of the V−XO reagent is quite good as compared with that in usual methods. The value of ΔA_c per 1 M glucose is found to be 2×10^{-4} (cf. Fig. 4). This value is 2.5 ~ 3 times larger than the molar absorptivities observed in the peroxidase-4 aminoantipyrine-phenol and the hexokinase-glucose-6-phosphate dehydrogenase methods, but somewhat lower than the value obtained in the peroxidase-3-methyl-2-benzothiazolinone hydrazone-dimethyl aniline method (*2*, *8*).

The present method can be widely applicable to he colorimetric determination of some other substances such as uric acid and amino acids, and may also be applied to the determination of activity of such enzymes as uricase and amino acid oxidases.

SUMMARY

The V–XO reagent (mixture of V(V) and xylenol orange) provided in our earlier studies is found very useful for the colorimetric determination of traces of hydrogen peroxide. In this paper the reagent is successfully extended to the assay of glucose in serum by the combined use of glucose oxidase.

The V–XO reagent exhibited a characteristic absorption maximum at 582 nm, and the presence of glucose together with glucose oxidase led to a significant decrease in the absorbance of the reagent. A linear relation was found between the magnitude of the decrease and glucose concentration in the range of 5 to 400 mg/100 ml. The average recovery of glucose added in serum was 98.6% and the data were reliable with a coefficient of variation below 3.0%. A good correlation to the method by 4-aminoantipyrine-phenol was found: $r = 0.970$. Ascorbic acid and uric acid did not interfere with the assay.

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MICROCHEMICAL JOURNAL 26, 250–261 (1981)

Comparison of a Graphite Tube Micromethod for the Determination of Serum Iron and Total Iron-Binding Capacity with Spectrophotometric Techniques

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INTRODUCTION

The voluminous literature since 1940 on methods for estimating iron in biological materials, especially serum, bears testimony to the difficulties inherent in this particular determination. Many of them appear to be connected with obtaining a full recovery of iron from serum. Several techniques have been suggested for dissociating iron from transferrin, reducing it from the ferric to the ferrous state (*3, 5, 6, 8–10, 16, 18*), and coupling it with a suitable chromogen before determination by colorimetric spectrophotometry. Reduction of the iron, either before or after addition of the chromogen, posed a problem as often did the development of turbidity, which interfered with the subsequent colorimetric determination. More recent techniques (*6, 9, 16, 18*) favor the reduction of iron before the addition of the chromogen while in the most recent technique Ceriotti and Ceriotti (*7*) added the releasing agent (HCl), the complexing agent (thiosemicarbazide), and the reducing agent (ascorbic acid) together with the chromogen (ferrozine) in a single solution to the serum.

Since most colorimetric techniques require at least 0.5 ml serum, it is essential that for pediatric cases and animal experimentation, where specimens are limited, a micromethod be used for the determination of serum iron and total iron-binding capacity. Flameless atomic absorption techniques are more sensitive by several orders of magnitude than the conventional flame techniques and therefore offer a unique opportunity for replicate determinations of serum iron in as little as 0.1 ml serum. Furthermore, the graphite tube technique does not suffer from interferences by other metals as do the flame techniques (*15, 17, 22*) and requires only 30 μl of prepared specimen for replicate serum iron determinations compared to the several milliliters required for flame atomic absorption spectroscopy.

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This study outlines a rapid micromethod using flameless atomic absorption spectroscopy for the determination of: (a) total iron in serum, (b) "serum iron," and (c) total iron-binding capacity (TIBC). The methods of Carter (*6*) and Olson and Hamlin (*14*) were combined to produce a protein-free filtrate and the results obtained for serum iron were compared with the colorimetric technique used by Carter (*6*) with ferrozine as the iron chromogen. In addition a comparison was made with the Boehringer Mannheim test kit method which uses Bathophenanthroline as the iron chromogen and no precipitation of the proteins is required. Comment is also made on the diurnal variation of serum iron found in normal men and women.

MATERIALS AND METHODS

Instrumentation

Atomic absorption spectroscopy. The Perkin–Elmer Model 603 atomic absorption spectrophotometer used for this study has been described by Baily *et al.* (*1*). In agreement with previous observations for copper (*2*), the tungsten-coated graphite tube was found unsuitable for iron determination. Therefore uncoated graphite tubes were used since tungsten-coated graphite tubes or pyrolytically coated tubes gave results which in most cases were 100% too high. The uncoated tubes could be used for up to 200 firings and, unlike the method for copper (*2*), could be used on subsequent days.

HGA-2200 graphite furnace operating program. The operating program of the HGA-2200 graphite furnace is shown in Table 1 and the manner in which the times and temperatures were arrived at is identical to that described for lead and copper (*1, 2*). However, the "maximum power" facility was not used for the present determinations.

Glassware and reagents. All glassware was acid-washed overnight in chromic acid and then repeatedly rinsed with water, double-distilled in glass. All reagents used, including those for precipitating serum proteins, were of analytical-reagent grade. The reducing agent, ammonium acetate buffer, trichloracetic acid precipitant (TCA), and the mixed ferrozine (Merck) color reagent were made up according to the method outlined by Carter (*6*). For a continuous supply of serum for these studies, a "pooled

TABLE 1
HGA-2200 OPERATING PROGRAM

	Drying	Ramp	Charring	Ramp	Atomization	Flow time stop	Integration
Time (sec)	60	50	60	50	7	7	7
Temperature (°C)	150		1300		2800	—	—

serum'' was obtained from the South African Institute for Medical Research and kept at 4°C. Standard solutions of iron were prepared by diluting a stock solution, 1000 ppm (m/v) in 0.01 *M* perchloric acid (Hopkins and Williams, Ltd., England), with glass-double-distilled water. The working solutions (1 μg ml^{-1}, 2 μg ml^{-1}, etc.) were prepared fresh daily and stored in iron-free polyethylene bottles.

Preparation of Serum Specimens

(*a*) *Total iron in serum.* For the determination of total iron in serum by the graphite tube method, the serum was diluted 10 times with glass-double-distilled water and 10 μl was dispensed into the graphite tube by means of the autosampler. A standard graph was obtained by adding the requisite amounts of a 1-ppm iron standard to aliquots of serum which were then mixed and finally made up with double-distilled water to give a 10-fold dilution of the serum. All iron determinations using the graphite tube were carried out at a wavelegth of 248.3 nm and a slit width of 0.7 nm.

(*b*) *Serum iron by graphite tube.* To 0.1 ml serum, 0.1 ml reducing agent (*6*) was added and after mixing, the solution was allowed to stand for 5 min at room temperature. Sufficient precipitant (11.3% TCA) was added to make the mixture up to a total volume of 1.0 ml (10× dilution). The solution was mixed thoroughly on a Whirlimixer and placed into a water bath at 90°C for 15 min. The precipitated proteins were centrifuged at 3000 rpm for 20 min and 10-μl aliquots of the clear supernatant were injected automatically into the graphite tube furnace for serum iron determinations. To simplify the addition of iron standards to serum, a standard graph was obtained by taking 0.5 ml serum and adding the requisite amounts of a 1-ppm standard iron solution before adding the other reagents as outlined above.

(*c*) *Total iron-binding capacity (TIBC) by graphite tube.* The procedure outlined in the Boehringer Mannheim test kit was followed for preparing the serum specimen. To 0.5 ml serum, 1.0 ml $FeCl_3$ solution (500 μg Fe/100 ml) was added and after mixing, the solution was allowed to stand for 30 min at room temperature. Approximately 100 mg $MgCO_3$ was then added and the mixture was allowed to stand at room temperature for 30 min during which period the suspension was thoroughly mixed at least five times. The mixture was next centrifuged at 3000 rpm for 10 min. A clear supernatant was obtained and 0.1 ml was treated as above for the determination of serum iron. The iron value multiplied by 3 (dilution factor) gave the total iron-binding capacity.

(*d*) *Serum iron by spectrophotometric techniques.* To test the validity of the present method for serum iron by graphite tube, determinations on the pooled serum were also carried out by two different spectrophotomet-

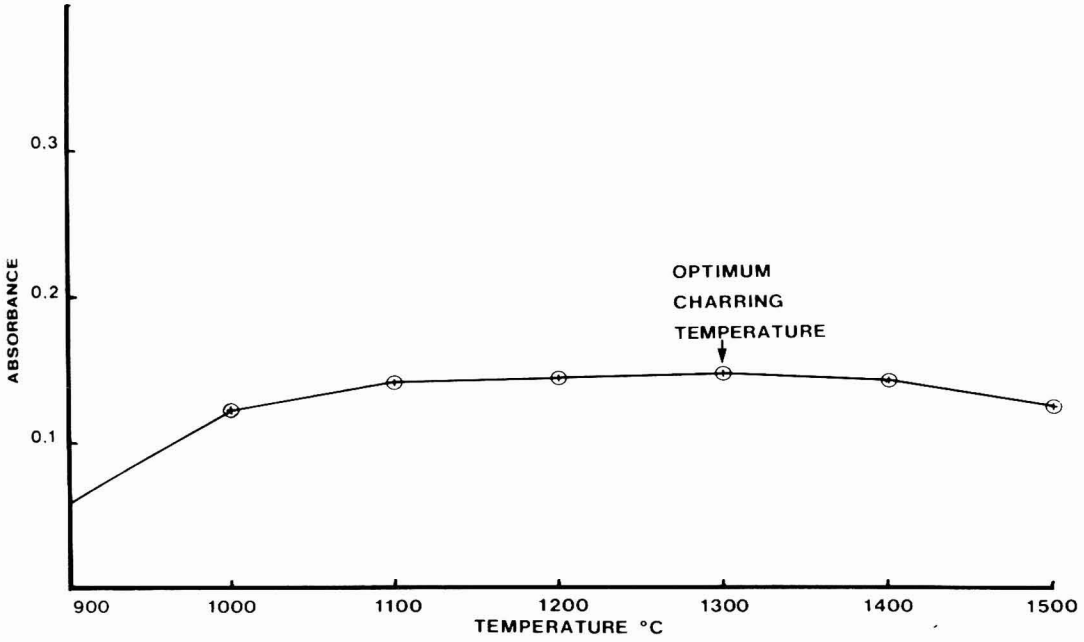

FIG. 1. Graph showing optimum charring temperature while keeping drying temperature (150°C) and atomization temperature (2800°C) constant.

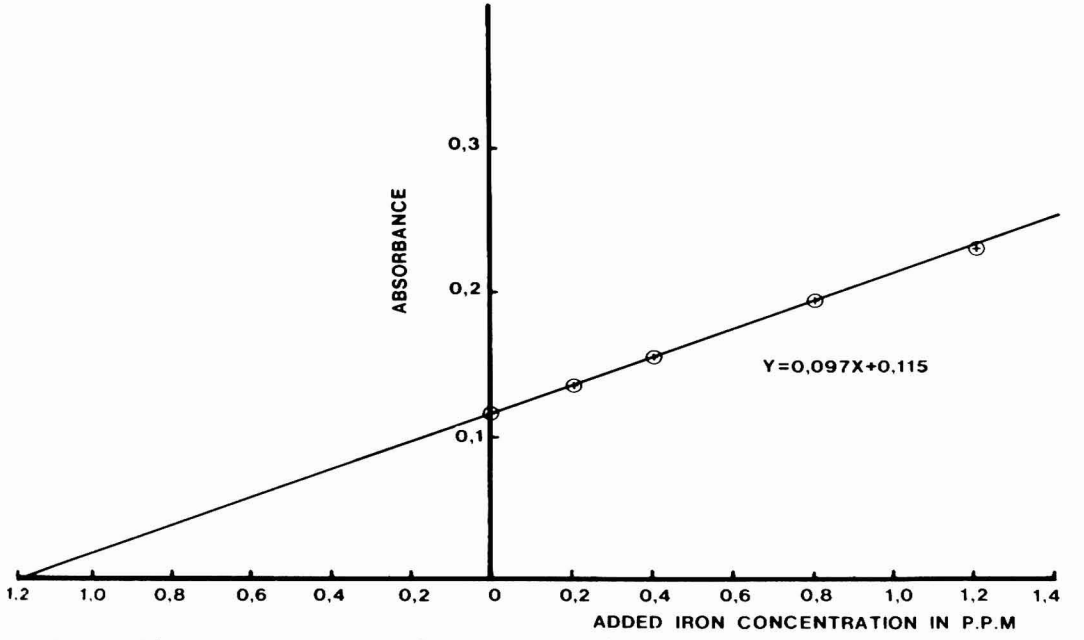

FIG. 2. Linear calibration graph for serum iron using combined methods of Carter (6) and Olson and Hamlin (*14*) for preparing serum (see text).

ric methods. In the first case, Carter's method (*6*) which removes the proteins, was followed except that after the addition of the precipitant (TCA), the mixture was heated at 90°C for 15 min as recommended by Olson and Hamlin (*14*). In the Boehringer Mannheim test kit method, the serum iron was determined without deproteinizing the serum and the entire determination was carried out at room temperature.

(*e*) *Iron in precipitated proteins (hemoglobin iron).* The precipitated proteins recovered after centrifugation as described in (*b*) above were dissolved in 0.1 ml, *N* NaOH. This mixture was then diluted to 1.0 ml with glass-double-distilled water (10-fold dilution of the 0.1 ml serum taken originally), and 10 μl dispensed into the graphite furnace. A standard graph (Fig. 3) was obtained by adding aliquots of a standard iron solution to 20 mg albumin dissolved in 0.1 ml 10 *N* NaOH. This solution was diluted to 1.0 ml with glass-double-distilled water. A correction was made for iron found in the albumin (Sigma Chemical Co.).

RESULTS

Figure 1 illustrates how the HGA-2200 graphite furnace operating program was determined. By keeping the drying temperature (150°C) and the atomization temperature (2800°C) constant, the most suitable charring

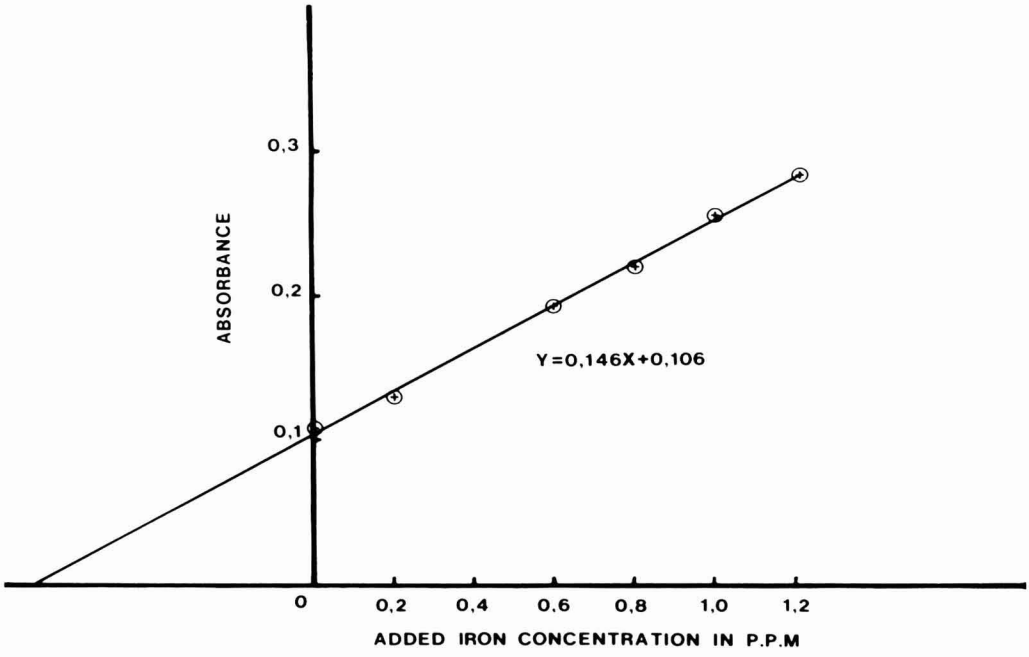

FIG. 3. Linear calibration graph of added iron in NaOH containing dissolved albumin for the determination of iron in precipitated proteins (hemoglobin iron).

TABLE 2
SERUM IRON: COMPARISON OF RESULTS FROM GRAPHITE TUBE AND SPECTROPHOTOMETRIC METHODS BY CARTER (6) AND BOEHRINGER MANNHEIM TEST KIT

Specimen number	Serum iron graphite tube (μg/100 ml)	Serum iron Carter (6) (μg/100 ml)	Serum iron Boehringer Mannheim kit (μg/100 ml)
1	120	121	127
2	121	119	123
3	98	116	114
4	127	121	116
5	133	123	120
Mean ± SD	120 ± 13.25	120 ± 2.40	120 ± 5.24
Percentage coefficient of variation	11.0	2.0	4.4

temperature was obtained (*1*). Similarly the optimum drying and atomization temperatures were obtained by keeping the other two factors constant. Figure 2 illustrates the linear calibration curve obtained for serum iron when the combined methods of Carter (*6*) and Olson and Hamlin (*14*) were used for preparing the serum. A similar graph was obtained when only Carter's method (*6*) was used for preparing the serum, but this gave somewhat higher results for serum iron, suggesting that all the hemoglobin iron had not been precipitated. Each point on the graph is the mean of 25 determinations since five separate sera were prepared on different days for each concentration and each determination was repeated five times.

The accuracy of the microtechnique for serum iron is illustrated in Table 2, where the results by this method are compared with the results obtained by Carter's spectrophotometric method (*6*) and the Boehringer Mannheim test kit method. Although the three techniques differ widely in the method for preparing the serum, the graphite tube determination, using only 0.1 ml serum and 30 μl of the prepared specimen for replicate determinations, gives almost identical results to the other two methods which require at least 1.5 ml serum for replicate determinations. A further test of the accuracy of the method is shown in Table 3, where the average percentage recovery of iron added to the serum was 100.6% ± 1.1.

Table 4 compares the results obtained for total iron-binding capacity (TIBC) by the present graphite tube method and the Boehringer Mannheim test kit method and Table 5 shows the diurnal variation of serum iron in six adult females and six adult males none of whom showed any clinical manifestations of iron-related disorders. Table 6 shows that total iron in serum, as determined by the present method, is the sum of "serum iron" and the iron in the precipitated proteins (hemoglobin iron). All

TABLE 3
PERCENTAGE RECOVERY OF IRON ADDED TO SERUM

Serum number	Added iron (μg/100 ml)	Iron expected (μg/100 ml)	Iron recovered (μg/100 ml)	Mean ± SD	Percentage recovery	Percentage recovery ± SD
1	0	—	120		—	—
2	0	—	121	123 ± 3.8	—	—
3	0	—	127		—	—
4	40	163	166		101.8	
5	40	163	166		101.8	102.2 ± 0.8
6	40	163	168		103.1	
7	80	203	210		103.4	
8	80	203	195		96.1	99.8 ± 3.7
9	80	203	203		100.0	
10	100	223	215		96.4	
11	100	223	226		101.3	100.3 ± 3.5
12	100	223	230		103.1	
13	200	323	323		100.0	
14	200	323	326		100.9	100.1 ± 0.8
15	200	323	321		99.4	

Mean percentage recovery 100.6 ± 1.1

determinations were carried out by the graphite tube method and a recovery of 97.8% was obtained.

DISCUSSION

The toxic effect of iron is demonstrated in patients with hemochromatosis or refratory anemia (*20*). In all cases of toxicity the method presented here lends itself to a rapid and accurate determination within minutes of the arrival of a patient at the hospital and may be performed on a minimum specimen (0.1 ml serum). In addition the total iron in the serum can be monitored during the addition of therapeutic chelators to the patients. Since the direct determination of total iron by atomic absorption spectrometry does not differentiate between serum or transferrin iron and hemoglobin iron (*22*) it is important to realize that sera without visible hemolysis may still contain hemoglobin. In the present study, the pooled serum was found to contain 1.5 mg hemoglobin per 100 ml serum, which meant a contribution of approximately 5 μg iron to the total value of 217 μg iron found per 100 ml serum. Tavenier and Hellendoorn (*19*) stated that "the determination of serum iron by means of atomic absorption can only

TABLE 4
COMPARISON OF RESULTS FOR TOTAL IRON-BINDING CAPACITY (TIBC) OBTAINED BY GRAPHITE TUBE AND BY BOEHRINGER MANHEIM COLORIMETRIC METHOD

Serum number	TIBC, graphite tube (μg/100 ml)	TIBC, Boehringer Mannheim test kit method (μg/100 ml)
1	387	413
2	419	400
3	399	385
4	430	431
5	396	426
Mean ± SD	406 ± 17.7	411 ± 18.9
Coefficient of variation	4.4%	4.6%

be performed when the haemoglobin iron plays hardly any role as compared to the total amount of iron present." This study draws a clear distinction between "total iron in serum" as determined in method (a) above and "serum iron" in method (b). In this study both determinations are carried out by flameless atomic absorption spectroscopy. However, the methods for preparing the serum specimens for the two determinations are completely different and the results for "serum iron" as determined here are fully comparable with the spectrophotometric methods (Table 2). Workers such as Olsen *et al.* (*13*), although recognizing that a TCA precipitation was necessary for serum iron, failed to distinguish between "total iron in serum" and "serum iron." Glenn *et al.* (*11*), however, made no correction for the hemoglobin iron and gave for their GRA method a serum iron mean value of 93.3 μg/100 ml serum.

TABLE 5
DIURNAL VARIATION OF SERUM IRON IN SIX FEMALE AND SIX MALE SUBJECTS WITH NO CLINICAL MANIFESTATIONS OF IRON-RELATED DISORDERS

Subject number	Females serum iron (μg/100 ml)		Males serum iron (μg/100 ml)	
	9 AM	3 PM	9 AM	3 PM
1	144	125	178	140
2	265	163	223	180
3	110	141	151	115
4	258	200	120	108
5	238	160	235	118
6	255	188	160	206

TABLE 6

TOTAL IRON IN SERUM IS THE SUM OF "SERUM IRON" AND "HEMOGLOBIN IRON"[a]

Serum number	Total iron in serum (by graphite tube) (μg/100 ml serum)	Serum iron (by graphite tube) (μg/100 ml serum)	Iron in precipitated protein (hemoglobin iron) (μg/100 ml serum)	Serum iron + protein iron (μg/100 ml serum)	Percentage recovery of total iron
1	233	127	97	224	96.1
2	217	121	92	213	98.2
3	210	120	88	208	99.1
4	187	98	86	184	98.4
5	237	133	97	230	97.1
Mean ± SD	217 ± 20.0	120 ± 13.3	92 ± 5.1	212 ± 11.8	97.8 ± 1.2

[a] All determinations were carried out by graphite tube.

The average value for the total iron in plasma was given by Rodgerson and Helfer (*17*) as 200 μg/100 ml plasma and the contribution of the plasma hemoglobin iron as 16 μg/100 ml. This gave these workers a "corrected" value for the total iron in plasma of 184 μg/100 ml plasma. In the present study the average iron content of the precipitated proteins (hemoglobin iron) was found to be 92 μg iron/100 ml serum (Table 6). This probably accounts for the discrepancy between values found by Rodgerson and Helfer's atomic absorption method (*17*) and values found by spectrophotometry.

Despite the widely differing techniques used for determining serum iron, viz, graphite tube, Carter's colorimetric method (*6*) with protein precipitation, and the Boehringer Mannheim test kit method, where no protein precipitation is employed, the results for serum iron (Table 2) show a remarkable degree of agreement. In a statistical analysis of the three methods, the graphite tube method was compared separately with each of the other two methods using the Student t test. This test is not subject to the limitation that sample variances within each method must be reasonably similar. No significant difference was found between the means obtained by any of the methods (graphite tube vs Carter, $t_8 = 0.102$ and graphite tube vs Boehringer $t_8 = 0.0904$, where neither of the results for t_8 are significant). Similarly a Student t test carried out on the results for total iron-binding capacity (Table 4) gave the result $t_8 = 0.415$, which is not significant. The variance of the graphite tube method is somewhat higher than that of the other two methods for serum iron, but is slightly lower for TIBC than is the Boehringer test kit method. The great advantage of the graphite tube method, however, is the small amount of specimen required.

Long *et al.* (*12*) have drawn attention to the considerable variation of reports on "diurnal variation" in serum iron concentration. The present study (Table 5) agrees with their findings that on the whole there is a decrease in serum iron concentration from morning to afternoon. Of the 12 subjects tested, 10 had higher serum iron concentrations in the morning by an average of 38% compared with an average of 21% found by Long *et al.* (*12*).

Iron deficiency anemia is prevalent among children under 2 years of age (*4*) and because relatively large quantities of venous blood have been required for assays of serum iron and total iron-binding capacity (TIBC), these tests are often omitted in diagnostic tests involving small children (*21*). The micromethod outlined in the present study will allow serum or plasma iron determinations to be carried out on finger-puncture specimens which will be beneficial in nutrition surveys of preschool children, a group in which iron deficiency is frequent (*21*).

SUMMARY

A rapid micromethod by flameless atomic absorption spectrometry is presented for the determination of total iron in serum or plasma. A clear distinction is drawn between "total iron in serum" and "serum iron." The results for serum iron are compared with existing spectrophotometric methods. Comment is made on diurnal variation in serum iron concentration.

ACKNOWLEDGMENT

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Spectrophotometric Study of the Reaction of Titanium with Bromopyrogallol Red in the Presence of Cetylpyridinium Bromide

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INTRODUCTION

Bromopyrogallol red yields intensely colored complexes with a number of metals and thus has frequently been used for the spectrophotometric determination of these metals (*3*). Many of these determinations are carried out in the presence of surface active substances, which considerably improve the conditions for the determination, especially the sensitivity.

We have already described the determination of titanium using bromopyrogallol red in the presence of gelatine (*9*). However, because the use of gelatine involves a number of disadvantages (the undefined composition of gelatine, low stability of gelatine solutions), we carried out a study of the reaction of Ti(IV) ions with bromopyrogallol red in the presence of cetylpyridinium bromide and developed a new spectrophotometric determination of Ti(IV). A mechanism for the reaction is proposed on the basis of a study of the kinetics of the reaction.

EXPERIMENTAL

Spectrophotometric measurements were carried out on a Unicam SP-800 recording spectrophotometer (Unicam Instruments Ltd., Cambridge, Great Britain) with quartz cuvettes with internal diameters of 1.00 and 4.00 cm. An external EZ-2 recorder (Laboratorni přístroje, Czechoslovakia) was used for kinetic measurements.

The pH values were measured on a PHM 62 pH-meter (Radiometer, Copenhagen, Denmark) with a combined GK 2401 B glass electrode. The instrument was calibrated using standard S 1316 buffer (pH = 4.00) and S 1326 buffer (pH = 7.00) from the same company.

The stock solution containing 2×10^{-2} *M* titanium was prepared by dissolving the spectral pure metal in concentrated hydrochloric acid in the presence of hydrogen peroxide (*7*); the final HCl concentration was 3 *M*.

The 5×10^{-4} *M* bromopyrogallol red stock solution (dibromopyrogallolsulfogalein, DG) was prepared by grinding the substance in ethanol and

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diluting with distilled water so that the ethanol content in the final solution was 20% (v/v). The solution must be stored in a closed vessel and used within 48 hr.

Cetylpyridinium bromide (CPB) was used as a 5×10^{-3} *M* solution in 20% methanol. The pH value was adjusted using glycine buffer (*I*), and the ionic strength was adjusted using 3 *M* potassium chloride.

RESULTS AND DISCUSSION

Absorption Spectra and Optimal Reaction Conditions

The absorption curves of the studied complex and its components are depicted in Fig. 1. A wavelength of 625 nm was chosen for further measurements, corresponding to the maximum of the differential curve.

Color formation in the ternary complex is complete after standing 20 min at laboratory temperature and the color is stable for 24 hr. Heating for 2–3 min at 100°C produces the same color intensity (the absorbance of the solution decreases on boiling for a longer time). These solutions are stable for 30 min.

It was found in a study of the effect of the pH on the complex color that the differential curves are constant in the pH interval 2.4 to 2.7. Subsequent measurements were carried out at pH 2.5. It was also found that a

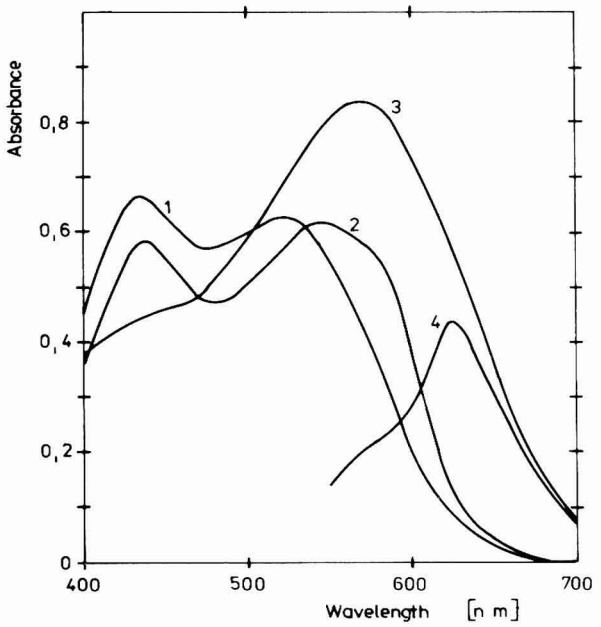

FIG. 1. Absorption spectra: (1) DG; (2) DG + CPB; (3) Ti(IV) + DG + CPB; (4) differential curve, (3) – (2). $c_{Ti} = 1 \times 10^{-5}$ *M*, $c_{DG} = 4 \times 10^{-5}$ *M*, $c_{CPB} = 5 \times 10^{-4}$ *M*, pH = 2.55, $I = 0.2$.

constant absorbance value is attained in the presence of at least a twofold excess of DG and a 30-fold excess of CPB with respect to the Ti(IV) concentration. The solution is cloudy up to concentrations of 1×10^{-4} *M* CPB, in agreement with literature data (the critical micelle concentration at an ionic strength of $I = 0$ (*4*) is $5.8-7.0 \times 10^{-4}$; this value decreases with increasing ionic strength (*2*)). A study of the effect of the ionic strength revealed that the solution absorbance is constant at $I = 0.1$ to 0.3, and that, at higher values, the absorbance increases and the maximum is shifted to longer wavelengths.

Complex Composition

The stoichiometric composition of the complex formed was studied by the Job method of continuous variations. It was found that titanium reacts with bromopyrogallol red in a ratios of 1:1 and 1:2.

Calibration Curve

For the optimal conditions found in this study ($\lambda = 625$ nm, pH = 2.5, $c_{DG} = 4 \times 10^{-5}$ *M*, $c_{CPB} = 5 \times 10^{-4}$ *M*), the dependence of the absorbance on the Ti(IV) concentration is linear in the range 0.05 to 0.5 μg Ti(IV) ml^{-1}. The parameters of the calibration curve, $A = a + b \times c_{Ti}$, were found by linear regression and have the values $a = 0.0075$ and $b = 0.9039$ ml μg^{-1}. The standard deviation is $s = 1.2 \times 10^{-2}$ and the sensitivity of the determination according to Sandell's definition is $S = 9.2 \times 10^{-4}$ $\mu g\ cm^{-2}$.

Interference

The determination can be carried out in the presence of Cu^{2+} ions up to a molar ratio of 1:10; Al^{3+} and F^- up to 1:50; Fe^{2+} and Co^{2+} up to 1:100; Cr^{3+}, Pb^{2+}, and I^- up to 1:200; Zn^{2+}, Ni^{2+}, and Br^- up to 1:1000; Mn^{2+}, SO_4^{2-}, and NO_3^- up to 1:3000. Cl^- ions do not interfere. NH_4^+, Fe^{3+}, Cd^{2+}, HPO_4^{2-}, $H_2PO_4^-$, NO_2^-, MnO_4^-, $Cr_2O_7^{2-}$, ClO_4^-, and EDTA interfere in ratios from 1:1.

Procedure

A solution containing maximally 12.5 μg Ti(IV) is pipetted into a 25-ml volumetric flash and the pH of the solution is adjusted after adding 1 drop of methanile yellow indicator solution by additions of 0.5 *M* sodium acetate or hydrochloric acid so that the color is the same as that of a reference methanile yellow solution at pH 2.5 (it was found that this indicator does not absorb at the wavelength used). Then 10 ml of glycine buffer, 2.5 ml of 5×10^{-3} *M* CPB solution, and 2 ml of 5×10^{-4} *M* DG solution are added and the flask is filled to the mark with distilled water. The absorbance is measured at 625 nm after standing for 20 min at laboratory temperature or heating for 2 or 3 min on a boiling water bath.

Reaction Kinetics

As study of the kinetics of a reaction frequently provides important information on the effect of the reaction conditions and on the reaction mechanism, a kinetic study of the reaction considered was carried out on the basis of the determined optimal conditions for the formation of the complex of Ti(IV) with DG in the presence of CPB.

The time changes in the concentrations of the individual components were followed spectrophotometrically. It was assumed that the 1:1 complex is formed under the conditions employed in the kinetic study (the 1:2 complex is formed at excess DG, which was not used in the kinetic measurements). The parameters of all the straight lines were found by linear regression.

The reaction order with respect to the individual components was found by differential method. The reaction order m with respect to DG was found from the slope of the straight line

$$\log v = \log k' + m \log c_{DG},$$

where v is the reaction rate and k' is the pseudo-first-order rate constant.

The measurement was carried out for two different initial DG concentrations ($c_{DG} = 5 \times 10^{-6}\ M$, $c_{DG} = 1 \times 10^{-5}\ M$) and for a constant excess concentration ($c_{Ti} = 1 \times 10^{-4}\ M$, $c_{CPB} = 5 \times 10^{-4}\ M$). Evaluation of the measured dependence yielded values of 1.08 and 1.09 for the reaction order, i.e., the reaction order with respect to DG is 1.

The reaction order with respect to Ti(IV) was found from the slope of the dependence of log k' on log c_{Ti} at excess Ti compared to DG. The dependence of the absorbance on time was carried out at constant DG and CPB concentrations ($c_{DG} = 5 \times 10^{-6}\ M$, $c_{CPB} = 5 \times 10^{-4}\ M$) and at constant Ti(IV) concentrations chosen so that the Ti:DG ratios were 10:1, 15:1, 20:1, and 25:1. Plotting of the determined k' value for individual Ti concentrations against the logarithm of the actual Ti concentration yielded a straight line with a slope of 1.02; thus the reaction is first order with respect to Ti.

The reaction order with respect to CPB was found in the same way. At constant DG and Ti concentrations ($c_{DG} = 5 \times 10^{-6}\ M$, $c_{Ti} = 1 \times 10^{-4}\ M$) and at a CPB concentration such that the CPB:DG ratios were 100:1, 120:1, 140:1, and 160:1, a dependence of log k' on log c_{CPB} was obtained, indicating that the reaction rate decreases with increasing CPB concentration, i.e., the reaction order with respect to CPB is negative (Fig. 2). It is apparent from the figure that the role of CPB in this reaction is not simple and that a stoichiometric ternary complex is apparently not formed. The negative value for the reaction order suggests that the actual complexation reaction is preceded by rapid equilibrium formation in-

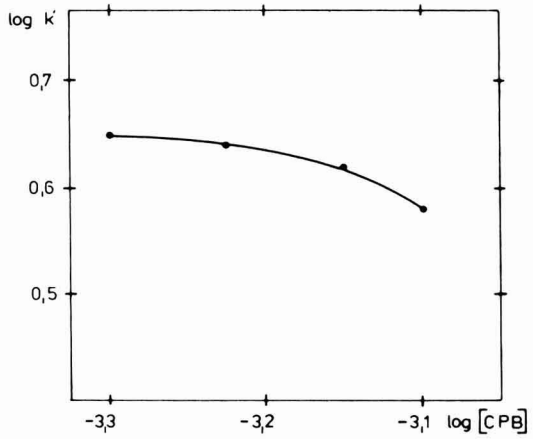

FIG. 2. Effect of the CPB concentration on the reaction rate. $c_{DG} = 5 \times 10^{-6}\ M$, $c_{Ti} = 1 \times 10^{-4}\ M$, pH = 2.5, $I = 0.2$.

volving CPB ions, probably equilibrium between the CPB micelles and bromopyrogallol red.

In a study of the effect of H^+ ions on the reaction rate (measurement carried out at $c_{DG} = 5 \times 10^{-6}\ M$, $c_{Ti} = 1 \times 10^{-4}\ M$, $c_{CPB} = 5 \times 10^{-4}\ M$), a reaction order with respect to H^+ ions of -3 was found. A negative value with respect to H^+ ions indicates that the actual complex formation is preceded by rapid equilibrium formation involving H^+ ions.

The experimental results formed a basis for proposal of a reaction mechanism. It follows from the distribution diagram for the soluble hydrated forms of Ti(IV) in dependence on the hydrogen ion concentration (*6*) that Ti(IV) is present as the $Ti(OH)_2^{2+}$ form in the stock solution employed (in 3 *M* HCl). At pH 2.5 where the studied reaction proceeds, the $Ti(OH)_3^+$ form predominates; literature data (*6*) indicate that this form reacts with complexing agents. The partial hydrolytic equilibrium

$$Ti(OH)_2^{2+} + H_2O \overset{K'_h}{\rightleftharpoons} Ti(OH)_3^+ + H^+ \qquad (1)$$

thus precedes the actual complexation reaction.

Similarly, the H_3DG^- form of bromopyrogallol red predominates at pH 2.5 and the Ti(IV)–HDG complex is formed by reaction of Ti(IV) with DG at this pH (*8*); thus the actual complexation reaction must be preceded by rapid equilibrium formation:

$$H_3DG^- \overset{K'}{\rightleftharpoons} HDG^{3-} + 2\ H^+. \qquad (2)$$

The actual complexation reaction can then be described by the relationship

$$Ti(OH)_3^+ + HDG^{3-} \xrightarrow{k} \text{complex}.$$

If the concentration of $Ti(OH)_3^+$ is expressed in terms of Eq. (1) and the concentration of HDG^{3-} in terms of Eq. (2), then the rate of this reaction is given by the expression

$$v = k \frac{K_h' K' [Ti(OH)_2^{2+}] [H_3DG^-]}{[H^+]^3}$$

in agreement with the reaction orders with respect to the individual components found in this study.

Further important information was obtained from a study of the effect of the ionic strength on the reaction rate (at concentrations $c_{DG} = 5 \times 10^{-6}$ M, $c_{Ti} = 1 \times 10^{-4}$ M, $c_{CPB} = 5 \times 10^{-4}$ M). It was found that the reaction rate increases with increasing ionic strength (at $I = 0.2$, the reaction half-life $\tau = 0.48$ min; at $I = 0.5$, $\tau = 0.34$ min), suggesting that the two reacting ions have the same charge. The findings of Nabivanets (*5*), who demonstrated in a study of the electromigration of Ti(IV) ions in HCl medium at a concentration corresponding to our conditions that Ti(IV) ions are positively charged, indicate that the charges of the two species cannot be negative.

Thus it can be assumed that the studied reaction involves interaction of $Ti(OH)_3^+$ ions with a positively charged associate formed from H_3DG^- ions bonded to positively charged cetylpyridinium bromide micelles.

SUMMARY

Optimal conditions were found for the reaction of Ti(IV) with bromopyrogallol red (λ = 625 nm, pH = 2.5, $c_{DG} = 4 \times 10^{-5}$ M, $c_{CPB} = 5 \times 10^{-4}$ M) and a new method was developed for the spectrophotometric determination of titanium in the concentration range 0.05–0.5 μg ml^{-1}. A mechanism was proposed for the studied reaction on the basis of a kinetic study.

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A Highly Effective One-Minute Thin-Layer Chromatographic Separation of Unconjugated Tri- and Dihydroxy Bile Acids

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INTRODUCTION

The quantitative determination of individual serum bile acids has been shown to be of diagnostic value for the differentiation of some hepatic and biliary tract diseases (*4, 6, 13, 17, 20, 27, 28, 33*). The cholic acid (C) to chenodeoxycholic (CDC) and deoxycholic (DOC) acids ratio, commonly known as the tri-/dihydroxy bile acids ratio, has been shown to distinguish between cholestatic and noncholestatic forms of liver disease (*4, 6, 27*). Similarly, the ratio of C to CDC has been described for the differentiation between biliary obstruction and cirrhosis of the liver (*6, 13, 17, 33*). However, the applicability of individual bile acid ratios as a routine laboratory analysis has been limited by the low concentrations of bile acids in serum samples, by the tedious extraction and concentration procedures, and by the highly sophisticated instrumentation required for accurate quantitation.

Commonly employed laboratory techniques for the preliminary purification and isolation of bile acids from serum samples include: preparative thin-layer chromatography (TLC) (*3, 8, 10, 14, 22*), liquid–liquid extraction (*3, 4, 8, 10, 16, 31*), liquid–solid extraction (*1, 2, 14, 17, 18, 22, 28*), and combinations of these in tandem (*3, 8, 10, 14, 22*). A variety of instrumental methods have been described for the quantitative determination of serum bile acids, e.g., gas–liquid chromatography (*1, 5, 8, 14, 17, 18, 23, 25, 26, 31*), uv spectrophotometry (*4, 15, 19, 27*), fluorometry (*8, 16, 22*), and radioimmunoassay (*7*).

Although conventional TLC procedures have widely been employed for the quantitative separation of bile acids, only recent procedures employing glass-microfiber chromatography sheets have been able to reduce the solvent migration time from hour(s) to minutes (*9, 11, 12, 29*) or even seconds (*30*). Furthermore, when the TLC separation is coupled with a sensitive fluorescent visualization technique, direct quantitation may be performed *in situ* with a spectrofluorometer equipped with a thin-layer scanning attachment (*29, 30*).

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In this paper, a simple, rapid, and sensitive TLC procedure, employing silica gel-impregnated glass-microfiber chromatography sheets (Commercial product, ITLC type SG sheets), is described for the separation of tri- and dihydroxy bile acid of serum samples. The serum bile acids were isolated on Amberlite XAD-2 resin, extracted, concentrated, and further purified by a simple 3-min TLC procedure. Complete separation of the tri- and dihydroxy bile acids was easily achieved within 1 min with the newly developed solvent system and miniature ITLC type SG sheets. The new separation procedure has been coupled with a sensitive fluorescent visualization technique.

EXPERIMENTAL

Materials

Reagent grades of formamide and dichloromethane (methylene chloride) were purchased from Fisher Scientific (Fair Lawn, N.J.). Amberlite XAD-2 resin was obtained from Eastman Kodak Co. (Rochester, N.Y.). Bile acid standards and additional reagents have previously been described (*30*). Human serum samples were supplied by the Regina General Hospital, Regina, Saskatchewan, Canada.

The silica gel-impregnated glass-microfiber chromatography sheets (ITLC, type SG), Seprachrom miniature chromatography chambers, Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment, and other additional materials have previously been described (*30*). Disposable Pasteur pipets were converted into miniature Amberlite XAD-2 columns (140 × 5 mm, approximate flow rate 0.1 ml/min). The eluants from the Amberlite XAD-2 column were concentrated onto blank ITLC chromatography disks by a Büchi Model R rotary vacuum evaporator (Brinkmann Instruments Inc., Rexdale, Ontario, Canada).

Rapid TLC Separation of Tri- and Dihydroxy Bile Acids Standards

A stock solution of C was prepared by adding 25 mg of C to a 25-ml volumetric flask which was filled to volume with absolute ethanol. A working standard containing 0.6 nmol of C per 5 μl of solution was prepared by pipetting an appropriate volume of the C stock solution into a 5-ml volumetric flask which was brought to volume with absolute ethanol. Stock solutions and working standards were similarly prepared for CDC, DOC, and LC. A working standard containing C, CDC, DOC, and LC, each at 0.6 nmol per 5 μl of solution, was prepared by pipetting an appropriate volume of each of the individual bile acid working standards into a 5-ml volumetric flask which was brought to volume with absolute ethanol. This resulted in a working standard with a tri-/dihydroxy bile acids ratio of 0.5.

Instant Thin-Layer Chromatography sheets (ITLC type SG, 20 × 20 cm) were cut with a razor blade to produce miniature chromatography sheets (9.9 × 6.5 cm) to fit the Seprachrom microchromatography chambers. The miniature ITLC sheets were heated in an oven at 120°C for 1 hr. Activated sheets were stored above silica gel in a desiccator.

The Popowicz (*24*) sample application technique was employed. Five blank chromatography disks (3-mm diameter) were excised from an activated miniature chromatography sheet with a Gem hand-punch. Holes were punched 8 mm from the bottom and at 11-mm intervals from the left edge of the sheet. Five microliters of the C working standard was applied to a blank disk. The standard disk was reinserted into the first position of the miniature ITLC sheet. Similarly, disks 2, 3, 4, and 5 were prepared to contain CDC, mixture of the four bile acids, DOC, and LC, respectively.

The chromatography solvent system was prepared by pipetting 2.0 ml of isooctane, 1.0 ml of diisopropyl ether, 0.07 ml of acetic acid, 0.13 ml of methanol, and 0.02 ml of formamide into a test tube. The solvent system was vigorously mixed and transferred into a Seprachrom chromatography trough. A prepared chromatography sheet was inserted into the Seprachrom chamber. The solvent system was allowed to migrate for 1 min. The chromatogram was removed, air dried for 4 min, and oven dried at 95°C for 3 min.

The chromatogram was sprayed with a solution containing 5% sulfuric acid in methanol and heated for 20 min in a 95°C oven (*32*). Fluorescent bile acid spots were observed under a Gelman Universal uv unit at 375 nm. The thin-layer chromatogram was visually evaluated. Completeness of separation was further evaluated by scanning the chromatogram with a Farrand Mark I Spectrofluorometer equipped with a xenon arc stabilizer and a thin-layer scanning attachment. The spectrofluorometer was employed in the fluorescence–reflectance mode. The Color Specification numbers for the primary and secondary filters were 7-54 and 3-73, respectively. Excitation and emission slits, having equivalent bandwidths of 5 nm and 10 nm, respectively, were employed in all studies. The excitation and emission maxima were 375 and 436 nm, respectively. The intensity of the reflected fluorescence of the bile acid spots was recorded on a Farrand Model 100 strip-chart recorder (Model SR-204, Heath Co., Benton Harbor, Mich.).

Establishment of Bile Acid R_f Values for Four Batches of ITLC Sheets

A working standard containing C, CDC, DOC, and LC, each at 0.6 nmol per 5 μl of solution, was prepared as previously described. The R_f values were determined for each of the unconjugated bile acid fractions.

Four different batches of ITLC type SG chromatography sheets were evaluated. The batches tested were as follows: Batch I, lot number 031077; Batch II, lot date October 10, 1977; Batch III, lot date October 14, 1978; and Batch IV, lot date October 15, 1978. Application, separation, and visualization of the bile acids were performed as described above.

Solvent System Stability Study

One hundred and fifty-one milliliters of the newly developed solvent system was prepared and stored in a dark brown bottle in the refrigerator at 4°C. The solvent system was mixed vigorously and 3 ml was pipetted into a Seprachrom trough. Chromatographic separation and visualization of the tri- and dihydroxy bile acids were performed as previously described. This test procedure was repeated daily for the first week and once every seventh day for an additional 3 weeks.

Isolation and Separation of Serum Bile Acids

A highly effective procedure was developed for the isolation of bile acids from human serum samples. Preliminary isolation was by column adsorption as described by Makino and Sjövall (*18*). Two milliliters of serum was diluted with 18 ml of 0.1 *M* NaOH in saline (0.9% NaCl). The mixture was passed through an Amberlite XAD-2 column (140 × 5 mm) at a rate of about 0.1–0.3 ml/min. The column was rinsed with distilled water until a neutral pH measurement was attained with pH 7–10 test paper (J. T. Baker Chemical Co., Phillipsburg, N.J.). The bile acids were eluted with 10 ml of ethanol and concentrated at 60°C by evaporation onto four blank chromatography disks in a 50-ml flask attached to a Büchi Model R rotary vacuum evaporator.

Two standard disks each containing a mixture of four unconjugated bile acids and two disks containing serum bile acids were inserted into four prepunched holes, which were 8 mm from the bottom and at 13-mm intervals from the left edge of an activated chromatography sheet. Four milliliters of methylene chloride was pipetted into a Seprachrom chromatography trough. The chromatography chamber was assembled and the solvent was allowed to migrate for 3 min. The sheet was immediately removed and air dried for 4 min. The disks were removed and the sheet was sprayed with 5% sulfuric acid solution followed by fluorescence development in a 95°C oven. This procedure minimized serum interference.

The four chromatogram disks were reinserted into another ITLC sheet. Thin-layer chromatographic separation of the tri- and dihydroxy bile acids was performed. The solvent was migrated for 2 min to allow complete separation between the trihydroxy and the remaining fractions of the serum sample. Fluorophore development was performed in a 95°C oven as previously described.

Alternatively, two-dimensional chromatography may be performed and thus obviate the necessity of a second miniature chromatogram. A bile acid sample disc was inserted into a prepunched hole, which was 18 mm from the bottom and 15 mm from the left edge of an activated chromatography sheet. A bile acid standard disk was also inserted into a second prepunched hole which was 10 mm immediately below the first one. Two-dimensional chromatographic separation was performed in a Gelman Seprachrom chamber. The first solvent, methylene chloride, was allowed to migrate for 3 min. The chromatogram was immediately removed and air dried for 4 min. The sheet was then cut to size (6.5 × 6.5 cm) to allow development of the second dimension. Chromatographic separation and visualization of the tri- and dihydroxy bile acids were performed as previously described.

RESULTS AND DISCUSSION

The Taylor *et al.* (*30*) chromatography solvent system was modified to achieve optimal separation of the tri- and dihydroxy bile acids from a mixture containing four unconjugated bile acids. The new solvent system consisted of isooctane–diisopropyl ether–acetic acid–methanol–formamide (2:1:0.07:0.13:0.02, v/v). The incorporation of methanol resulted in faster migration of the bile acids, while the presence of formamide prevented trailing of the bile acid spots (*21*). Complete separation of each of the bile acid fractions, LC, CDC and DOC, and C, was easily achieved with a 1-min chromatographic solvent migration time (Fig. 1). The solvent front migrated an average of 33.5 mm from the origin. Reproducible chromatographic separation of each of the bile acid fractions was observed within the same lot number of ITLC sheets (see Table 1). Fur-

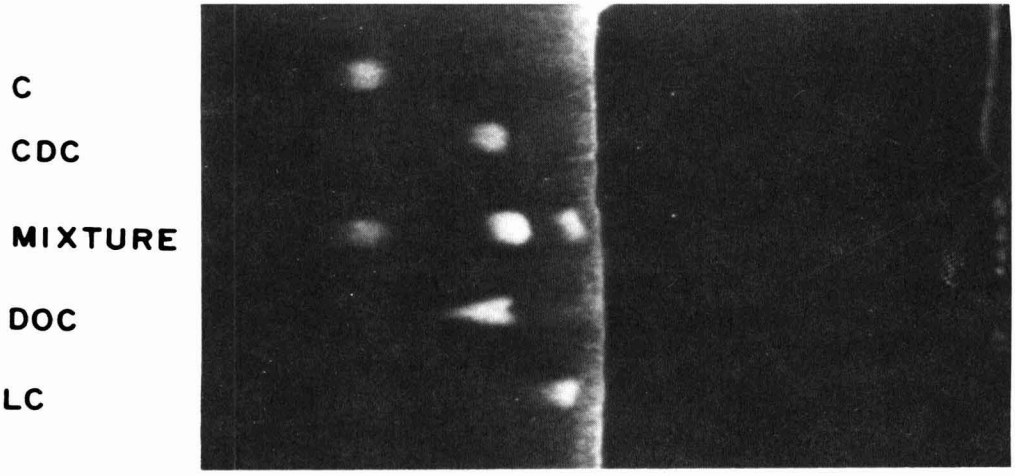

FIG. 1. One-minute thin-layer chromatographic separation of tri- and dihydroxy bile acids. Refer to text for complete details.

TABLE 1
SEPARATION OF UNCONJUGATED TRI- AND DIHYDROXY BILE ACIDS ON FOUR BATCHES OF ITLC TYPE SG CHROMATOGRAPHY SHEETS

Bile acids	$R_f \times 100 \pm$ C.V.[a]			
	Batch I	Batch II	Batch III	Batch IV
LC	80 ± 2.4%	74 ± 2.5%	89 ± 1.6%	86 ± 2.9%
Dihydroxy	51 ± 4.9%	45 ± 4.1%	71 ± 2.9%	68 ± 3.0%
Trihydroxy	24 ± 11.0%	17 ± 8.2%	39 ± 4.8%	36 ± 5.4%
Solvent migration distance (mm)	32	32	34	35

[a] Each value reported represents an average of 12 test results. Refer to text for complete details.

thermore, variations observed with different lot numbers of ITLC sheets did not affect separation of the tri- and dihydroxy bile acids fractions.

The newly developed solvent system was observed to be stable for approximately 3 days when stored in a dark brown bottle at 4°C. After 3 days of testing, the solvent front band on the chromatogram was observed to broaden and engulf the LC spots. Similar broad solvent front bands were observed when aged diisopropyl ether was used in the solvent system. This appears to indicate that peroxide formation is responsible for broadening of the band at the solvent front. However, separation of the tri- and dihydroxy bile acids remained unaffected. After 1 week of testing, the chromatographic separation between the tri- and dihydroxy bile acids was observed to deteriorate. This deterioration is attributed to alteration of the solvent system by evaporation of the volatile components. Thus for optimal results, it is recommended to use peroxide-free diisopropyl ether and to prepare the solvent system fresh prior to use.

The surface texture of the ITLC sheets was observed to vary for the different lot numbers tested. Batches I and II had smooth surfaces, while batches III and IV were relatively coarse. The batch-to-batch average solvent migration distances varied between 32 and 35 mm for 1-min solvent migration times. However, despite these differences complete separation was always achieved for each of the bile acids fractions. Average R_f values and corresponding coefficients of variation were tabulated for each of the ITLC batches tested (Table 1).

The Brühl and Schmid (*32*) procedure for visualization of the bile acids required spraying the chromatograms with 5% sulfuric acid in methanol, heating in a 95°C oven for 20 min, and viewing the developed fluorophores under a long-wave uv light source. This detection procedure had been employed to quantitate as little as 10 ng of LC by direct measurement of

the reflected fluorescence intensity of fluorescent spots with a spectrofluorometer equipped with a thin-layer scanning attachment (*30*).

In the current investigation, reproducible quantitative measurements of CDC, DOC, and C were not achieved. The development and stability of the corresponding bile acid fluorophores were observed to be dependent upon the amount of the sulfuric acid spray, the oven temperature, and the incubation time. Under optimal fluorophore development and chromatogram storage conditions, the bile acid spots could be observed more than 6 months after fluorophore development. In contrast, overspraying with the detection reagent would result in visualization of the bile acid spots on both sides of the chromatogram. The spots were observed to diffuse and the fluorescence intensity decreased rapidly and disappeared within a few hours.

The developed chromatograms were scanned with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. The spectrofluorometer was modified as previously described (*30*). Broad excitation and emission spectra were observed for each of the four unconjugated bile acids. The excitation maxima varied between 375 and 377 nm, while the emission maxima varied between 435 and 437 nm. Because of the broad spectral characteristics, it was possible to detect all of the bile acid fluorophores at the same excitation and emission wavelengths, 375 and 436 nm, respectively. The reflected fluorescence intensity of the bile acid spots was monitored and recorded on a Farrand Model 100 strip-chart recorder (see Fig. 2).

The present technique has been applied to the isolation and separation of unconjugated tri- and dihydroxy bile acids from normal human serum samples. The low concentration of bile acids in serum, further complicated by the presence of interference materials, necessitates the use of an

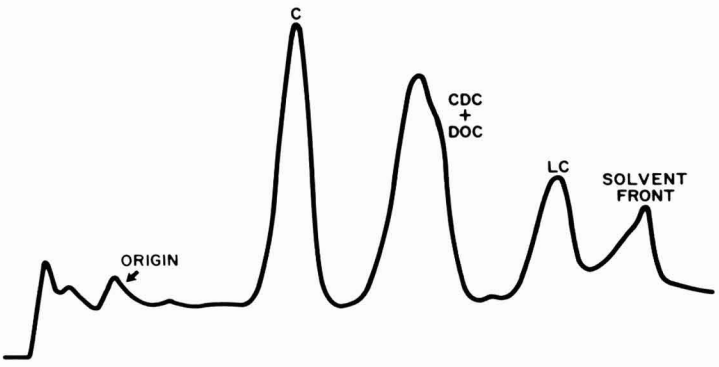

FIG. 2. Spectrofluorometer scan depicting the reflected fluorescence intensity of the bile acid spots. The chromatogram solvent migration time was 1 min. Refer to text for complete details.

isolation and concentration process prior to analysis. Anion-exchange resins, such as Amberlite XAD-2, have been reported to be desirable for the quantitative isolation of bile acids (*18*). The conversion of disposable Pasteur-type pipets into miniature Amberlite XAD-2 columns was found to be convenient and economical. Serum bile acids and some unidentified interference materials, possibly of lipid origin, were adsorbed and eluted from the columns. The concentrate was further purified by a rapid 3-min TLC procedure. Interference materials and some LC had migrated with the methylene chloride solvent system (see Fig. 3). The unconjugated tri- and dihydroxy bile acids remained at the origin. The ITLC disks, containing the purified bile acids, were removed and reinserted into another activated miniature chromatography sheet. Although excellent chromatographic separation of the tri- and dihydroxy bile acids standards was achieved with a 1-min solvent migration time, a 2-min separation time was optimal for complete separation of the serum bile acids fractions (see Fig. 4). Further preliminary investigation in this laboratory has performed the above TLC purification and separation procedure by two-dimensional chromatography, employing miniature ITLC type SG sheets. This eliminates the disc transfer process and the necessity of a second chromatogram. Bile acid reference standards were incorporated adjacent to the serum bile acids disc and separated with the second solvent migration.

The procedure described in this paper is rapid, sensitive, and highly effective for the isolation and separation of unconjugated tri- and dihydroxy bile acids of serum samples.

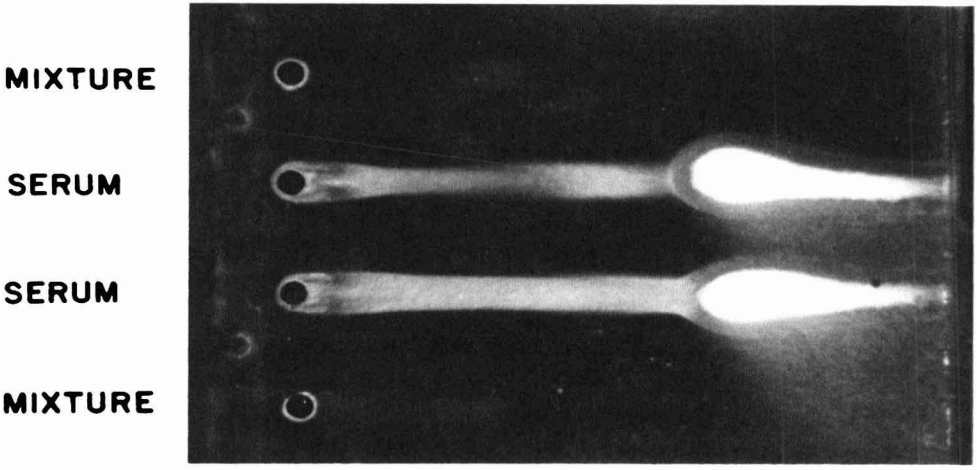

FIG. 3. Thin-layer chromatogram depicting the migration of serum interference materials. The solvent migration time was 3 min. The tri- and dihydroxy bile acids remained at the origin. Refer to text for complete details.

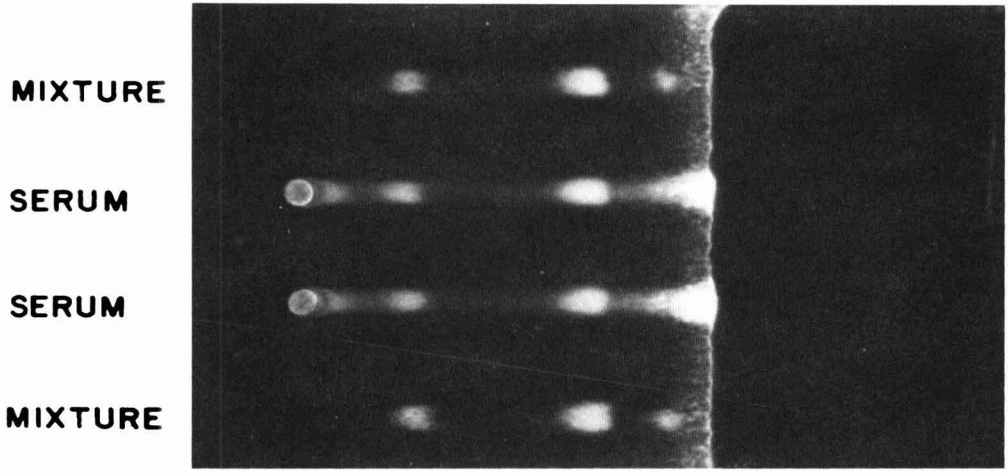

FIG. 4. Two-minute thin-layer chromatographic separation of unconjugated tri- and dihydroxy bile acids of serum. Refer to text for complete details.

SUMMARY

A rapid, miniature thin-layer chromatographic procedure has been developed for the separation of unconjugated tri- and dihydroxy bile acids. The new solvent system consisted of isooctane:diisopropyl ether:methanol:acetic acid:formamide (2:1:0.13:0.07:0.02,v/v). Complete separation of tri- and dihydroxy bile acid fractions was easily achieved within 1 min on ITLC type SG chromatography sheets. A sensitive fluorescent visualization technique was employed. The reflected fluorescence intensity of the bile acids fluorophore spots was measured with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. The excitation and emission wavelengths were 375 and 436 nm, respectively. The above procedure has been adapted for the separation of unconjugated bile acids from serum samples. The bile acids were adsorbed onto Amberlite XAD-2, eluted with ethanol, and concentrated. Further purification was performed by a simple 3-min thin-layer chromatographic technique. This new procedure for the separation of tri- and dihydroxy bile acids is rapid, sensitive, and less complex than conventional procedures presently employed in clinical laboratories.

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Study and Application of the Complex of Sn(II) with Glycerol in Volumetric Analysis

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Research in the application of oxidation–reduction volumetric methods is governed by attempts to (a) increase the selectivity of determinations, (b) find and test new reagents, and (c) suitably affect the formal redox potential of known reagents using complexing agents. The problem described under (c) has been studied most extensively in "ferrometry" (*1, 3–7, 9, 10–13*).

This work is concerned with the opposite effect—stabilization of strong reducing agents which are unstable in air, e.g., Sn(II), using complexing agents, here glycerol (*2, 8*).

As the literature is rather confusing in this area, the results are evaluated here and the possibility of applying the glycerol complex of Sn(II) in volumetric analysis is described.

EXPERIMENTAL

Apparatus. Potentiometric (EP), bipotentiometric (BiP), and biamperometric (BiA) titrations were carried out using a PHM 64 pH meter from Radiometer, Denmark. In the BiP and BiA titrations a titration adapter was employed; this was constructed in the Research Shops of the Czechoslovak Academy of Sciences, Prague. In the BiA method the signal was given in mV on the scale of the pH meter. The indicator electrode was either a platinum foil (0.35 cm^2) or a pair of identical platinum foils (0.35 cm^2); the reference electrode was an SCE. The work was carried out in a vessel designed to work under an inert atmosphere. In the titrations either a 10-ml burette, with divisions of 0.02 ml, or an ABU 12 automatic burette connected to a TTT 60 automatic titrator (Radiometer, Denmark) was employed. The polarographic and voltammetric measurements were carried out on a PO-4 polarograph from Radiometer, Denmark, using a Kalousek vessel and a dropping mercury or rotating platinum disc electrode and an SCE.

Reagents. $SnCl_2 \cdot 2H_2O$ p.a., $K_2Cr_2O_7$ p.a., H_2O_2 p.a., I_2 p.a., KI p.a., chloramine-T p.a., Na_2CO_3 p.a., $NaHCO_3$ p.a., NH_4OH p.a., NaOH p.a., HCl p.a., H_2SO_4 p.a., EDTA p.a., and glycerol p.a., ethanol (pure) were

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the products of Lachema, n.p., Brno. $K_3Fe(CN)_6$ was from Merck, GFR, and $AgNO_3$ was the product of Evans Medical Supplies Ltd., England.

Preparation of 0.1 *N* and 0.01 *N* $SnCl_2$ in glycerol and ethanol (further Sn(II)–glyc.): 5.6413 g $SnCl_2 \cdot 2H_2O$ was dissolved with constant stirring (1 hr) in 375 ml glycerol which had been previously freed of oxygen. Then the solution was diluted with oxygen-free absolute ethanol to 500 ml (final dilution must be carried out after 1 hr). The preparation of 0.01 *N* Sn(II) was the same: an amount of 0.22565 g of $SnCl_2 \cdot 2H_2O$ to a final volume of 200 ml. Further a 0.1 *N* Sn(II)–glyc solution was prepared with a glycerol:ethanol ratio of 1:1.

The tin content in the $SnCl_2 \cdot 2H_2O$ used was found by titration of the 0.1 *N* $SnCl_2$ solution in 0.8 *N* HCl with potassium bichromate in 1 *N* HCl medium. The product was found to contain 99.60% $SnCl_2 \cdot 2H_2O$.

RESULTS

Determination of the Titer of 0.1 N Sn(II)–Glyc Using Bichromate and Potassium Hexacyanoferrate

Determination of the titer of 0.1 *N* Sn(II)–glyc was carried out using a standard $K_2Cr_2O_7$ solution in the following media: sat. $NaHCO_3$, sat. Na_2CO_3, sat. Na_2CO_3 + conc. NH_4OH (24:1), 12.5% NH_4OH and in a medium of 2 *M* carbonate buffer (pH 9.3–11.3). The results of the determination are given in Table 1.

Procedure. To 45 ml of the electrolyte 5 ml of 0.1 *N* $K_2Cr_2O_7$ was pipetted and the solution was bubbled for 10 min with N_2. Then the titration was carried out with constant stirring and bubbling with N_2. Because of the high viscosity of the Sn(II)–glyc solution (measured viscosity = 463 cP), it is necessary when using the glass burette to add the reagent at such a rate that the solution flows completely from the burette walls. It follows from the results obtained that the value of the titer of $SnCl_2$ in 0.8 *N* HCl corresponds to the value of the titer of Sn(II)–glyc in saturated Na_2CO_3 medium.

TABLE 1
DETERMINATION OF THE TITER OF 0.1 *N* Sn(II)–GLYC USING POTASSIUM DICHROMATE[a]

Electrolyte	Added (mg) Cr(VI)	Found (mg) Cr(VI)	Titer (0.1 *N*) Sn(II)–glyc	EP (mV/ml) (0.04 ml)
Sat. $NaHCO_3$	8.666	8.5273	0.1016	4,550
Sat. Na_2CO_3	8.66	8.6833	0.0998	8,800
Sat. Na_2CO_3 + NH_4Cl(24:1)	8.66	8.7700	0.988	10,500
12.5% NH_4OH	8.66	9.0646	0.0956	9,400

[a] Titer of 0.1 *N* $SnCl_2$ in 1 *M* HCl medium = 0.0998.

In 0.5 *M* acetate buffer (pH 6) a green-blue precipitate of chromium (III) hydroxide is formed; a precipitate is not formed in the same buffer at pH 4, but a visible potential change is not apparent on the curves.

In Britton–Robinson buffer medium, 0.1 *M*, pH 11.6, the measuring error is 1%. Practically the same results were obtained in the determination of the titer of 0.1 *N* Sn(II)–glyc using 0.1 *N* $K_3[Fe(CN)_6]$. The most suitable medium is saturated Na_2CO_3. The reversibility of the $Fe(CN)^{4-}_6$–$Fe(CN)^{3-}_6$ system results in more rapid potential equilibration than in titration with dichromate.

Study of the Stability of the Standard Solutions

It has been mentioned (*2*) that standard solutions of Sn(II)–glyc are stable for up to 2 months in the air. We studied the stability of solutions of 0.1 *N* Sn(II)–glyc, 0.01 *N* Sn(II)–glyc, and 0.1 *N* Sn(II)–glyc (ethanol to glycol ratio of 1:1). These solutions were titrated at certain time intervals with a solution of dichromate in an inert atmosphere. The results are depicted graphically in Fig. 1. The 0.1 *N* Sn(II)–glyc solution stored under an inert atmosphere exhibited the greatest stability.

Titrations carried out in the air exhibited a greater reagent consumption (+error). If, however, the titrated solution is freed of oxygen and then the titration is carried out in the air, the positive error falls to 1%.

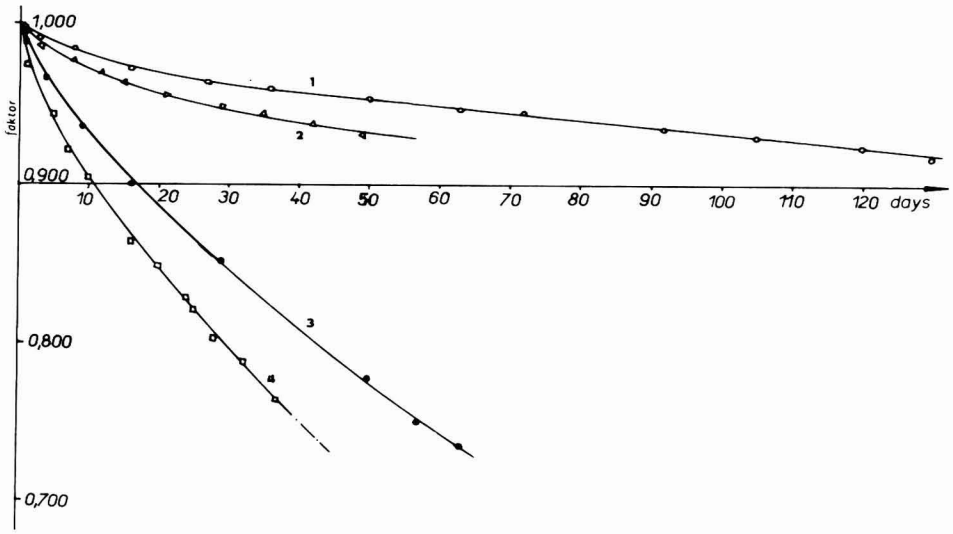

FIG. 1. Study of the stability of standard 0.1 *N* and 0.01 *N* Sn(II)–glyc solutions. (1) 0.1 *N* Sn(II)–glyc solution stored under an inert atmosphere; (2) solution of 0.1 *N* Sn(II)–glyc exposed to atmospheric oxygen; (3) 0.1 *N* Sn(II)–glyc solution stored under an inert atmosphere, prepared from 1:1 glycerol and ethanol; (4) 0.01 *N* Sn(II)–glyc solution exposed to atmospheric oxygen.

The Determination of Sn(II)–Glyc by Titration with Potassium Dichromate and Potassium Hexacyanoferrate

In the determination of some substances it is necessary to add an excess of the reagent and then to titrate the unconsumed amount. Thus a number of determinations of 0.1 *N* Sn(II)–glyc were carried out under an inert atmosphere in various media using standard solutions of 0.1 *N* $K_2Cr_2O_7$ and 0.1 *N* $K_3[Fe(CN)_6]$. It follows from the results obtained that the most suitable medium is saturated Na_2CO_3. The potential stabilization is rapid and reproducible.

The formal potential of the Sn(IV)–glyc/Sn(II)–glyc system read from the titration curves for the determination with potassium dichromate in a medium of sat. Na_2CO_3 is −800 mV, in sat. $NaHCO_3$ −580 mV, and in 12.5% NH_4OH −840 mV (SCE). It can be assumed from the titration course of the potentiometric curves that the Sn(IV)–glyc–Sn(II)–glyc redox system is irreversible and thus a more suitable method for indication of the equivalence point will be titration with two polarizable electrodes. The irreversibility of this system was confirmed polarographically and voltammetrically. The presence of glycerol in alkaline medium has such a marked affect on the electrode reaction on the rotating platinum disk electrode that a well-developed polarographic curve is not obtained. It was demonstrated by logarithmic analysis of the polarographic curves of the studied system that it is irreversible. This conclusion was confirmed oscillopolarographically. On the other hand, the Sn(IV)/Sn(II) system in a 0.8 *M* HCl medium is reversible both at a dropping mercury and at a rotating platinum disk electrode. It is apparent from the results that the value of the polarization current of potential used for BiP or BiA for indication of the equivalence point will have to be determined experimentally.

Determination of Some Inorganic Redox Systems

The individual determination were carried out using the following general working procedure: to 45 ml of sat. Na_2CO_3 were pipetted *x* ml of the determined substance and up to 10 ml of a further solution. Then N_2 was introduced into the solution for 10 min with continuous stirring. During this time the potential of the indicator platinum electrode almost always stabilized. Then the titration with 0.1 *N* Sn(II)–glyc was carried out with constant solution bubbling with N_2.

The amount of substance determined (in mg), the error (percentage), the standard deviation, and the slope (mV/ml) for 0.04 ml when using the EP and BiP methods and a note where applicable are given in Table 2. The titration curves of potassium dichromate are depicted in Fig. 2.

TABLE 2

REDUCTOMETRIC DETERMINATIONS OF SOME INORGANIC AND ORGANIC SUBSTANCES WITH 0.1 $\dot{N}$ Sn(II)–GLYC IN ALKALINE MEDIUM

Compound	Amount (mg)	Error (%)	Standard deviation	EP (mV/ml)	BP (mV/ml)	Note
Cr(VI) Cr(III)	0.866–26.1	0.1–1.24	0.01–0.10	9,000	25,000	
Fe(III) Fe(II)	2.79–83.76	−0.20–0.55	0.02–0.30	11,500	22,000	
H_2O_2	1.70–25.5	−1.10–0.28	0.01–0.09	6,000	20,500	
Iodine	3.17–63.5	−3.24–−1.88	0.01–0.26	10,000	27,000	Sat. $NaHCO_3$
Chloramine-T	7.0–141.0	−0.57–0.77	0.01–0.15	9,000	18,000	
$Ca(ClO)_2$	3.58–35.75	−0.34–0.65	0.01–0.50	7,000	15,500	
Ag^+ Ag^0	5.39–161.8	−0.55–0.26	0.01–0.60	12,500	24,000	Na_2CO_3 + NH_4OH
N-Bromsukcinimid	15.8–79.4	−0.50–1.25	0.08–0.31	9,000	22,000	
Pyridylazoresorcinol	6.4–63.8	−0.70–1.82	0.20–0.32	1,500	7,000	

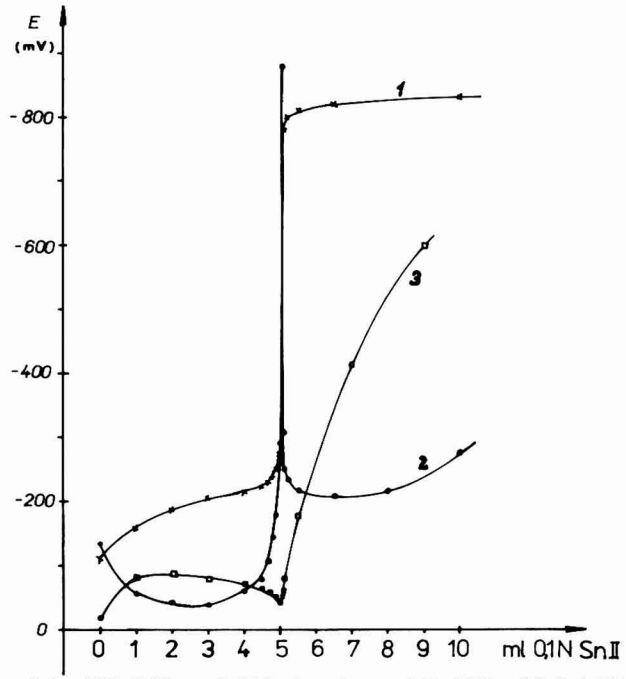

FIG. 2. Course of the EP, BiP, and BiA titrations of Cr(IV) with 0.1 *N* Sn(II)–glyc. in sat. Na_2CO_3: 45 ml of sat. Na_2CO_3 + 5 ml 0.1 *N* $K_2Cr_2O_7$, inert atmosphere. (1) EP, (2) BiP, (3) BiA indication.

DISCUSSION OF RESULTS

Contrary to the literature data, the "stabilization" of a standard solution of tin(II) chloride in glycerol and ethanol media results in increased formal redox potential values E_f^{φ}. Thus the tin salt complexed in glycerol is a much weaker reducing agent than tin(II) chloride in acid medium. This follows not only from the relatively high stability of the Sn(II)–glyc standard solutions in the air but also from the fact that this reagent can be used to reduce quantitatively only a few oxidizing agents. A certain disadvantage is also the possibility of simultaneous oxidation of glycerol. It is apparent from the dependence of the titer on the pH that the optimum medium for the titration is a saturated sodium carbonate solution. At pH values below 11.6 the results are accompanied by a negative error, at higher pH value by a positive error. Titrations with a standard Sn(II)–glyc solution are not disturbed by complexing agents such as glycerol, tartaric acid, citric acid, EDTA, etc. provided that these substances do not take part either in the reduction reaction with the determined substance or do not unfavorably affect the E_f^{φ} value of the system by a further reaction with formation of a complex (Sn(IV)/Sn(II)). The

titration can be carried out in air assuming that the analyzed solution is freed of oxygen before the reaction. Provided that the reaction is complete within 30 min, the titration determination error is +1% as a result of the "noninertness" of the atmosphere. The standard deviation value is not higher than 0.10.

The titer determination can be reliably carried out in saturated sodium carbonate medium using potassium dichromate or potassium hexacyanoferrate in an inert nitrogen atmosphere. For back titration of Sn(II)–glyc in saturated sodium carbonate with 0.1 *N* potassium dichromate the determination error was −1%, apparently as a result of imperfect inertness of the atmosphere. The high viscosity of the Sn(II)–glyc solution makes it preferable to use a piston burette.

The voltammetric and polarographic studies indicated that the Sn(IV)–glyc/Sn(II)–glyc system behaves irreversibly. Thus the equivalence point was indicated both by equilibrium potentiometry and by bipotentiometry and, in some cases, by biamperometry.

In this way volumetric determinations of potassium dichromate, potassium hexacyanoferrate, iodine, chloramine-T, and calcium chlorate, hydrogen peroxide, some inorganic peroxides, and silver were studied and developed.

The carbonate complexes of Ce^{4+}, MoO_4^{2-}, VO_3^-, WO_4^{2-}, and tungstosilicate react with Sn(II)–glyc but the reaction is very slow even at increased temperatures. The reaction was not quantitative even after adding large amounts of Sn(II)–glyc in excess. This is also true for Fe^{3+}, MnO_4^-, BrO_3^-, IO_3^-, ClO_2^-, SeO_4^-, and SeO_3^{2-}. The behavior of divalent copper (Cu(II)citrate) and BiY^- is interesting as they seem to be quantitatively reduced to the metal. However, attainment of the equivalence point in both direct and indirect titration with an Sn(II)–glyc solution is not accompanied by a change in the electrode potential. TeO_4^{2-} and TeO_3^{2-} are also reduced to the metal. Solutions of Ir(IV) and Pd(II) react with glycerol (Ir(IV) → Ir(III), Pd(II) → Pd). The course of these reactions and the possibility of reducing Fe(III) in the presence of dimethylglyoxime will be studied further. Table 2 gives a list of substances determined for which quantitative reaction was achieved.

In conclusion, it can be stated that assumptions on the stabilization of Sn(II) solutions in glycerol while maintaining the same E_f^0 value as in acid medium are unfounded. It follows from the experiments carried out so far that the Sn(II)–glyc solution belongs among medium strong relatively stable reducing agents and will probably find application in the determination of some organic substances and in organic preparative chemistry.

SUMMARY

The preparation of standard Sn(II) solutions in glycerol and ethanol media was studied and the most suitable conditions were found for their standardization using dichromate and

potassium hexacyanoferrate. Further, the long-term stability of standard Sn(II)–glyc solutions was studied and it was found that the titer of 0.1 *N* Sn(II)–glyc does not change in an inert atmosphere over a period of 4 months. The solution undergoes partial oxidation in the air (Fig. 1). It was demonstrated polarographically and voltammetrically that the studied redox system is irreversible. The formal redox potential value $E_f^0 = 0.80$ V (SCE) 0.1 *N* Sn(II)–glyc can be back-titrated with 0.1 *N* $K_2Cr_2O_7$ in a sat. Na_2CO_3 solution under an inert atmosphere. Generally it is possible in sat. Na_2CO_3 medium to determine with great precision dichromate and potassium hexacyanoferrate, iodine, chloramine-T, chlorine lime, hydrogen peroxide, inorganic peroxides, and silver (Table 2). Equilibrium potentiometry, bipotentiometry, and biamperometry can be used for indication of the equivalence point.

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A Highly Sensitive Spectrophotometric Determination of Gallium with Chromal Blue G in the Presence of Cetyltrimethylammonium Chloride

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INTRODUCTION

Recently, some spectrophotometric methods for metals based on the formation of a ternary complex on a micelle surface formed in the presence of a quaternary ammonium salt have been suggested (*1*, *2*, *5*, *6*, *8*). Continuing the study on the sensitizing effect of a quaternary ammonium salt on the reaction of chromal blue G (CBG) with various metal ions (*12–14*), it was found that microgram amounts of gallium react very sensitively with CBG in the presence of cetyltrimethylammonium chloride (CTMA). Chromal blue G (sodium-2″-chloro-4″-nitro-4′-hydroxy-3,3′-dimethylfuchsone-5,5′-dicarboxylate, Color Index 43835), a triphenylmethane dye, has been used as a spectrophotometric reagent for scandium (*10*), beryllium (*11*), and palladium (*12*).

This paper describes a highly sensitive method for the determination of gallium based on the formation of a colored complex of gallium with CBG in the presence of CTMA. Several conditions under which microgram amounts of gallium can be determined, influence of diverse ions, and the composition of the complex are discussed.

EXPERIMENTAL

Reagents and Apparatus

Standard gallium solution. A stock solution of gallium was prepared by dissolving 0.9899 g of gallium metal (99.999% pure) in diluted hydrochloric acid and then diluting it with distilled water to 1 liter (1.42×10^{-2} *M*). The concentration of hydrochloric acid in this solution was 1 *M*. This standard stock solution was diluted further as required.

CBG solution. The dyestuff (CBG) was purified by recrystallization from ethanol before use, and an aqueous 0.1% solution was prepared.

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CTMA solution. The CTMA solution (3.0×10^{-3} *M*) was prepared by dissolving 0.960 g of it in 1 liter of distilled water.

Buffer solution (pH 6.5). A buffer solution of pH 6.5 was prepared by mixing 0.2 *M* sodium hydroxide solution and 0.2 *M* potassium dihydrogenphosphate solution.

All the other chemicals used were of a guaranteed reagent quality.

The absorbance measurements were made with a Hitachi automatic recording digital spectrophotometer, model 624, with matched 1.00-cm quartz cells. The pH measurements were made with a Hitachi–Horiba glass-electrode pH meter, model F-7LC.

Standard Procedure

Transfer the sample solution containing 1–15 μg of gallium to a 50-ml Erlenmeyer flask. Add 0.5 ml of the CTMA solution, 1.0 ml of the CBG solution, 5 ml of the buffer solution, and a small amount of water to make the volume about 20 ml. Heat the solution in a water-bath at 60–70°C for 10 min. After cooling to room temperature, transfer to a 25-ml volumetric flask, dilute to the mark with distilled water, mix, and measure the absorbance of the solution at 662 nm against a reagent blank obtained in the same way.

RESULTS AND DISCUSSION

Absorption Spectra

Absorption spectra of CBG and its gallium complex are shown in Fig. 1. Curves A and B show the absorption spectra of CBG and the binary complex obtained with gallium at pH 6.5, respectively, while curves C

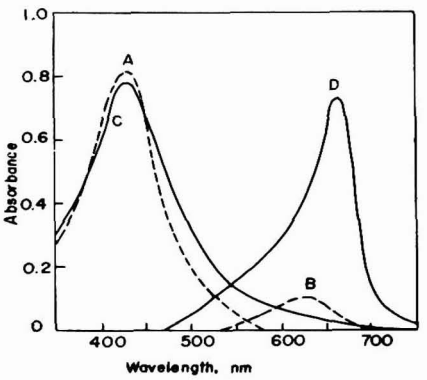

FIG. 1. Absorption spectra of CBG and its gallium complex at pH 6.5. (A) Reagent blank of (B), reference:water. (B) Ga–CBG complex (Ga. 1.1×10^{-5} *M*, CBG 8.6×10^{-5} *M*), reference: reagent blank. (C) Reagent blank of (D), reference:water. (D) Ga–CBG complex (Ga. 5.1×10^{-6} *M*, CBG 8.6×10^{-5} *M*, CTMA 6.0×10^{-5} *M*), reference: reagent blank.

and D show the effect of the addition of CTMA to these solutions at the same pH value. The formation of the ternary complex is accompanied by a marked increase in the absorbance and a bathochromic shift in the maximal absorption of the complex from 631 to 662 nm.

The spectra of the complex at various pH values are shown in Fig. 2. The maximal absorbance of the complex is found at 662 nm above pH 5.9, and at shorter wavelength at lower pH.

Effect of pH on Color Development

The effect of the pH on the color development of the complex was examined by measuring the absorbance of the colored solutions at 662 nm. The results are shown in Fig. 3, from which it can be seen that the maximal absorbance can be obtained in the pH range from 6.2 to 6.8. A sodium hydroxide–potassium dihydrogenphosphate buffer solution, pH 6.5, was found to be satisfactory for this purpose.

Effect of CBG Concentration

The effect of varying the concentration of CBG on the color development was examined by measuring the absorbance of solutions kept at constant concentration of gallium and CTMA. Addition of 1.0 ml of a 0.1% CBG solution in a final volume of 25 ml sufficed for less than 0.6 ppm of gallium.

Effect of CTMA Concentration

The effect of changes in the concentration of CTMA on the absorbance was determined. The maximal absorbance can be obtained over the range $(3.0-9.0) \times 10^{-5}$ *M* of CTMA. The optimum amount of CTMA was 0.5 ml of a 3.0×10^{-3} *M* CTMA solution in a final volume of 25 ml.

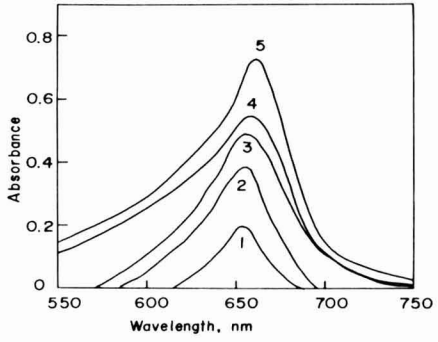

FIG. 2. Absorption spectra of gallium complex at various pH. Ga 5.1×10^{-6} *M*, CBG 8.6×10^{-5} *M*, CTMA 6.0×10^{-5} *M*, reference: reagent blank; pH:(1) 3.5, (2) 5.0, (3) 5.5, (4) 7.0, (5) 6.5.

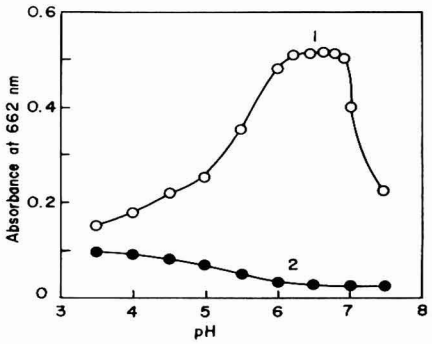

FIG. 3. Effect of pH. (1) Ga–CBG complex (Ga 3.6×10^{-5} *M*, CBG 8.6×10^{-5} *M*, CTMA 6.0×10^{-5} *M*), reference: reagent blank. (2) Reagent blank, reference:water.

Rate of Color Development

Figure 4 shows the color development curves for the heated and unheated solutions of the complex. At room temperature (20°C), the color of the complex develops gradually; the full color is obtained in 2 hr after preparation. When a prepared solution is heated in a waterbath at 60–70°C, however, the reaction is greatly accelerated, and the maximal color development is obtained in 10 min. The absorbance is stable for 24 hr.

Calibration Curve, Sensitivity, and Reproducibility

A calibration curve for the gallium determination was prepared by the standard procedure. Beer's law was obeyed over the range of 1–15 μg of gallium in 25 ml of the solution. The color reaction has a molar absorptivity of 1.44×10^5 liter mol^{-1} cm^{-1} at 662 nm, and the spectrophotometric sensitivity was estimated to be 4.8×10^{-4} μg Ga cm^{-2}, corresponding to $\log I_0/I = 0.001$.

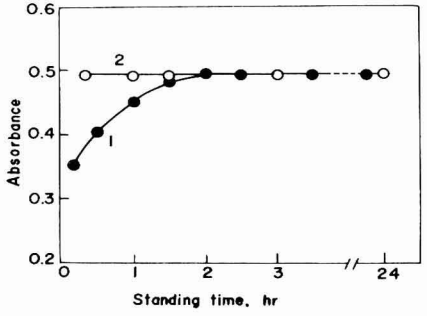

FIG. 4. Effect of standing time. Ga 3.5×10^{-6} *M*, CBG 8.6×10^{-5} *M*, CTMA 6.0×10^{-5} *M*, Ph 6.5, reference: reagent blank; (1) unheated solution, (2) heated solution (60–70°C).

The present method is very sensitive. Its sensitivity is much greater than that of the method based on Xylenol orange (molar absorptivity, $\epsilon = 2.29 \times 10^4$) (*7*), PAR ($\epsilon = 1.02 \times 10^5$) (*3*), Eriochrome cyanine R ($\epsilon = 2.0 \times 10^4$) (*4*), and Chromeazurol S and CTMA ($\epsilon = 1.15 \times 10^5$) (*9*).

A standard solution containing 5 μg of gallium was analyzed 10 times by the standard procedure; the average absorbance was 0.413 with a standard deviation of 0.002 absorbance unit and a relative error of $\pm 0.8\%$.

Effect of Diverse Ions

The influence of diverse ions on the determination of gallium was examined under the conditions of the standard procedure. The most commonly encountered ions were added individually to a solution containing 6.2 μg of gallium. The following metal ions did not interfere up to 30-fold weight excess: barium (II), calcium(II), cadmium(II), cobalt(II), lead(II), magnesium(II), manganese(II), mercury(II), nickel(II), palladium(II), scandium(II), thorium(IV), uranium(VI), and zinc(II). Aluminum(III), beryllium(II), copper(II), and iron(III) interfere seriously. Chloride, acetate, nitrate, fluoride, sulfate, and phosphate are without effect even in large amounts. Fluoride effectively masks the more seriously offending ions, aluminum(III) and beryllium(II). However the influence of copper(II) and iron(III) cannot be eliminated by the addition of fluoride. Therefore, these ions must be separated by suitable procedure.

Complex Formation

The method of the continuous variation was employed to establish the stoichiometry of the complex in the presence of CTMA. The constant concentration of CTMA was 6.0×10^{-5} *M*, and measurements were made at pH 6.5 and 680 nm. The results indicated that a 1:4 complex between gallium and CBG is formed in the presence of CTMA, and a 1:3 complex in the absence of CTMA. These results were confirmed by the mole-ratio method. From the data obtained the formation constant for the complex was estimated to be 1.4×10^{12} for the 1:4 complex.

SUMMARY

A new spectrophotometric method for the determination of gallium with chromal blue G in the presence of cetyltrimethylammonium chloride is described. The sensitivity of color reaction between gallium and chromal blue G is greatly increased by the sensitizing action of cetyltrimethylammonium chloride. The gallium complex has maximal absorbance at 662 nm and pH 6.2–6.8. Beer's law is obeyed over the range 0.04–0.6 ppm of gallium. The molar absorptivity of the complex is 1.44×10^5 liters mol^{-1} cm^{-1} at 662 nm, and the spectrophotometric sensitivity is 4.8×10^{-4} μg Ga cm^{-2}. The mole ratio of gallium and chromal blue G in the complex is estimated to be 1:4. The formation constant and effect of interfering ions are described.

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Separation of Monofunctional Isomeric Naphthalene Derivatives by Means of Liquid Chromatography

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INTRODUCTION

Separation of isomeric organic compounds is an important analytical and industrial problem to be resolved chromatographically. A number of papers (*1–4, 6, 7*) give evidence to the fact that liquid along with gas and thin-layer chromatography enables separation of the selected structural and stereoisomers. Particularly valuable are those chromatographic separations which provide the relative order of washing out the analyzed isomers unchanged. This can contribute to the successful identification of the discussed isomers.

In this paper we present separation of the selected alpha and beta monofunctional naphthalene derivatives by means of liquid chromatography. An additional effort was undertaken to explain their relative chromatographic sequence.

EXPERIMENTAL

Investigations presented in this paper deal with the selected monofunctional derivatives of naphthalene, which are listed in Table 1. Separation of substances was performed with the help of a liquid chromatograph produced by Laboratorni Pristroje (Czechoslovakia).

A. Sorbent: SEPARON SE. The column length: 250 mm; temperature: 20°C; mobile phase: *n*-heptane + 0.05% isopropanol (v/v); the mobile phase flow rate: 1 cm^3/min; detector: UV at 254 nm.

B. Sorbent: SEPARON SIVSK. The column length: 250 mm; temperature: 20°C; mobile phase: *n*-hexane + chloroform + methanol = 35:15:4 (v/v/v); the mobile phase flow rate: 1 cm^3/min; detector: UV at 254 nm.

DISCUSSION

The results of chromatographic separations are given in Table 1. Some exemplary chromatograms obtained with the B working conditions are given in Figs. 1a–d.

As revealed from the presented data, retention times of those naphthalene derivatives with the functional groups substituted in the β

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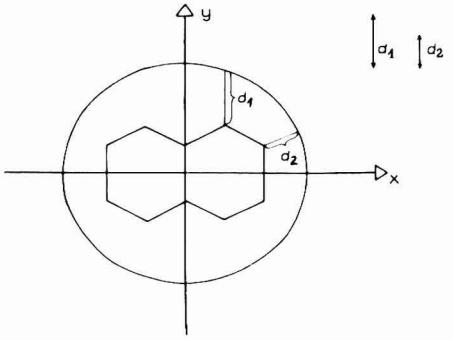
TABLE 1
SEPARATION OF THE SELECTED MONOFUNCTIONAL ISOMERIC NAPHTHALENE DERIVATIVES BY MEANS OF LIQUID CHROMATOGRAPHY

Number	Compound	Isomer (position)	Retention time	
			A	B
1	Naphthoic aldehyde	1	6 min	3 min
		2	8 min	4 min
2	Naphthol	1	7 min 50 sec	7 min 55 sec
		2	9 min 20 sec	8 min 20 sec
3	Naphthoic acid	1	20 min	13 min
		2	20 min	14 min 15 sec
4	Naphthylamine	1	15 min	9 min
		2	15 min	9 min 50 sec
5	Acetylnaphthalene	1	8 min 10 sec	5 min
		2	10 min	5 min
6	Bromonaphthalene	1	5 min 10 sec	6 min 10 sec
		2	5 min 10 sec	6 min 10 sec

position always surpass the corresponding number values of the α-isomers. This fact has to do both with the spacial structure of the discussed isomers and with their dipole moment values.

Considering the spacial structure of naphthalene it ought to be stressed that the hydrogen atom in β position (and analogously a substituted functional group) appears in a more favorable situation to interact with the sorbent layer than its α counterpart. Certain support to this statement can be given by means of the following scheme presenting the naphthalene molecule (placed in the middle of a circle upon a system of two coordinates).



SCHEME 1. The naphthalene molecule with the marked distances (d_1 and d_2) between the hydrogen atoms and the chromatographic sorbent surface.

The above presented scheme makes evident that $d_1 > d_2$, which explains a greater tendency of the β isomer to adsorb upon the sorbent layer.

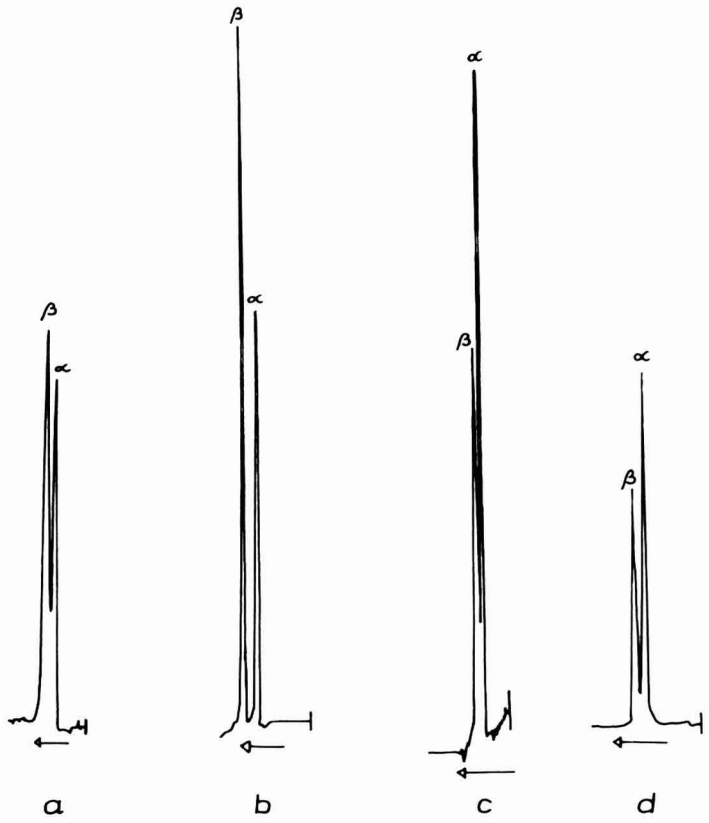


FIG. 1. The chromatographic separation of the following monofunctional isomeric naphthalene derivatives: (a) α- and β-naphthol; (b) α- and β-naphthoic aldehyde; (c) α- and β-naphthylamine; (d) α- and β-naphthoic acid.

As seen from literature (*5*), all the dipole moment values are greater with the β monofunctional naphthalene derivatives than with the α ones.

Lack of satisfactory separation in the case of α- and β-bromonaphthalene shows that the applied A and B working conditions are insufficient to provide the positive chromatographic result. Nevertheless it can be assumed that with the changed chromatographic conditions the relative retention time of the β isomer should surpass that of the α isomer.

On the basis of the presented results it can be concluded that the relative retention times of the α and β monofunctional naphthalene derivatives depend mainly upon their spacial structure and the dipole moment values.

The differentiated retention times of the α and β isomers with the latter ones significantly higher enable good analytical conditions to perform identification of the discussed substances by means of liquid chromatography.

SUMMARY

An effort was undertaken to establish principal regularities enabling liquid chromatographic identification of the selected monofunctional isomeric naphthalene derivatives. An explanation was offered taking advantage both of the structural and the dipole moment characteristics, aiming to ground a significant differentiation between the chromatographic behavior of the α and β isomer series.

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Erratum

Volume 25, No. 4 (1980), in the article, "A Sensitive Reaction for Dilute Cholesterol Determinations," by J. D. Artiss and B. Zak, pp. 535–542: Page 535, line 13, the word "determinant" should read "determinand."