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REVIEW

APPLICATIONS OF POLYACRYLAMIDE GEL ELECTROPHORESIS AND POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING IN CLINICAL CHEMISTRY

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CONTENTS

1. BACKGROUND

Fractionation of plasma proteins has had two major goals. The first has been to understand the function and structure of blood plasma, body fluids, and tissue proteins, and the second to use this knowledge in diagnosis and therapy in the field of clinical medicine. These two areas of endeavor began with the first efforts of Howe [1] in 1921 to separate plasma proteins by means of salting-out techniques. This was followed by the development of free-boundary electrophoresis by Tiselius [2] and of paper zone electrophoresis by Konig [3] in 1937, followed by the improvements of Durrum [4]. This led to the first practical use of boundary and zone electrophoresis in both research and clinical laboratories. The development of cellulose acetate electrophoresis by Kohn [5] in 1957 provided a practical, simple method of serum protein separation for the clinical laboratory with a resultant proliferation of use and development that continues to grow, even today. These techniques, however, as routinely practiced resolve only 5—10 of hundreds of plasma protein components present.

The immunoelectrophoretic techniques of Grabar and Williams [6] combining immunodiffusion and agar gel electrophoresis provided a method where 20—30 fractions could readily be demonstrated, thus, markedly expanding the qualitative information obtainable with procedures that could readily be carried out by clinical laboratory personnel.

A number of excellent books and monographs have been published which fully describe the plasma protein changes in many disease states. The reader is referred especially to the more comprehensive works in this area [7—12] for background reading.

The development of high-resolution starch gel electrophoresis by Smithies in 1955 [13] and the moving boundary buffer system on starch gel by Poulik in 1957 [14], followed a year later by the development of the zymogram technique by Hunter and Burstone [15] for characterizing multiple molecular forms of enzymes, provided a method of greater resolution for in-depth qualitative studies of plasma and tissue proteins, and enzymes. Because of technical complexity these procedures became more useful in research areas and did not find ready clinical applications. They have been utilized widely by geneticists, and have provided much of the basis for the further studies on genetic polymorphism. These advances were followed by the development of polyacrylamide gel electrophoresis first reported by Raymond and Weintraub in 1959 [16].

The purpose of this chapter is to present an overview of the application

of polyacrylamide gel systems in the field of clinical medicine. Toward this aim the material is separated into the areas of polyacrylamide gel electrophoresis (PAGE) and polyacrylamide gel isoelectric focusing (PAGIF). Material in each section is presented in terms of the familiar serum protein patterns, followed by other body fluids and tissue proteins. Histochemical procedures for the enzymes of clinical importance are similar in both PAGE and PAGIF and thus are combined and presented in tabular form.

2. PROTEIN SEPARATION IN POLYACRYLAMIDE GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL

As noted previously, polyacrylamide gel electrophoresis was introduced by Raymond and Weintraub in 1959 [16]. Ornstein and Davis in 1962 [17] greatly advanced resolution in acrylamide with the development of the disc technique. They utilized a discontinuous buffer and pH system designed to produce initial zone stacking between a leading and trailing buffer ion which resulted in a concentration effect and narrow starting zones. The procedure has been employed both in the originally described gel cylinders and on flat gel slabs. This system is hereafter designated as system one. Allen et al. [18] developed a discontinuous buffer and ionic strength system at a continuous pH in flat slab pore gradient gels which produced thin starting zones without a sample or stacking gel to provide greater flexibility, particularly for isoenzyme studies. This system is hereafter designated as system two. These two systems have been widely used for both clinical and research applications. Computer derived discontinuous buffer systems to produce selective stacking and unstacking were developed by Jovin [19, 20] to provide an optimal resolution in the separation of components of similar size and charge. Separation based directly on molecular weight differences was developed by Maizel [21], who treated samples with sodium dodecyl sulfate (SDS), to produce a unit charge on each of the macromolecules present, allowing them to separate in continuous or gradient pore size gels based solely on molecular weight.

The first serum patterns in PAGE [16, 17] had the initial effect of implying that it was a high-resolution technique. However, the resolution of multicomponent systems, containing several hundred components, such as serum, remains unsolved with this technique. Improvements between 1969 and 1973 with various multiphasic buffer systems [18—20, 22], gel gradient strategies and the theoretical considerations developed, particularly those presented by Robard and Chrambach [23] and Kapadia et al. [24] clearly demonstrate that PAGE is limited in its resolving capability to some 30—35 serum proteins.

The development of synthetic polyamino carboxylic ampholytes by Vesterberg in 1967 [25] and polyamino sulfonic and carboxylic ampholytes by Pogacar and Jarecki [26] in 1974 and Grubhofer [27] in 1972 provided a new approach to protein separation, that of isoelectric focusing. This procedure is, for all practical purposes, an electrophoretic equilibrium method for separating proteins according to their isoelectric point and offers unique advantages for both preparative and analytical methods. By utilizing various two-

dimensional gel methods further resolution may be obtained. For example, by coupling isoelectric focusing in the first dimension with SDS electrophoresis in the second dimension O'Farrell [28] was able to increase resolution of the plasma proteins, identifying up to 1000 radioactively labelled serum proteins in a single specimen. Obviously, the ability to increase resolution of a mixture of proteins by over two orders of magnitude in recent years has not as yet even been partially exploited in the field of clinical medicine. Such an information explosion has perhaps, rather led to considerable perplexity and a wait-and-see attitude by many clinical laboratory workers to determine the practical potential of this tool in clinical diagnosis and therapy control.

Current literature seems to indicate that as PAGE replaced starch gel electrophoresis, PAGIF has already replaced PAGE in many applications as the method of choice for the separation and isolation of complex mixtures of proteins.

For clinical applications it should be stressed that no single set of conditions or procedures has yet been found optimal for all protein and isozyme separations. The potential use of PAGE must be considered and compared with the more recent developments with PAGIF, to select an optimal system for a given task.

For both methods, a rigorous standardization of all steps involved in the practical performance of the technique is a major prerequisite for utilization of their full potential as diagnostic tools. It is essential to adopt a standard set of conditions for sample preparation and handling, gel purification, polymerization, separation conditions, staining, and quantitative evaluation. Pattern deviations due to methodological effects must be minimized to achieve an acceptable level of reliability and reproducibility. A brief review of some of the more pertinent points is, therefore, appropriate before presenting specific applications of these methods in clinical medicine.

3. STANDARDIZATION OF SAMPLE COLLECTION AND HANDLING

Blood sample collection and handling procedures in each hospital are usually processed on a strict routine and this area might not seem to warrant comment; however, it should be emphasized that the higher the resolution techniques, the more minor alterations resulting from handling and processing inconsistencies will be magnified, leading to greater variation in results. Many of the tests most applicable to these techniques, thus, must be handled as non-routine, special chemistries. Precautions must be taken to assure that the sample arrives at the laboratory as soon as possible after collection.

Serum should be drawn from the blood samples immediately after clotting and the clot may be best allowed to contract in the cold. Plasma for enzymes, clotting factors, and lipoproteins should be obtained from chilled blood samples and run as soon as possible, or stored frozen at $-70°$, with the exception of samples for lipoprotein analysis, which may be stored at $4°$ for not more than 48 h. On the other hand, hemoglobin samples may be treated with potassium cyanide and glycerol and held at $-20°$ for subsequent analysis for periods of two to three weeks without affecting separation patterns quali-

tatively or quantitatively. Urine may be concentrated by freeze dialysis and then stored at −70°. Long-term storage of samples for future reference, or for comparative serial studies, should be made in aliquots to avoid repeated freezing and thawing and again held at −70° or lower.

PAGE systems employing electrochemical conditions where the sample is photopolymerized in a sample gel at pH 6.8 may result in the denaturation of isoenzymes [29] or provide a final pH environment below the isoelectric point for some of the protein macromolecules of interest. In the latter instance, these will be trapped in the opaque sample gel and not migrate. Lactic dehydrogenase isoenzyme 5 is a prime example of this problem. Maintaining the sample prior to and during the initial phase of electrophoresis at pH 9.0 may be necessary not only to assure that the majority of proteins are above their p*I* but also to prevent precipitation of proteins in complex mixtures such as tissue extracts and body fluids [30]. PAGE system two lends itself to this requirement since the system operates at a continuous pH of 9.0 and requires no stacking or sample gel. Adjustment of sample pH to 9.0 prior to PAGIF also may be made and will not affect resultant resolutions.

4. STANDARDIZATION OF APPARATUS AND OPERATIONS

The usual clinical requirement for the ability to process a number of samples and controls simultaneously favors the choice of equipment which minimizes the risk of apparatus-inherent complex manipulations. Equipment designed to produce gel slabs offers a number of advantages over that designed for separation in cylindrical gels, or gel rods.

(i) On flat slabs many samples, e.g., 12—30 with PAGE apparatus and up to 60 with PAGIF systems, can be applied side by side and separated under identical conditions. Standards may be included on the same gel for exact side-by-side comparison and to act as internal control checks.

(ii) Flat slabs, as normally used, vary from 0.5 mm to 3 mm thick and have more efficient dissipation of the Joule heat produced during separation than the usually employed 5—6 mm diameter cylindrical or rod gels. (The use of capillary tube gels is not considered in this context since they have a very limited, practical application in the clinical laboratory.)

(iii) Due to their rectangular cross-section, flat gels are better evaluated by quantitative microdensitometry with a much decreased risk of optical artifacts.

(iv) Flat gels allow two-dimensional analysis where a combination of techniques such as cellulose acetate—PAGE or PAGIF, or PAGIF—SDS may be used for expanded resolution in analytical studies and in addition these dry more easily for storage or autoradiography.

(v) Less preparation time is needed for multisample analysis and for both PAGE and PAGIF prepackaged gels of various types and pH ranges are now commercially available..

5. STANDARDIZATION OF GEL FORMATION AND SEPARATION CONDITIONS

A number of aspects for the control required to achieve reproducible gels

prepared "in-house" have been reported [31]. Reagent purification and polymerization conditions have been reported by Allen et al. [29] and Chrambach and Robard [32]. Catalysts and polymer cross-linking concentration effects have been discussed by Watkins and Miller [33].

Constant power application provides the most efficient and controllable form of power to effect separations in both methods. However, the high voltage gradients required to focus proteins optimally in PAGIF are well beyond the capabilities of earlier 500V constant power supplies and require a device similar to that recently developed by LKB capable of delivering 2000 V.

Normally, PAGE and PAGIF systems utilize glass plates as a support system for the polyacrylamide gel slabs, thus limiting heat transfer due to the characteristics of glass. Cooling efficiency can be increased over 200-fold by substituting ceramic plates such as beryllium oxide (Berlox K-150) for glass to improve heat transfer as described by Allen et al. [34]. Shorter run times and higher voltage gradients may thus be achieved without heat denaturation of labile proteins.

6. POLYACRYLAMIDE GEL ELECTROPHORESIS: APPLICATIONS IN CLINICAL MEDICINE

6.1. Plasma proteins

Both systems one and two have been extensively utilized to study plasma protein changes effected by a variety of diseases and physiological stresses. Hoffmeister [35] has standardized method one and has studied over 10,000 patient samples with the technique. Densitometric traces of serum proteins separable by the two methods are shown for orientation and comparative purposes in Figs. 1 and 2, and the proteins separable and quantifiable by these methods that are important in disease in Table 1.

6.1.1. Immunoglobulins

A number of reports has been published concerning the differentiation of the myeloma proteins and Waldenströms macroglobulinemia [36—39]. Normally, it requires considerable experience to detect unequivocally abnormal proteins in the immunoglobulin region, particularly if the immunoprotein concentration is in the same range as neighboring normal proteins, or if the patient belongs to an unusual haptoglobin type. It has been demonstrated also with method one by post-electrophoretic immunoprecipitation that certain immunoglobulin components with an isoelectric point above 8.5 remain in the turbid stacking gel [39], which is generally discarded. Therefore, with this technique a myeloma protein could be missed, which is an intolerable risk for the clinical chemist. Use of a clear sample gel, or system two, obviates this problem and even those proteins which migrate cathodically at pH 9.0 are well defined in the clear cap gel region [40]. On the other hand, the concentrating feature of PAGE, with both systems one and two, allows the detection of very low paraprotein concentrations, particularly in type 1—1 haptoglobin serum, and has detected paraproteinemia in cases with a

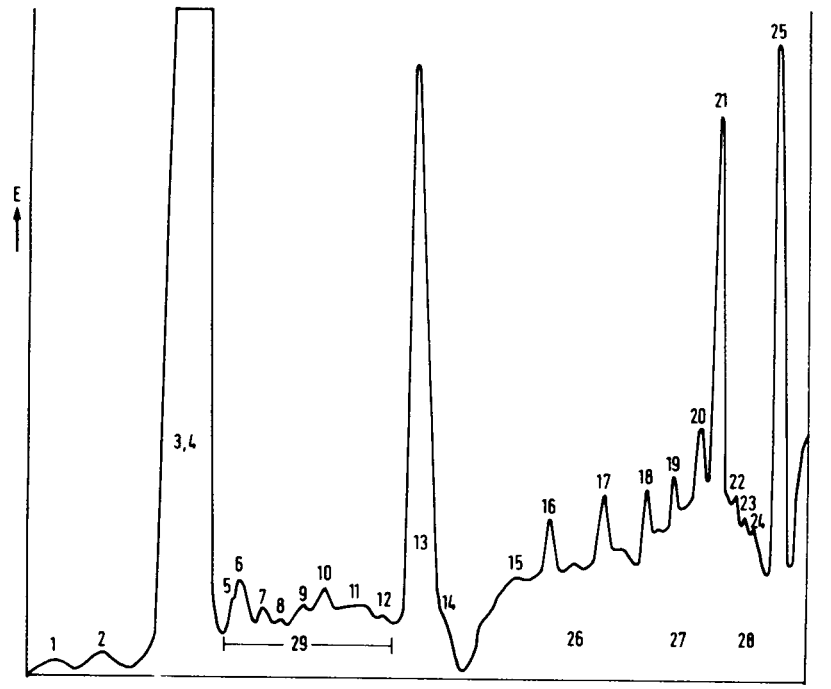

Fig. 1. A densitometric trace of the most important serum proteins separated by PAGE system one. 1, Prealbumin; 2, acid α_1-glycoprotein; 3, albumin; 4, α_1-antitrypsin; 5,7, Gc-globulins; 6, α_1 HS-glycoprotein, α_1-antichymotrypsin; 8, unknown; 9, unknown; 10, ceruloplasmin; 11, unknown; 12, hemopexin; 13, transferrin; 14, inter-α_1 trypsin inhibitor; 15, β_1-A-globulin; 16—19, haptoglobin polymers type 2—2; 20, β-glycoprotein; 21, α_2-macroglobulin; 22—24, haptoglobin polymers type 2—2; 25, β-lipoprotein; 26, I_gA; 27, I_gG; 28, I_gM; 29, α_1-lipoprotein. (After Hoffmeister [35].)

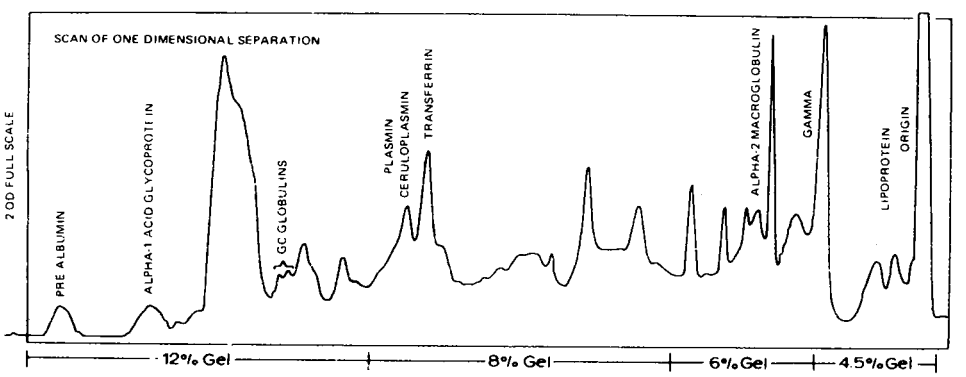

Fig. 2. A densitometric trace of a 2—2 haptoglobin-type serum separated by system two on 4.5—6—8—12% pore-size gradient at a continuous pH of 9.0 using a discontinuous sulfate—borate buffer system. The schematic of this separation is shown in Fig. 8.

TABLE I

HUMAN SERUM PROTEINS SEPARATED BY PAGE

Protein	Biological function	Importance in diagnosis
Acid		Increase: neoplasms, inflammations, rheumatoid arthritis
α_1-Glycoprotein	?	Decrease: liver diseases
Prealbumin	Thyroxine-binding globulin, retinol-binding globulin	Decrease: liver diseases
Albumin	Osmot. function, protein pool, binding of ions, dyes, etc.	Decrease: kidney diseases, neoplasms, liver diseases, chron. inflammations
α_1-Antitrypsin	Proteinase-inhibitor	Increase: inflammations liver diseases
Gc-Globulin	Three genetic types	Decrease: liver diseases
Ceruloplasmin	Copper-binding globulin, oxidase	Increase: pregnancy, neoplasms Decrease: Morbus Wilson
C-Reactive protein	Phagocytosis promoting activity	Increase: inflammatory conditions
Hemopexin	Heme-binding	Decrease: hemolytic anemias
Transferrin	Iron-binding	Decrease: neoplasms, inflammations, paraproteinemias, nephrosis
β_1 A-Globulin	Complement-factor	Increase: infections, toxoplasmosis Decrease: autoimmune diseases
Haptoglobins	Binding of hemoglobin three genetic types	Increase: inflammations, neoplasms, infections, PCP, nephrosis
Type 1—1		Decrease: liver diseases
Type 2—1		
Type 2—2		
α_2-Macroglobulin	Proteinase-inhibitor	Increase: liver diseases, diabetes, nephrosis
α-Lipoprotein HDL	Transportation of cholesterol	Increase: lipid metabolism disturbances Decrease: liver diseases, Tangier disease
Pre-β-lipoprotein LDL	Transportation of lipids	Increase: type-3,4 hyperlipoproteinemia
β-Lipoprotein LDL	Transportation of lipids	Increase: nephrosis, type-2 hyperlipoproteinemia Decrease: liver diseases
Chylomicron VLDL		Increase: type-1, hyperlipoproteinemia
γA-Globulin	Antibody	Increase: liver diseases, chron. infections, PCP Decrease: paraproteinemias, antibody deficiency syndrome
γG-Globulin	Antibody	Increase: liver diseases, chron. infections, myeloma, PCP Decrease: paraproteinemias, antibody deficiency syndrome
γM-Globulin	Antibody	Increase: chron. infections, macroglobinemia Waldenström, liver diseases

clinical myeloma suspicion, but where a normal immunoelectrophoretic pattern was present [35]. In the hands of a skilled person it is probably the most reliable zone electrophoretic method for early detection of the paraproteinemias. The distinct protein peaks may also reveal whether monoclonal or polyclonal paraproteins are involved.

6.1.2. β-Globulins

β_1 A-Globulin, or C_3, may be found to be decreased in autoimmune diseases, such as lupus erythematosis, and increased in infections, especially toxoplasmosis. These changes may be seen most readily in 1—1 haptoglobin types and/or by staining with PAS. Levels may be verified with immunoelectrodiffusion techniques [41].

Transferrin is very apparent and well separated from ceruloplasmin in system one and two although their relative positions are not the same (see Figs. 1 and 2). Decreases are seen in this protein in nephrosis and malignant neoplasms, especially bronchial carcinoma [35] and in various inflammatory diseases. Quantification of this protein is readily made by densitometric techniques with an average value of 0.222 ± 0.022 g/dl and a reference range of 0.178—0.266 g/dl.

The β_2 lipoproteins may be separated readily in both systems one and two following prestaining with Sudan black B. Both the pre-β, very low density lipoprotein (VLDL) and the β, low density lipoprotein (LDL) may be quantified on these systems. In system one the chylomicron region remains in the opaque sample gel while in system two it will penetrate into the first millimeter of the 3.0% separating gel allowing microdensitometric quantification. The rapid separation time 30—35 min, with visualization during the separation, makes this a very useful and relatively simple technique for the differentiation of the familial hyperlipoproteinemia types 2a, 2b, and 3, and in differentiating type 4 from type 5 [42]. Fig. 3 shows a typical series of patterns obtained using system two.

Hemopexin in system one normally appears adjacent to transferrin, and anodally to ceruloplasmin in system two. While this protein appears as a minor component in serum its decrease, like that of the haptoglobulins, is apparent in hemolytic anemias.

6.1.3. α_2-Globulins

α_2-Macroglobulin, which contains about 5% of the proteinase-inhibiting activity of the serum, is also important in binding hormones. It is strongly increased in liver diseases, diabetes, and in nephrotic syndrome and its clear separation and location in relation to γ-globulin allows ready differentiation of Waldenströms macroglobulinemia from myeloma.

Ceruloplasmin, a copper binding α_2-globulin is markedly increased in malignant neoplasms and in pregnancy, while it is decreased in Wilson's disease. Ceruloplasmin is more readily separable as a discrete zone in system two, employing a gel pore size gradient, than in system one, using a continuous gel pore size.

The haptoglobins are particularly well separated by PAGE techniques. The addition of hemoglobin to a serum sample readily identifies haptoglobin

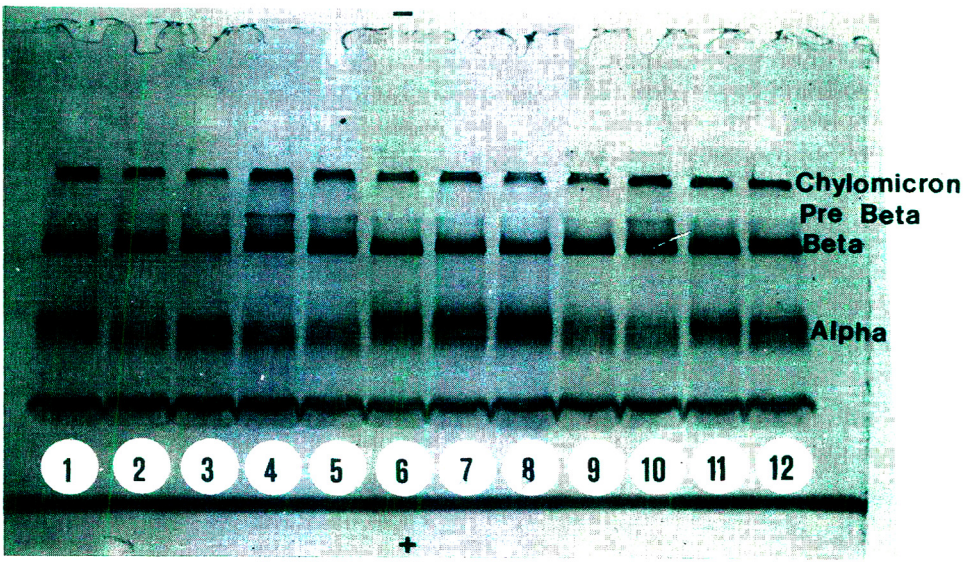

Fig. 3. Separation of pre-stained serum lipoproteins stained with Sudan black B. The separation was carried out in system two on a 3—6—9% T-pore-size gradient at a continuous pH of 9.0 using a discontinuous citrate—borate buffer system. Sample 6, 7, and 8, are female as indicated by the increase HDL level. Samples 1, 2, 3, and 11 show the four typical HDL types seen with this method and sample 4 indicates an increased pre-β band. Albumin is marked with the tracking dye added to the cathodical buffer.

types without staining, although benzidine-peroxide staining as shown in Fig. 4 may be carried out to enhance visualization. Hoffmeister [35] found that haptoglobins were markedly elevated in over 90 confirmed cases of bronchial carcinoma. He also found that tumors in a progressive stage demonstrated a progressive decrease of transferrin and γ-globulin, while tumors of the female genitalia, the stomach, pancreas, and intestine showed less significant change in the haptoglobin levels. Haptoglobins were found to be elevated in primary chronic polyarthritis, the nephrotic syndrome, and toxoplasmosis. In contrast to neoplasms and chronic inflammatory processes, transferrin will remain normal and the γ-globulins increase in the sera of patients with PCP and toxoplasmosis. The latter also show an increase in β_1 A-globulin.

6.1.4. α_1-Globulins

The α_1 high-density lipoproteins (HDL) may be prestained with Sudan black B and separated with both PAGE systems one and two. In system two polymorphisms in the HDL are apparent as in the increased HDL levels in females as illustrated in Fig. 3. In Tangier disease HDL bands are absent. The Gc globulins show three distinct genetic types on both systems one and two. While these proteins are more of an aid to identification in forensic medicine, they do increase in liver diseases. The α_1-acid glycoprotein (orosomucoid) is also well resolved on both PAGE systems and is increased in inflammatory conditions, rheumatoid arthritis and malignant neoplasms.

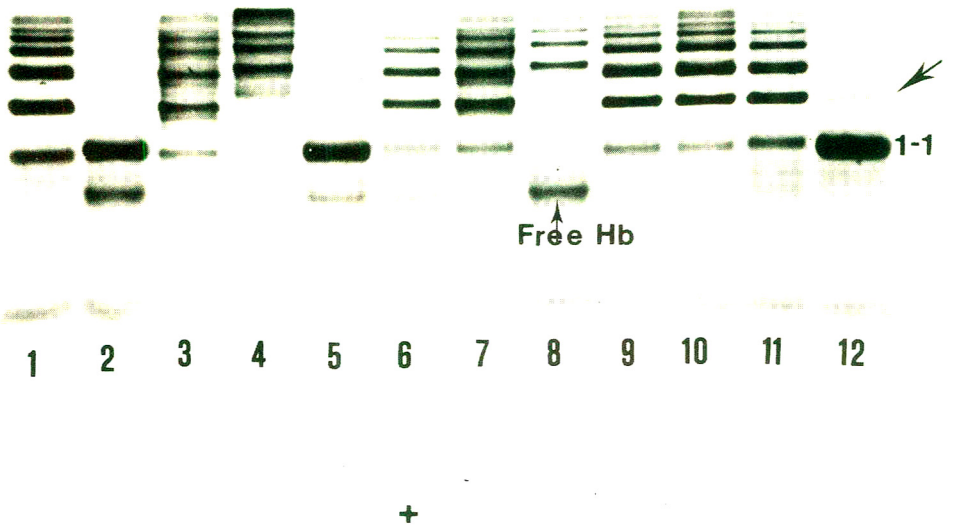

Fig. 4. Serum mixed with hemoglobin and separated on system two in a 4.5—6—8—12% T-pore-size gradient at a continuous pH of 9.0 in a discontinuous citrate—borate buffer system. The separation was stained with benzidine-peroxide. Samples 1, 3, 6, 7, 9, 10, and 11 are haptoglobin type 2—1, samples 4 and 8 are haptoglobin types 2—2, and samples 2, 5, and 12 are haptoglobin types 1—1. The pipette tip was purposely not rinsed or changed between samples 11 and 12 to show sensitivity of carry over of the major 2—2 haptoglobin marked by arrow. Fig. 4 with permission of Ortec.

6.1.5. Albumin

Albumin is not separated in either system from the α_1 proteinase inhibitors (α_1 Pi) unless the gel monomer concentration exceeds 22%. The 50,000 dalton α_1 Pi migrates in routinely used gel strengths with the forward edge of the albumin increasing the albumin value by 3—4% in normal individuals. In Pi-deficient individuals or in normal individuals with inflammatory processes where α_1 Pi is elevated, albumin variations of 5—6% may be due solely to the α_1 overlap. Decreases seen in albumin in kidney diseases, neoplasms, liver disease, and chronic inflammation normally will be readily apparent, although not as a true reflection of the albumin actually present. Prealbumin is resolved well by both systems one and two and shows a concurrent decrease with albumin in liver diseases.

6.2. Proteins of other body fluids

6.2.1. Cerebrospinal fluid

The concentrating effect of both systems one and two is of particular advantage for the analysis of cerebrospinal fluid (CSF) as compared with other zone electrophoretic methods. A clear cut decision can be made with PAGE to determine whether elevated protein levels in the CSF are caused by a

disturbance of the blood—CSF barrier, or by synthesis of proteins within the central nervous system. Disturbance of that barrier will produce an approximation of the CSF pattern to that of the serum pattern. A pattern of CSF showing a breakdown of the blood—brain barrier or blood contamination is characterized by the following alterations [39]:

(i) The high molecular weight proteins are markedly increased, i.e. the α_2-macroglobulin, β-lipoprotein and haptoglobin polymers.

(ii) The low molecular weight proteins like prealbumin constitute a smaller relative proportion of the total protein.

(iii) CSF bands like the third post-albumin became undetectable.

Those diseases, where new proteins are synthesized in the central nervous system become detectable by CSF electrophoretic analysis. They produce a protein profile different from the patient's serum. These include neurosyphilis, multiple sclerosis, subacute sclerosing leucoencephalitis, infectious meningitis, brain tumors, intervertebral disk profusion, cerebral infarction, and Bence-Jones proteins [43, 44].

Takeoka et al. [43] have divided the PAGE CSF-protein pattern into 6 zones based on mobility. These are the prealbumin region, albumin region, the post-albumin region divided into two zones between albumin and transferrin, a post-transferrin zone and a γ-globulin zone. Utilizing this technique, and staining with Amido Schwarz 10-B, normal values of CSF of the prealbumin zone were 10.97 ± 2.31%, albumin zone 40.74 ± 5.72%, A_1 zone 5.25 ± 0.72%, A_1 zone 8.27 ± 1.24%, transferrin 10.82 ± 2.18%, post-transferrin 7.94 ± 1.93% and γ zone 16.05 ± 2.49%.

Several authors have also described the abnormal and pathological CSF protein patterns [45—48] and Papadopoulos and Suter [49] have reported that PAGE furnished useful supplementary information for the differential diagnosis of neuropathies (encephalitis, meningitis, Gullain-Barre syndrome, etc.). Weiss et al. [37] have shown the difference between α_2 and α_{1a} myeloma and macroglobulinemia (Waldenström) using system one.

6.2.2. Urine

Generally urinary proteins need to be concentrated for PAGE, for example by ammonium sulfate precipitation [50], concentration by dialysis, ultrafiltration, lyophilization or benzoic acid adsorption [51]. Anderson [52], on the other hand, has found that freeze dialysis is an excellent way to concentrate urine in order to preserve enzyme activity and to minimize protein denaturation.

Ammonium persulfate precipitation has been employed by Adams-Mayne and Jirgensons [53] to extract Bence-Jones proteins which they then investigated by the use of the acid PAGE at pH 4.3.

Pesce et al. [54] have employed SDS PAGE on concentrated urine samples to differentiate the proteinurias of glomerular and tubular origins based on the molecular weight of the proteins present. Glomerular proteinuria is characterized by the presence of albumin and plasma proteins of a molecular weight larger than albumin, while tubular proteinuria is characterized by albumin and proteins of molecular weights below albumin. Urinary chorionic gonadotropin has been analyzed in urine and followed during the course

of pregnancy by a number of investigators [55—57]. Both chorionic gonadotropin and leuteinizing hormone have been studied by Kaplan et al. [58].

6.2.3. Saliva

Up to 21 different proteins have been identified in parotid and submaxillary saliva by PAGE using system one [59—62]. Differences observed among individuals were found to be due to biological and genetic variation. However, within individuals good reproducibility was found. Salivary amylase may be analyzed by system one [63] and the method is particularly favorable when compared with starch gel, since acrylamide is not hydrolyzed by amylases.

6.2.4. Exudates, transudates, and other body fluids

While a number of studies investigating other body fluids in pathological conditions have been reported using PAGE, these methods have not apparently found any widespread use in the field of clinical chemistry. System one has been used to study synovial, ovarian, and follicular cysts, and ascites in carcinomatous peritonitis [64—66]. System one has also been used to differentiate radicular cysts from granulomas in root canal fluids [67].

6.3. Tissue proteins

Tissue proteins have not been studied extensively by PAGE for clinical purposes, however characteristic patterns of different ferritins in human HeLa and KB cells have been described by Richter [68, 69]. Structural proteins of amyloid fibrils in human amyloides livers have been described [70,71] as has thyroglobin from congenital goitre [72], while Sykes et al. [73] have studied the collagens of human dermis.

A number of enzyme and isoenzyme studies have been made also on a variety of tissue and cell extracts, and on body fluids. These will be listed separately in tabular form for both PAGE and PAGIF.

7. ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL: APPLICATIONS

7.1. Plasma proteins

The theory and fundamental aspects of isoelectric focusing have been reviewed by a number of authors [74—76] and have been particularly well presented in a comprehensive study by Righetti and Drysdale [77], to which the interested reader is referred.

Isoelectric focusing in polyacrylamide gel is beginning to experience considerable interest and use as a clinical tool. The method has the high-resolution capacity that PAGE seemed to promise, but in the light of experience was found to lack. The lag between research development and routine clinical application with this technique is perhaps to be expected as with any other new technique. While apparatus costs like those for PAGE are considerably higher than for agar gel and cellulose acetate systems, they are well below those of a number of routine instruments normally found in the clinical chem-

istry laboratory. Additional complications with PAGIF are certainly present in terms of interpretation and assessment of patterns. Analysis of serum, for example, by electrophoresis on cellulose acetate or agar gels can reveal 10—12 components and three times this on various PAGE systems. However, in PAGIF the pattern complexity may increase five-fold on extended pH range gels. The interpretation and quantification of this many components would at first appear to present a formidable task, but use of this technique with narrow pH range ampholytes, immunological, and enzyme techniques simplifies the problem.

Some 50 protein components are revealed in the serum as shown in the schematic presentation of a two-dimensional cellulose acetate—PAGIF pH 3—10 gel of a 1—1 haptoglobin type illustrated in Fig. 5. Expansion of the region between pH 3.5 and 5.0 indicates that eight components are present in the α_1 region characteristic of a Pi MM phenotype. A comparative schematic presentation of a two-dimensional cellulose acetate—PAGIF gel of serum from a 2—2 haptoglobin type is shown in Fig. 6. Similarly, many other classical serum components may be optimally resolved using such a scheme. The normal γ-globulins, for example, are best resolved on a pH gradient of 5.5—7.5.

Since the review of Latner in 1975 on PAGIF as a clinical tool [78] numerous advances have been made in the area of clinical pathology and methodology. Banding positions, or isoelectric points, of an ever-increasing number of clinically important proteins have now been identified. Likewise, a number of reports of multiple molecular forms of enzymes from serum, CSF, urine, saliva, and tissues have been published, providing a foundation for the cataloging by isoelectric point of clinically important body fluid and tissue enzymes and proteins.

Two important advantages of PAGIF on horizontal flat slabs as compared to PAGE have allowed, and hold great promise in the area of the identification and quantification of protein components. The first is the previously mentioned ability to separate large numbers of samples side by side. The second is that the focused sample components are presented at the surface of the gel allowing the application of two recent techniques which greatly expand the analytical potential of this technique.

The immunoprint technique developed by Arnaud et al. [79], primarily to study the α_1 Pi system, allows separated sample components to be identified immunologically directly from the gel without resorting to a more complex crossed immunoelectrophoresis procedure. The immunoprint technique has been used also to study transferrin and haptoglobin heterogeneity [80]. Basically, the immunoprint is accomplished by soaking a cellulose acetate strip in appropriately diluted specific antibody and then laying it on the focused gel. A glass plate is applied to sandwich the cellulose acetate under slight pressure against the gel. After ½ h the plate is removed and the strip placed in phosphate-buffered saline for several changes over 24 h to wash off unprecipitated proteins. The strip is then stained with Coomassie RB250 and the mirror-image bands reflect the insoluble, specific antigen—antibody complex as shown in Fig. 7. Thus, any of the many serum proteins for which

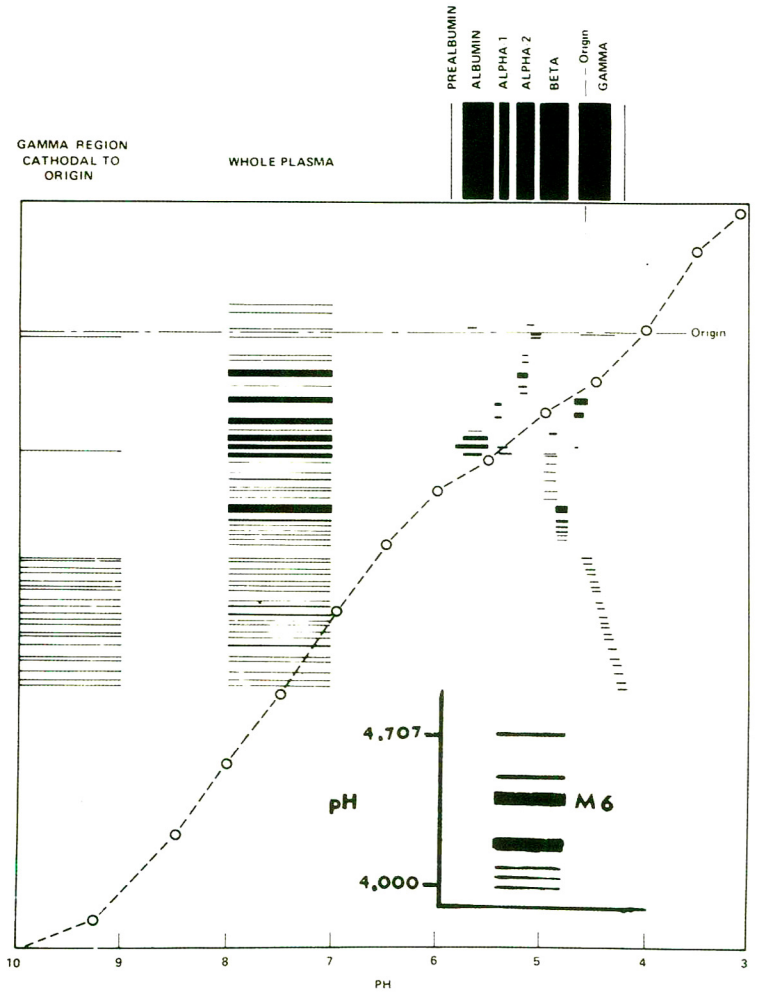

Fig. 5. Schematic separation of serum from a 1—1 haptoglobin type separated in the first dimension on cellulose-acetate and in the second dimension PAGIF on a pH 3.5—10 gradient. The insert in the lower right hand corner is the same serum separated on a narrow range 3.5—5.0 pH gradient indicating a typical MM α_1 Pi phenotype. Coomassie RB250 staining was used on both support media.

specific immunological reagents are available, may be directly analyzed with this technique.

The replicate printing technique developed by Narayanan and Raj [81] has not only the potential of the application just described, but also the ability to perform a number of additional biochemical tests on each separation. This technique consists of taking the focused gel and imprinting it sequentially onto a 1-mm thick agar layer on glass plates. While each print is weaker than the preceding one, up to 5 or 6 prints may be made in practice from a given separation. Thus, proteins, glycoproteins, various isoenzyme tests, and immunoprints can be made from a given gel separation. The exact location

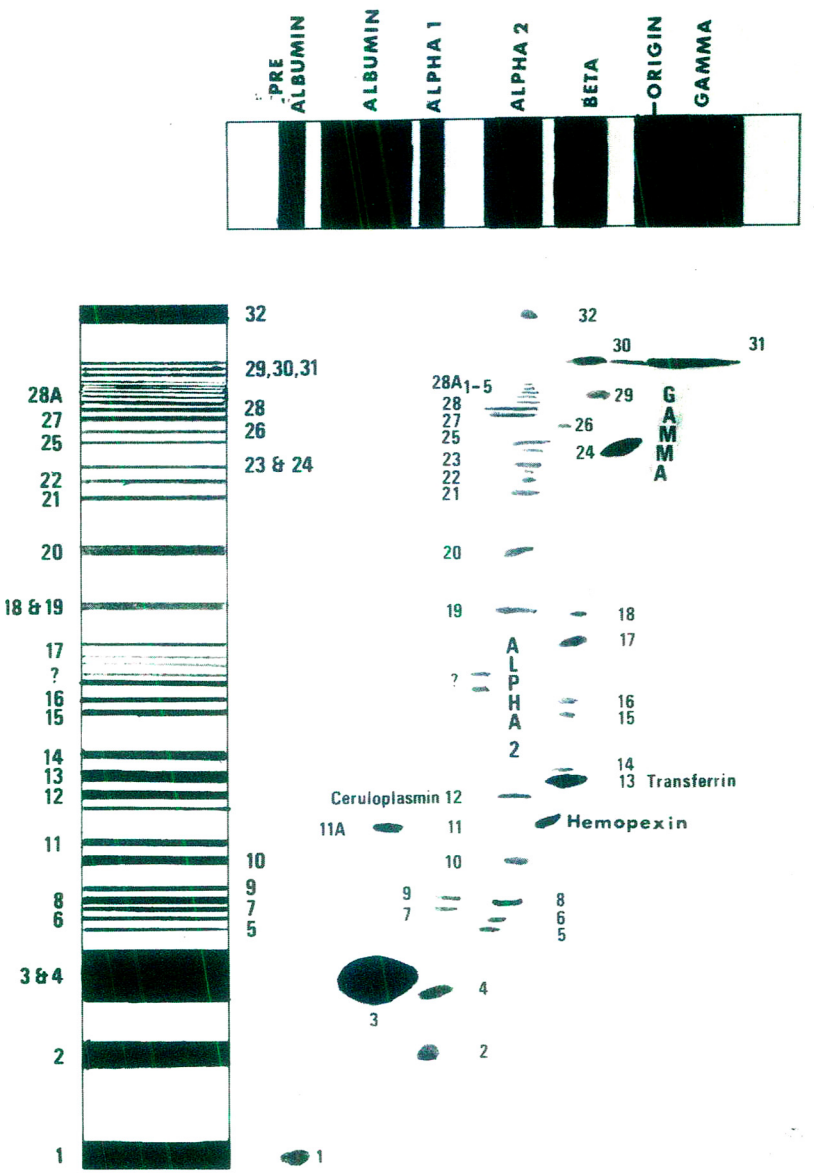

Fig. 6. Schematic separation of serum from a 2—2 haptoglobin type separation in the first dimension on cellulose acetate and in the second dimension on a discontinuous sulfate—borate buffer system in a 4.5—6—8—12% T-pore-gradient gel slab. Note the cloud formation and consequent lack of resolution in both the α_2- and γ-globulin regions. Coomassie RB250 staining was used on both support media. The unidimensional separation on the left is also given in the densitometric trace in Fig. 2. 1, Prealbumin; 2, α_1-acid glycoprotein; 3, albumin; 4, α_1 antitrypsin; 5, 6, 8, α_2-globulin; 7, 9, Gc globulins; 10, unknown; 11, hemopexin; 11a, unknown; 12, ceruloplasmin; 13, transferrin; 14, unidentified β-globulins; 15, 16, β-a_2-globulins; 17, 18, unidentified β-globulins; 19—23, 25, $28A_{1-5}$, haptoglobin polymers; 24, β-α-globulin; 26—28, α_2-macroglobulins; 29, unidentified β-globulins; 30, β_1-lipoproteins; 31, I_gM; 32, chylomicron.

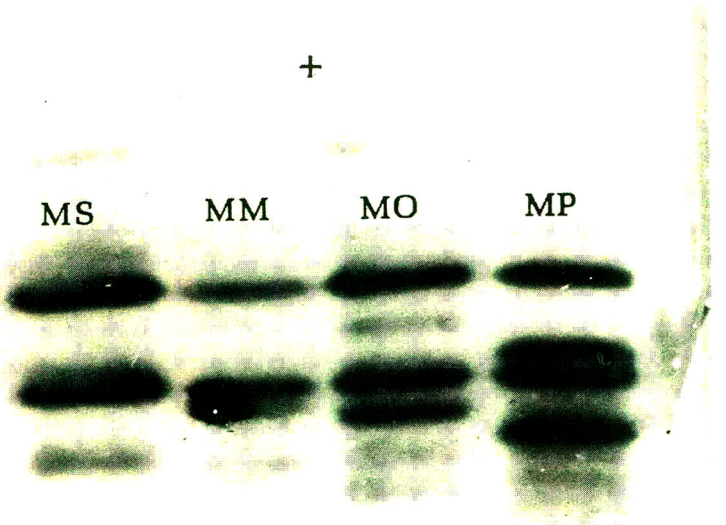

Fig. 7. Immunoprint fixation on cellulose acetate of various α_1 Pi types separated by PAGIF on a pH 3.5—5.0 gradient. The specific immunoprecipitate was stained after washing and fixation with Coomassie RB250.

of each band is the same on all plates allowing multiple characteristics of each component to be determined simultaneously. The development of commercially available thin gels backed on flexible Mylar film by LKB (Rockville, Md., U.S.A.) has provided the critical step necessary to both make printing feasible and allow cross-comparison, since the Mylar-backed master does not shrink or swell during fixation and staining procedures following printing.

7.1.1. Immunoglobulins

PAGIF has been successfully applied in the analysis of the extensive heterogeneity of the immunoglobulins. This technique has been used to study both the heterogeneity resulting from distinct structural genes and that generated by post-synthetic modifications of biosynthetically homogeneous protein. With thin-layer PAGIF Williamson et al. [82] have shown in pH 5.5—7.5 gradient gels, with a resolving power of 0.005 pH units, that there are some 400 theoretical focusing positions for a single antibody band using the method developed by A· 'eh et al. [83]. However, impressive as this resolution may seem, Kreth and 'liamson [84] have determined that a minimum statistical estimate of 8000 m، ɔclonal antibodies against the haptene 3-nitro-4-hydroxy-

5-iodophenylacetyl could be produced in the C_3H mouse. In a multiple-band spectrum of monoclonal antibodies, therefore, it is theoretically possible to distinguish 5×10^4 different isoelectric spectra in this pH range [82]. As an additional tool in differentiating such spectra Keck et al. [85, 86] have developed autoradiographic methods on thin-layer gels where the antibodies are first immobilized by treatment with sodium sulfate and then glutaraldehyde. The bands were then treated by ^{125}I-labeled antigen and analyzed by autoradiography.

From a more practical clinical viewpoint, PAGIF has been used by Cornell [87] to show that in some instances the method would, like PAGE, detect monoclonal proteins in serum before they were detectable by cellulose acetate or immunoelectrophoresis. Awdeh et al. [88] have shown that the origin of the microheterogeneity from a plasma cell tumor, which produces a single molecular species of I_gG_2, is due to lability after synthesis. Brendel et al. [89] have reported also that non-myelomatous monoclonal I_gG proteins possess the same individuality and limited microheterogeneity as myelomatous monoclonal I_gG proteins.

Trieshmann et al. [90] have analyzed a series of human I_gG autoantibodies and have found them to consist of I_gA, I_gM, and I_gG. All demonstrated single peaks by liquid column focusing with isoelectric points from pH 3 to 4.5 in contrast to normal immunoglobulins which focus between 5.5 and 7.5. Assessing sera for such components is also feasible on I_gM acid components in large pore gels which would provide a more definitive method to detect microheterogeneity than liquid column isoelectrofocusing. Wilson et al. [91] have reported that in the sera of cystic fibrosis patients and in heterozygous carriers, a heat-labile γ-globulin with an isoelectric point of 8.46 is present. Their finding of a similar band in one out of twelve normal individuals suggests that the diagnostic value of this band, however, must be viewed with caution since the I_gG acts only as a carrier [92]. Dale et al. [93] have demonstrated that it is possible to distinguish I_gA myelomatosis from I_gG myelomatosis using two-dimensional gel techniques and even the I_gA light chain can be demonstrated in the serum.

Cwynarski et al. [94] have described I_gG paraproteins from patients with various gammopathies, both malignant and benign, and present the possibility that a number of "benign-paraproteinemias" may in fact represent premalignant phases.

Bouman et al. [95] have investigated the microheteroneity of I_gG from plasmacytomas and found the monoclonal I_gGs to display 3 to 13 bands. They also indicated that post-synthetic deamidation was not found to occur spontaneously and consequently is not the cause of the microheterogeneity observed.

7.1.2. β-Globulins

The allotyping of complement components C_4, C_2, C_5, C_6, C_7, and factors B and D in whole serum separated by PAGIF followed by an *in-gel* hemolytic assay has been reported by Hobart and Lachmann [96]. Polymorphisms in C_2 were found to be unlinked to HLA although C_2 deficiency is known to be linked genetically to this system.

Transferrin which migrates as a single zone in PAGE has been shown by Hovanessian and Awdeh [97] to be separable into two zones by isoelectric focusing with isoelectric points of 5.6 and 5.2, respectively. The former is monoferric transferrin and the latter diferric transferrin. Should distinct functions exist for the mono- and diferric transferrin or for the two sites of transferrin then the ratio of Fe-transferrin:Fe_2-transferrin might become a valuable index of serum iron metabolism.

Fibrinogen heterogeneity has been demonstrated by Gaffney [98] and Soria et al. [99], who have also employed PAGIF to study abnormal fibrinogens. Arnesen [100] has employed this technique to the study of fibrinogen degradation and fibrin split products.

The diagnosis of Type 3 hyperlipoproteinemia (Broad-β Disease) is based on the demonstration in fasting plasma of a low-density lipoprotein which in zone electrophoresis migrates in the β region. Godolphin and Stinson [101] have demonstrated a Sudan black B-staining band in untreated serum with an isoelectric point of pH 5.44 which appears to be characteristic of the disease. Utermann et al. [102] have shown that this disease is further characterized by lack of an apo-lipoprotein designated E III and suggest that the disease is the result of an autosomal recessive inheritable trait. These authors have also developed a rapid screening method which does not require prior ultracentrifugation of the serum for such analyses.

7.1.3. α_2-Globulins

The α_2-macroglobin has been studied by Jones et al. [103] in relation to its subunit structure following thiol reduction. Microheterogeneity has been studied by Frenoy and Bourrillon [104], and Ohlson and Skude [105] have demonstrated semi-quantitatively the determination of complexes between various proteases and human α_2-macroglobulins.

Ceruloplasmin may be identified by means of its oxidase activity on *p*-phenylene diamine or by using standard immunological reagents for ceruloplasmin.

The haptoglobins may be separated by PAGIF and identified as to genetic type by the addition of hemoglobin with or without enhancement by benzidine staining. Chappuis-Cellier [80] has used the immunoprint technique to localize the haptoglobins. The potential advantage of PAGIF over PAGE for the study of the haptoglobins would appear to be in elucidating greater microheterogeneity and possible subtypes in conjunction with simplified set-up procedures.

The α_2-HS glycoprotein microheterogeneity has been observed by Hamber et al. [106], who reported an isoelectric point range of 4.7—5.1 which is just outside of the p*I* range of the α_1 proteinase inhibitors but overlaps that of the heterogeneic plasma glycoprotein kininogens. Thus, the α_2-HS glycoproteins may require differentiation from the kininogens by techniques such as the immunoprint method.

7.1.4. α_1-Globulins

The α_1 Pi systems have received considerable interest in the last few years with well over 300 separate reports appearing in the literature. Deficiency states have been associated with a predisposition to emphysema [107], in-

fantile cirrhosis [108], juvenile arthritis [109], non-allergic asthma [110], and other inflammatory diseases. Allen et al. [111] have recently found that the more serious heterozygous deficiency states MZ and MS predispose individuals to periodontal disease marked by erosive bone loss unless meticulous oral hygiene is practiced. Pi typing by PAGIF developed by Allen et al. [112] and Arnaud et al. [113] allows ready differentiation of the 30 identified alleles and some 56 phenotypes presently described. The microheterogenic system first demonstrated on the so-called acid starch gel system of Fagerhol and Laurell [114], is capable of being fully resolved only by PAGIF. Sixty samples can be analyzed per gel slab within 3 h and Pi typing is a more accurate and practical way of detecting heterozygote deficient individuals than radial immunodiffusion (RID) and trypsin inhibitory capacity techniques. Since α_1 Pi is an acute phase reactant, serum levels in heterozygote deficiency states are often raised during infection or in patients treated with steroids, particularly estrogens, and therefore, individuals with deficiency states could present with serum levels in the low normal or normal range. Over 30% of MS Pi types show low normal or normal levels even in the absence of infection or steroid treatment [115], and thus, would be assessed as normal by commonly employed tests to determine serum levels. The microheterogeneity and subgroup analysis possible with PAGIF are shown in Fig. 8.

One-step verification of Pi allele products in the region of isoelectric points from 4.40 to 4.72 may be accomplished by the protease probe techniques of adding trypsin to the serum which clears the Pi bands, since the complexes formed have much higher isoelectric points, as described by Allen et al. [34] and by immunoprint fixation procedure described by Arnaud et al. [79].

Separation of lipoproteins on gel slabs in which whole serum is placed on filter paper tabs results in considerable surface smearing of the VLDL and LDL which do not penetrate the gel. Gel rods containing a 5% monomer concentration have been used by Kostner et al. [116] to separate the lipoproteins with the HDL region showing four distinct bands. Kostner et al. [117], utilizing a modified system employing photopolymerized gel rods containing 23% ethylene glycol, were able to separate 8 bands from prestained sera. While HDL_2 and HDL_3 heterogeneity has been demonstrated by sucrose gradient column studies by Sodhi et al. [118], Blaton et al. [119] and Eggena et al. [120], PAGIF has not been widely used. Removal of VLDL and LDL from serum by heparin and magnesium as described by Burstein and Scholnick [121] will provide samples on which HDL may be separated without resorting to prior density gradient ultracentrifugation. Scanu et al. [122] have studied the HDL in depth by analytical PAGIF and have found that focusing is most valuable in the characterization of products separated and purified by prior chromatographic procedures.

The thyroxin-binding globulin has been shown by PAGIF to exhibit microheterogeneity similar to the other α_1-globulins. Some 9 bands have been found with PAGIF by Marshall et al. [123], with 4 major and 5 minor bands from purified material obtained from pooled plasma with a p*I* region from 4.2 to 5.2. Although this reported range of isoelectric points overlaps that of the proteinase inhibitors, the low concentration of thyroxin-binding glob-

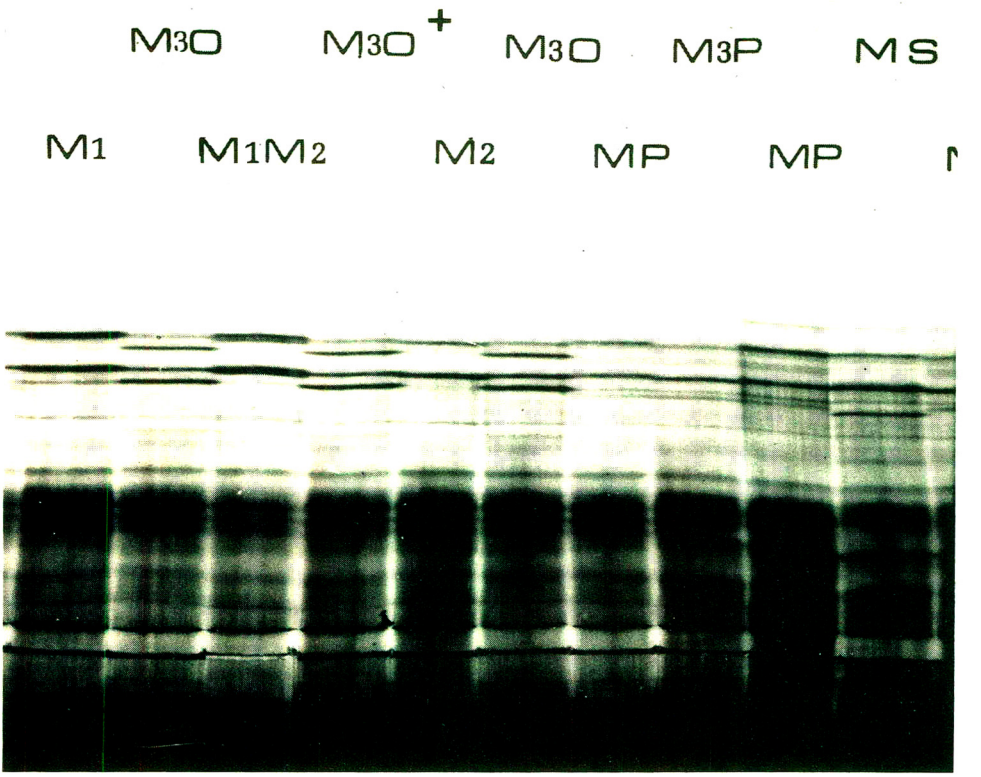

Fig. 8. Serum samples separated by PAGIF on pH gradient 3.5—5.0 on 1-mm-thick gels showing the complexity of M variants in the Pi system as well as the common S allele and the rare O and P allele products.

ulin, 1—2 mg per 100 ml serum, in practice, causes no interference with Pi typing. Detection of the thyroxin-binding globulins by immunoprint techniques may be difficult due to their very low concentration. To date, radiographic techniques applied directly to the gel have not proven satisfactory for localizing these proteins.

α-Foetoprotein normally undetectable within days after birth reappears in the serum of patients with primary liver carcinoma and embryonal carcinoma. It has been isolated and its microheterogeneity studied by PAGIF. Two major α-foetoprotein peaks with isoelectric points of 5.08 and 5.42 have been described by Alpert et al. [124] in both hepatomas and foetal serum. Two components of α-foetoproteins have also been described in the ascites fluid from a patient with primary carcinoma by Sokolov et al. [125] with isoelectric points of 4.78 and 5.20, respectively.

7.2. Hemoglobin

Aside from their clinical importance, the hemoglobins hold a special place in isoelectric focusing. They have played perhaps the most important role in

the early development of the technique. As proteins bearing a chromophoric heme group, they were used extensively to demonstrate not only the resolving power of isoelectric focusing, but also as markers to indicate completion of pH gradient information. They have also served as a model system for subunit exchange, binding studies and cooperativity as reported by Park [126]. High-voltage, controlled low-temperature systems have provided a considerable advance in the study of the hemoglobinopathies as well as in the study of human hemoglobin phenotypes as demonstrated by Bunn [127], while Drysdale et al. [128] have compared human and animal hemoglobins.

Chromatographic techniques have been previously used to study heterogeneity of both normal and abnormal types [129, 130], but the ability to separate rapidly multiple samples by PAGIF on pH 6—8 gradient gels for both quantitative and qualitative studies offers a valuable tool for clinical studies. Jeppsson and Bergland [131] showed that thin-layer PAGIF was able to separate Hb-Malmo from Hb-A, and Hb-F from Fb-F_{11}, which was not possible with previous zone electrophoretic techniques. Another high oxygen affinity hemoglobin with a mutation at B97 and associated with familial erythrocytosis was reported using this technique by Taketa et al. [132]. Monte et al. [133] have recently mapped hemoglobin mutants by PAGIF. Altland [134] has developed a screening process for hemoglobinopathies using a portion of the dried blood samples obtained for PKU screening. He employed silicon rubber strips, with holes punched at intervals to serve as sample wells, into which the samples dissolved in 50 μl of 0.02 *M* KCN were applied in two rows of 48 samples to a single gel. A center anode strip is used with a cathode strip at both the top and bottom of the gel so that 96 samples per run may be analyzed.

Koenig et al. [135] have shown that the measurement of Hb-AIc provides a reliable index of control of carbohydrate metabolism in diabetes, and Trivelli et al. [136] have reported that Hb-AIc is increased two-fold or more in patients with poorly controlled diabetes mellitus. Spicer et al. [137] using high-voltage, constant-power PAGIF have shown that Hb-AIc and the other fast hemoglobins Hb-AIa and Hb-AIb may be separated and accurately quantified by microdensitometry on pH 6—8 gradient gels as illustrated in Fig. 9. This method is rapid with the added advantage that abnormal hemoglobin phenotypes are also detected if present. For pediatric patients, these authors have utilized standard heparinized micro-hematocrit tubes to obtain blood from a finger or ear prick for both Hb-AIc analysis and for Pi typing or other plasma protein or enzymes analyses as may be desired.

7.3. Cerebrospinal fluid

Vesterberg [138] has shown that patients with multiple sclerosis have a relative increase in the CSF of alkaline globulins, i.e. those with an isoelectric point greater than 7.5 at 10°. Delmotte [139] has also reported similar findings. Stibler and Kjellin [140] have demonstrated the focusing patterns in CSF of degenerative neurological diseases. The same authors have also studied the protein patterns in hereditary ataxia, hereditary spastic paraplegia, muscular dystrophy, and spinal muscular atrophies.

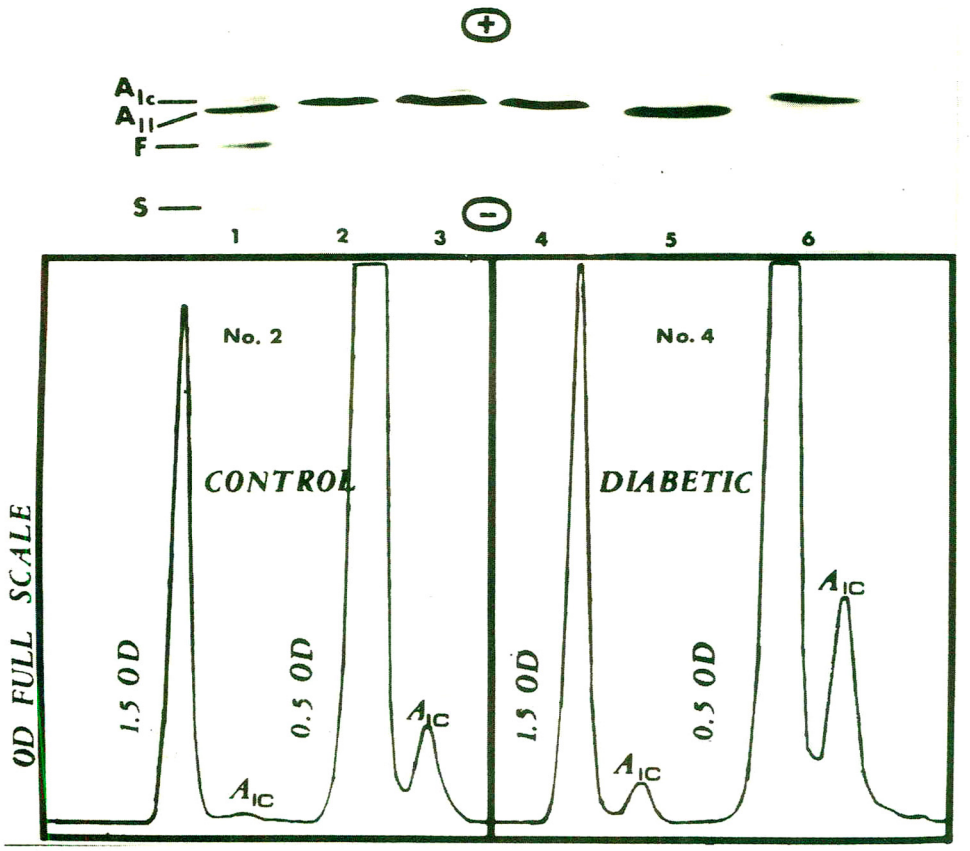

Fig. 9. Separation of hemoglobin by PAGIF on pH 6.0—8.0 gradient. Sample 1 is a mixture of F, S, AII, and A_{Ic}. Sample 2 is from a normal individual, samples 3 and 4 from diabetics. Sample 5 is twice rechromatographed AII from a Biorex column and sample 6 is similarly rechromatographed HbA_{Ic}. Separations are directly fixed in 12.5% TCA and subjected to quantitative microdensitometry as shown below.

Two-dimensional gel techniques have been used by Fossard et al. [141] for a variety of studies of CSF and Latner [142] has reported that in an unusually high proportion of patients suffering from multiple sclerosis an abnormal protein spot was found which had a p*I* slightly more acid than transferrin.

7.4. Urine

Rotbol [143] appears to have first applied isoelectric focusing to human urinary proteins on polyacrylamide gel. He found some 30 protein zones to be present in normal urine. Vesterberg [138] has identified the location of the β_2-microglobulin and found more than 40 protein zones in urine from patients with advanced renal damage. Hall and Vasiljevic [144] have characterized further the β_2-microglobulin and described two homologies, one with

TABLE 2

PAGE AND PAGIF ZYMOGRAM METHODS

Enzyme	Substrate/reaction mixture	PAGE	PAGIF	Source	Application	Reference
Acid phosphatase	α-Naphthol phosphate—Fast Garnett GBC	x	x	White cell	Hairy cell leukemia	160
	4-methyl-umbelliferyl phosphate		x	extract		157
Alkaline phosphatase	α-Naphthol phosphate	x	x	Serum	Lymphatic leukemia, infectious mononucleosis	161
Amylase	Contact print		x	Saliva		162
	overlay Phadebus Rtest system (Pharmacia)	x		urine,	Rise in 1 & 2	163
	starch iodine			sera	pancreatic disease injury	
Acid and glucosidase	4-Methylumbelliferyl α-D-Glucopyranoside		x	Muscle, liver	Pompe's disease	164
α-L fucosidase	4-Methylumbelliferyl α-L-Fucopyranoside		x	Liver	Mucopolysaccharidosis F	165 166
Glucose-6-PO_4 dehydrogenase	Glucose-6-phosphate monosodium salt NBT, NADPT, PMS	x	x	Red cells	Genetic variants, hemolytic episodes	167 168
N-Acetyl-β-D-hexosaminidase	α-Naphthol-As-BI-N-acetyl-,3-D-glucosimide—Fast; Garnett GBC salt		x	Serum, tissue, amniotic fluid	Tay-Sachs	169 170 171
β-Galactosidase	4-Methylumbelliferyl N-acetyl-β-galactosamine		x	Liver	Mucopolysaccharidosis Type 1, 2, 3	172
Esterases	α-Naphthol butyrate		x	Skin,	Wound esterase	173
	α-Naphthol acetate	x		serum,	differentiation of	174
	Fast Red TR				bacterial and viral pneumonias,	
			x	kidney,	platinate drugs,	175
			x	spleen	histiocytic neoplasms	176
Tyrosinase	L-Dopa (L-3,4 and 1-hydroxyphenylalanine)		x	Melanocytic tissue	Melanoma	156

Peroxidase	DAB-H_2O_2	x	x	Red cells, uterine fluid	Estrogen-induced growth	177 178
Creatinine phosphokinase	Creatine PO_4—PMS NBT	x	x	Serum	Myocardial injury	179 180
Lactic dehydrogenase	Lithium lactate	x	x	Serum, red cells, urine	Myocardial injury pyelonephritis	181 182 183
Proteases	Casein agar overlay		x	Tissue cells		184
Pyruvate kinase	Phosphoenol pyruvate NADH, LDH, ADD—MTT		x	Red cells	Hereditary hemolytic anemia	185

a p*I* of 5.3 and the other with a p*I* of 5.7. This protein, which has a molecular weight of 11,500, has been established to be the smaller of the two polypeptides of the HLA antigen and is elevated in the majority of myeloma patients. The highest β_2-microglobulin levels are found in patients with tubular deficiencies and in patients following renal transplantation. Patients afflicted with Balkan nephropathy are a special example of renal tubular disorder in which high levels of β_2-microglobulin are found [144].

Boulton and Huntsman [145] have used both PAGE and PAGIF to distinguish myoglobin from hemoglobin in non-fresh urine of kidney donors where conversion of myoglobin to met-myoglobin makes spectroscopic recognition of this pigment unreliable. Hultberg et al. [146] have studied the isoenzymes of four acid hydrolases in both kidney and urine and have found that there was a predominance of isoenzymes with a low isoelectric point in the urine. In contrast, in kidney tissue extracts, isoenzymes with higher isoelectric points predominated. Kellar et al. [147] have studied the colony stimulating factor from human leukemia urine and identified a glycoprotein demonstrating marked microheterogeneity with isoelectric points between pH 3.3 and 4.1.

7.5. Salivary proteins

Beeley [148] has employed PAGIF in gel rods to separate the salivary proteins and Benninck and Cornell [149] have used the method as a criterion in the purification and partial characterization of four proteins from human parotid saliva. Chisholm et al. [150] have found additional protein bands with acid isoelectric points in the saliva of individuals with Sjögren's syndrome and those with rheumatoid arthritis. Pronk [151] found three different protein type patterns in parotid saliva which were shown by family studies to be phenotypic expressions of one autosomal locus with two co-dominant alleles. These were found to be correlated with α-amylase activity.

7.6. Enzymes

The significance of enzymes present in the plasma, originating from healthy and diseased tissue, as indicators of disease and therapy control is readily apparent in clinical medicine. Implicit in such correlations of enzyme levels with disease is that their elevation or decrease is representative of events occurring in a particular disease state at the cellular level. Alterations in the level of a particular enzyme or isoenzyme may result either from the tissue or from a deficit in clearance. Moreover, genetic and physiological factors can singly, or in combination, alter plasma levels in the absence of disease.

Many enzymes may be analyzed by both methods using suitable histochemical stains, a number of which have been compiled by Raymond [152], Righetti and Drysdale [77], and Maurer [153]. PAGE requires the gel to be pretreated in a sufficiently concentrated buffer to equilibrate the gel to the proper pH for a given reaction. PAGIF requires similar treatment, however, these gels with ampholyte concentrations of 20—50 m*M* make it quite easy, in most cases, to utilize a 100—200 m*M* buffer directly with the substrate

and complexing agents. Wadström and Smith [154] have reported ways to overcome adverse pH conditions in PAGIF gels. Additionally, enzymes may lose activity after PAGIF due to chelation of necessary metal cofactors. For example, Latner et al. [155] have reported 90% loss of activity of alkaline phosphatase unless the zinc cofactor was added back to the reaction mixture.

7.7. Tissue proteins and enzymes

PAGE and PAGIF have not been widely used as clinical tools to study soluble proteins and enzymes obtained from tissues or cells. However, this potential deserves mention as it is certainly within the microanalytical capability of both systems. For example, only a portion of wedge biopsy material is necessary to provide sufficient material for soluble protein extraction. Studies such as the detection of carcino-foetal liver ferritins by Alpert et al. [156]. The multiple molecular forms of tyrosinase in melanomas have been described by Burnett and Seilor [157] and tartrate resistant acid phosphatases from leucocytes in hairy-cell leukemia by Yam et al. [158].

Latner [78] has also shown differences in kidney tissue in renal carcinoma and in the cervical mucosa of pap-smear-positive women. These few examples suggest tissue and cell extracts particurlarly when separated by PAGIF, in combination with replicate print and immunoprint techniques, may in the future play a valuable role in clinical diagnosis.

Some selected clinically significant enzymes which may be qualitatively and quantitatively analyzed with both these techniques are given in Table 2 along with their diagnostic significance and source of origin.

8. OTHER HIGH-RESOLUTION METHODS

Two-dimensional electrophoresis employing PAGIF in the first detection and SDS—PAGE in the second described by O'Farrell [28] provide a more exotic method of high resolution than the earlier PAGIF—PAGE techniques of Latner [142]. This technique deserves special mention, not so much for its present applicability as a routine clinical tool, but for its potential to study gene products at the molecular level. This method has been used by Ivarie et al. [159] to detect missense mutations in structural genes and in monitoring gene expression in response to environmental stimuli.

9. SUMMARY

The use of polyacrylamide gel electrophoresis and polyacrylamide gel isoelectric focusing techniques in the area of clinical medicine is reviewed. The two techniques are compared both to previous electrophoretic techniques and to each other with regard to simplicity of operation and degree of resolution required for a given laboratory task. Plasma, body fluids and tissue proteins, and isozymes that presently may be utilized in clinical diagnosis and clinical research are presented with emphasis attempted toward describing the simplest procedure providing adequate resolution for a given task.

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DETERMINATION OF KYNURENINE BY A SIMPLE GAS—LIQUID CHROMATOGRAPHIC METHOD APPLICABLE TO URINE, PLASMA, BRAIN AND CEREBROSPINAL FLUID

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SUMMARY

A simple, sensitive and specific method for the determination of kynurenine is described. This is based on alkaline cleavage of kynurenine, followed by solvent extraction, trifluoroacetylation and gas—liquid chromatography with electron capture detection. Using this method kynurenine has been determined in urine and plasma, and for the first time in brain and cerebrospinal fluid. Increases in kynurenine in brain, plasma and urine are demonstrated following tryptophan administration to man and rat.

INTRODUCTION

Kynurenine is the first major metabolite of tryptophan on the pathway initiated by the enzymes tryptophan pyrrolase (tryptophan-2,3-dioxygenase) and indoleamine-2,3-dioxygenase. The former enzyme is found in the liver and is specific for L-tryptophan, the latter has a much wider distribution in the body, and a wider substrate specificity [1, 2]. Estimation of kynurenine production, sometimes following a tryptophan load, has been used as an index of the activity of this pathway [3, 4] which has been shown to be altered in a number of pathological states [3].

We have previously presented [5] a convenient modification of the method of Tompsett [6] which could be applied to urine and to plasma, provided that interference from tryptophan was prevented during the alkaline cleavage of kynurenine to *o*-aminoacetophenone (OAAP). In these methods the product was determined colorimetrically following diazotisation and coupling with naphthylethylene diamine. OAAP derived from urinary kynurenine has previously been determined by gas—liquid chromatography (GLC) using flame ionisation detection [7]. In the present method the previous procedure [5] has been modified so that the OAAP produced can be determined after trifluoroacetylation by GLC using electron capture detection. Kynurenine can

readily be determined without prior tryptophan loading in 200 μl of human or 100 μl of rat plasma. In addition the method can be used for urine, and also to study the kynurenine pathway in brain via determinations in brain and cerebrospinal fluid (CSF) material.

MATERIALS AND METHODS

L-Kynurenine sulphate, kynuramine dihydrobromide and L-tryptophan were obtained from Sigma (London, Great Britain), *o*-aminoacetophenone from Koch-Light (Colnbrook, Great Britain, α,α′-dichloro-*p*-xylene (DCPX) from Ralph N. Emanuel (Wembley, Great Britain), N-formyl-L-kynurenine from Calbiochem (Bishops Stortford, Great Britain) and trifluoroacetic anhydride (TFAA) and other chemicals (AR grade where available) from BDH (Poole, Great Britain). Tiron was obtained from Fisons (Loughborough, Great Britain).

Procedure

Brain tissue (1—2 g) was homogenised in 5 volumes of acid butanol (0.85 ml conc. HCl per litre *n*-butanol). After centrifugation (2500 *g*, 10 min) the supernatant, together with an equal volume of *n*-heptane, was back-extracted with 1—2 ml of 0.1 *M* HCl. Plasma could be processed similarly using 10 volumes of acid butanol, or the supernatant from extraction with 10 volumes of 10% trichloroacetic acid (TCA) could be used. A suitable volume of the 0.1 *M* HCl extract, the TCA extract, or undiluted CSF or urine was made alkaline with one third of its volume of 10 *M* NaOH, and 100 m*M* Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid, disodium salt) added to a final concentration of 5 m*M* [5]. After extraction with butyl acetate, the alkaline aqueous phase was heated in a boiling water-bath in a closed glass tube for 20 min and cooled to room temperature. The product was extracted into a small volume of butyl acetate (routinely 300 μl) containing 200 ng/ml DCPX as internal standard. A suitable volume (routinely 200 μl) of the organic phase was mixed briefly with an equal volume of alkali-borate buffer (0.2 *M* borax in 1.5 *M* NaOH) in a 1.5-ml Eppendorf reaction tube. After a brief interval, 1/8 volume (routinely 25 μl) of trifluoroacetic anhydride was added. The contents were mixed immediately and centrifuged to separate the phases. One μl of the supernatant organic phase was injected into a Hewlett-Packard 5713A gas chromatograph. Conditions: injection port, 250°; on-column injection onto 3 ft. × 1/4 in. coiled glass column packed with 10% OV-1 on HP Chromosorb W at 120°; exit directly into ^{63}Ni electron capture detector at 300°; carrier gas, argon—methane (95:5) at 60 ml/min.

Tryptophan in plasma or brain was determined as previously described [5] on a further aliquot of the 0.1 *M* HCl phase from the above procedure.

RESULTS

Development of the method

In the previously described method [5] OAAP formed by heating kynurenine in strong alkali was extracted into amyl alcohol. This was clearly not a

suitable solvent for trifluoroacetylation since it could react with TFAA. Chloroform, as used by Naruse et al. [7], is not compatible with electron capture detection. Ethyl acetate appeared suitable, but was hydrolysed to some extent by the strong alkali used. Butyl acetate was not hydrolysed, and extracted the OAAP efficiently.

Since removal of excess TFAA by evaporation under a stream of gas might lead to loss of the rather volatile trifluoroacetyl derivative of OAAP, it was removed by hydrolysis with an alkaline buffer. NaOH (2.5 moles/l) alone resulted in some loss of the derivative, but the amount of alkali-borate buffer used resulted in a maximal yield, while keeping the aqueous phase alkaline so that the trifluoroacetic acid produced remained in the aqueous phase. Since the addition of the highly volatile TFAA was likely to be subject to some error the adequacy of the buffering capacity of the alkali-borate was checked by varying the amount of TFAA added. 15 μl TFAA resulted in a lower yield, but the yield was essentially the same for 20 to 30 μl TFAA (Table I). The reaction of TFAA with OAAP in butyl acetate appeared to be almost instantaneous, and no advantage was obtained by incubating with TFAA before addition of the alkali. Prior addition of the alkali resulted in equally high yields and was adopted because of its convenience. The final volume of butyl acetate used for extraction was kept low in order to maximise the final concentration of OAAP while avoiding evaporation. Under these circumstances a reduction in the volume of the aqueous phase to be extracted with butyl acetate was sought. This was achieved by the use of acid butanol with subsequent back extraction into 0.1 *M* HCl, and resulted in a ten fold reduction in the volume of aqueous phase compared with the perchloride acid extraction used in the previous method [5]. The recovery of kynurenine with acid butanol at the initial extraction is lower, but the improved recovery at the butyl acetate extraction step more than compensates for this, resulting overall in higher peaks for a given amount of OAAP or kynurenine. In addition acid butanol extraction enables tryptophan, 5-hydroxytryptamine, 5-hydroxytryptophan and 5-hydroxyindole acetic acid [8, 9] to be determined concurrently on a single sample. Another advantage of the acid butanol technique is the reduction in interference (see below).

TABLE I

VARIATION IN PEAK HEIGHT RATIO OF OAAP—TFA TO INTERNAL STANDARD WITH VOLUME OF TFAA USED IN ASSAY

Methods: as described in text using butyl acetate containing 200 ng DCPX and 244 ng standard OAAP per ml.

TFAA volume (μl)	Peak height ratio
15	68.4
20	112.5
25	118.6
30	119.2

Evaluation of the method

DCPX internal standard carried through this method alone yielded a single sharp symmetrical peak with a retention time of 6.4 min (Fig. 1). Standard OAAP carried through the final stage of derivative formation, or standard kynurenine carried through the whole method yielded a similar peak with a retention time of 7.2 min (Fig. 1). Quantitation was achieved by expressing the height of the OAAP peak as a ratio of the internal standard peak height for each chromatographic run. The method was simple and convenient in practice, the use of differential extraction, before and after heating, resulting in very clean GLC traces. Blanks were normally less than 1% of internal standard peak, corresponding to 5 ng kynurenine added initially (a peak height of 20% internal standard corresponded to 100 ng kynurenine). The linear yield of product with varying amounts of kynurenine is shown in Fig. 2. Reproducibility of duplicate determinations was 5% of their mean.

Incubation of standards or tissue samples in strong alkali for 20 min at room temperature in place of the boiling water-bath (see Materials and Methods) resulted in a reduction of the OAAP detected to 3%. Fig. 3 shows a mass spectrum of the GLC peak obtained from rat plasma carried through the method compared with that from standard OAAP, which identifies the substance produced from rat plasma as OAAP. However, the possibility that the OAAP produced by heating in strong alkali comes from sources other than kynurenine must be considered. Other possible sources include tryptophan, acetyl-kynurenine, kynuramine and formyl-kynurenine. In the presence of Tiron, tryptophan does not interfere detectably at 3 times its normal level in brain, and interferes only to a small extent (Table II) at 30 times its normal level (500-fold molar excess over kynurenine). The other three substances will interfere, in that when heated in alkali they do yield OAAP, kynuramine and formyl kynurenine on approximately a mole for mole basis, and acetyl

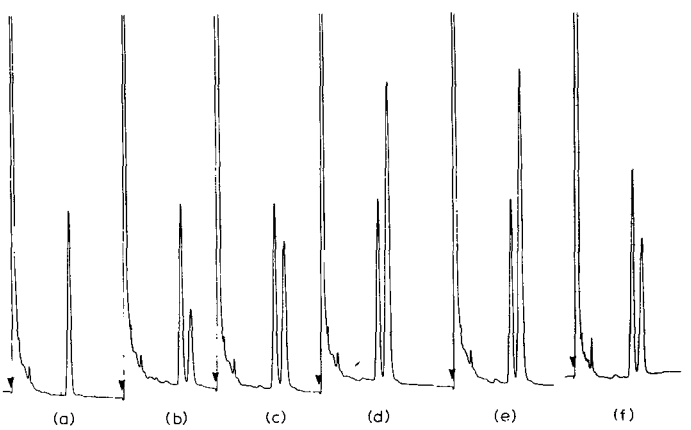

Fig. 1. GLC records from kynurenine analysis. (a) Blank, (b) 150 ng, (c) 300 ng, (d) 600 ng added kynurenine standard, (e) autopsied human brain cortex and (f) rat brain. Injection is indicated by arrow; the first major peak is DCPX internal standard; the second is the OAAP derivative.

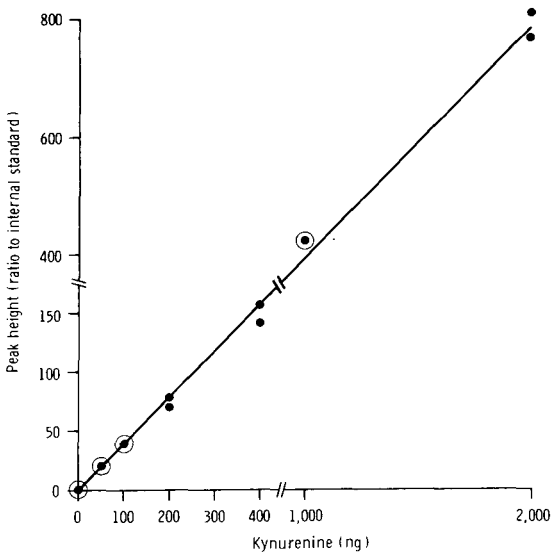

Fig. 2. Standard curve for kynurenine assay. Duplicate determinations at each point; ⊙ indicates co-incident results.

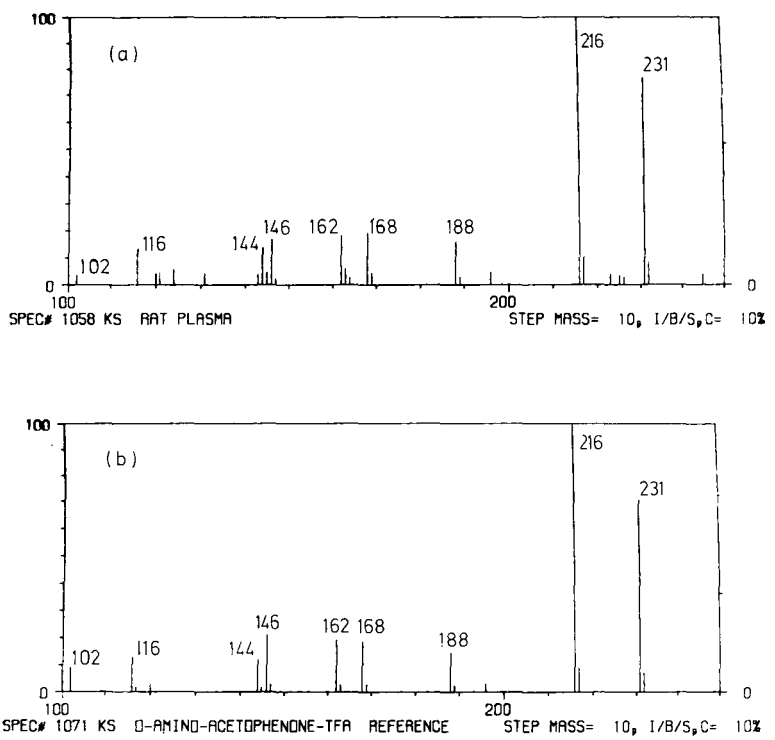

Fig. 3. Mass spectra of material derived from (a) rat plasma and (b) OAAP standard. Mass spectra were obtained using the Varian MAT-112 mass spectrometer and 100 MS Spectro-System.

TABLE II

INTERFERENCES AND RECOVERIES OF STANDARDS

All figures are percentages.

Compound	Interference* (added to alkali before heating)	Recovery through		Overall interference*
		Butyl acetate	Acid butanol	
L-Tryptophan				0.03
Acetyl-L-kynurenine	21.1	91.6	54.4	11.8
Formyl-L-kynurenine	86.6	90.5	32.6	28.6
Kynuramine	92.2	14.3	75.5	11.2
L-Kynurenine	(100)	83.7	106.6	(100)

*Expressed as per cent of kynurenine on an equimolar basis.

kynurenine at about one fifth on a molar basis (Table II), a result similar to that reported previously [5]. However, when carried through the whole method kynuramine interference is reduced to 11% since it is efficiently extracted by the butyl acetate wash prior to heating (Table II). If present, kynuramine can readily be detected, and indeed quantified, by adding an equal volume of heptane to the butyl acetate and back-extracting with 0.1 *N* HCl. If the HCl phase is now heated with alkaline Tiron, as in the kynurenine method described, OAAP is produced, which can be quantified by the same procedure. The recovery through this procedure of kynuramine added to brain is 90.1%, and endogenous kynuramine has not been detected in any rat or human brain samples analysed to date (limit of detection about 5 ng/g).

When acetyl-kynurenine and formyl-kynurenine are carried through the whole method their interferences are reduced as shown in Table II since they are not so well recovered from the acid butanol phase. The residual interferences are of little importance where kynurenine formation is being studied, since both are likely to be produced in parallel with kynurenine. Indeed, in using the method to assess pyrrolase activity this could be a positive advantage since formyl-kynurenine and kynurenine will both be measured if the acid butanol stage is omitted, and thus the assay will be independent of the presence of excess formamidase. This would be particularly useful in purification studies on tryptophan pyrrolase.

Should it be necessary to unequivocally identify kynurenine as the source of OAAP a small column of Dowex 50W produces distinctive elution profiles for kynurenine and acetyl kynurenine [10, 11] and also for formyl kynurenine and tryptophan [12]. In this way we have established [11] that the OAAP produced from rat or human brain tissue carried through the method is derived from kynurenine and not from acetyl-kynurenine, formyl-kynurenine or kynuramine.

The levels of kynurenine and recovery values found by applying the described methods to tissues and fluids from several species are shown in Table III. The values for rat brain confirm those given in preliminary reports from this laboratory [13] and from another [14]. The values for human urine and human and rat plasma agree reasonably well with those previously published from this laboratory and others [7, 15, 16]. The results for rat plasma

TABLE III

CONCENTRATIONS OF KYNURENINE FOUND IN VARIOUS TISSUES AND FLUIDS

Concentrations are expressed in ng kynurenine per ml or g wet weight and represent means ±standard deviation. The number of observations is given in parentheses.

Tissue	Species	Specification	Concentration	Recovery (%)
Urine	Human	Early morning sample	573±99(6)	85.3
Plasma	Human	Fasting sample at 09.00—10.00 h	404±158(6)	—
	Rat		807±111(10)	76.3
	Goat		143	—
CSF	Human	Lumbar	7.1 ±1.7(5)	71.4
	Pig	Cisternal	38.7	68.5
	Goat	Cisternal	13.4	—
	Beagle	Cisternal	7.2	—
Brain	Human	Putamen	317±176(8)	—
		Temporal cortex-autopsy	216±176(8)	—
		Temporal cortex-biopsy*	114±20(3)	—
	Rat	Whole brain	161±16(7)	80.3

*Frozen immediately after removal at therapeutic neurosurgical operation.

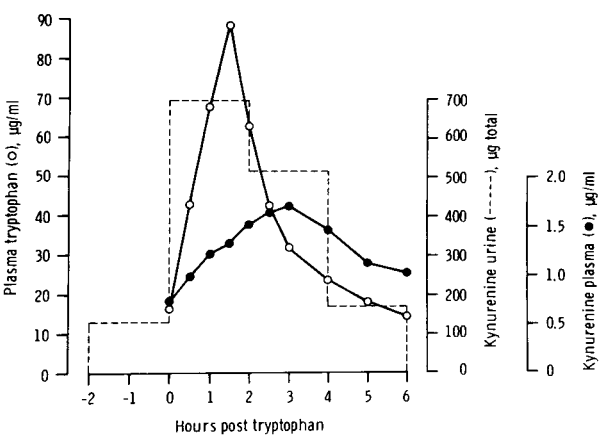

Fig. 4. Time course of plasma tryptophan and kynurenine and of urinary kynurenine following a tryptophan load in man. Tryptophan (3 g) suspended in orange juice was administered at time 0. Nicotinamide (100 mg) had been administered four times the previous day, and at 2 h before tryptophan.

reported here are a little lower than those we reported previously [4], but the present method is more specific than the method used in that study.

Tryptophan administration to man (3 g orally) results in increased kynurenine in plasma and urine (Fig. 4), the plasma results agreeing broadly with those previously presented from this laboratory [5]. The high sensitivity of this method allows a much more detailed study of the dynamics of kynurenine formation and excretion in man than was previously possible, and we

TABLE IV

TRYPTOPHAN AND KYNURENINE IN BRAIN AND PLASMA FOLLOWING TRYPTOPHAN ADMINISTERED TO RATS

Tryptophan was dissolved in 0.33 *M* HCl and neutralised with 0.155 *M* sodium carbonate to yield a neutral solution in isotonic saline. It was administered i.p in a volume of 1 ml per 200 g rat. Controls were injected with vehicle alone. Animals were decapitated 2 h later.

Administration	Number of observations	Brain (mean±S.D.)		Plasma (mean ±S.D.)	
		Tryptophan (μg/g)	Kynurenine (ng/g)	Tryptophan (μg/ml)	Kynurenine (ng/ml)
Control	6	1.90±0.35	115±38	20.8±3.0	1064±237
Tryptophan (50 mg/kg)	8	4.00±1.06*	201±35**	34.4±9.3*	1334±261
Tryptophan (100 mg/kg)	6	5.02±1.50**	228±94	37.3±5.3**	2481±347**

*Different from control $p<0.01$.
**Different from control $p<0.001$.

are currently applying this approach in studies on psychiatric patients and normal controls.

Tryptophan administration to rats (50 or 100 mg/kg i.p. to male Sprague Dawley rats, 200—250 g) leads to an increase in kynurenine in plasma and in brain (Table IV). The demonstration of kynurenine in rat and human brain [11, 13, 14], together with the demonstration of the necessary enzyme in rat brain [2, 17] and the synthesis in rat brain of labelled kynurenine from labelled tryptophan [14] make it likely that at least a part of the kynurenine normally found in brain is synthesised there, and that at least part of the increase in rat brain kynurenine following tryptophan loading is synthesised within the brain. Since the amount of kynurenine found in rat brain is about half and the amount in human brain about double that of the amounts of 5HT found in the same brain samples [12] the physiological significance of the kynurenine pathway in brain may be considerable.

In summary the use of this method offers the possibility of detailed studies on the kynurenine pathway in man and other species in a variety of tissues including the brain. It also offers the possibility of sensitive and direct assays of tryptophan pyrrolase and indoleamine-2,3-dioxygenase.

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technical assistance with some of the experiments described. Acetyl-kynurenine standard was generously provided by Dr. R.R. Brown (Madison, Wisc., U.S.A.).

NOTE ADDED IN PROOF

A fuller account of this work has now appeared [18]. The kynurenine levels reported in whole rat brain agree with the present report, but these authors report a lower level of kynurenine in rat plasma, and also report kynuramine in rat brain and plasma, which could not be detected in the present study.

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Biomedical Applications

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DETERMINATION OF URINARY THIAMINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY UTILIZING THE THIOCHROME FLUORESCENT METHOD

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SUMMARY

A sensitive, reproducible, and specific method for the determination of urinary thiamine has been established. Unique to this method is the use of high-pressure liquid chromatography (HPLC) to separate the fluorescent thiamine derivative from interfering fluorescent compounds. Urine samples were passed through a Decalso cation-exchange column, washed with 0.5 *M* KCl to remove some interfering compounds, and eluted with 3.4 *M* KCl. The eluted thiamine was converted to the fluorescent derivative, thiochrome, by reaction with alkaline potassium ferricyanide. The reaction mixture was extracted with isobutanol and subjected to HPLC monitored by a fluorescent detector.

Within-day and day-to-day coefficients of variation proved to be 2.5% and 1.2%, respectively. Recovery of added thiamine (range 0.04 to 2.0 μg/ml) averaged 99.9 ± 5.3%. The sensitivity of this method was 0.03 μg/ml.

INTRODUCTION

The evaluation of possible thiamine deficiency has been attempted by several methods. Erythrocyte transketolase assays [1] have been used even though the assay is difficult and its results considered inadequate by some investigators. The determination of urinary thiamine excretion has been employed to evaluate

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the nutritional status of thiamine in human surveys [2—4]. Other studies suggest that thiamine-to-creatinine ratios might provide a better index of thiamine deficiency [5, 6].

The urinary thiamine assays incorporate conversion of thiamine to thiochrome, which was first described by Jansen [7] in 1936. The early studies incorporating this reaction [8, 9] suffered from low recoveries and non-specific interfering substances. Subsequent investigations attempted to eliminate these problems by modifying the early procedures. Cyanogen bromide has been used in place of potassium ferricyanide to optimize the oxidation of thiamine [10]. Burch et al. [11], using whole blood and blood cells, corrected for non-thiochrome fluorescence by measurement before and after ultraviolet destruction of thiochrome. Haugen [12] eliminated the chromatographic separation [8, 9] by incorporating a blank tube containing benzenesulfonyl chloride which destroys the thiamine[13]. Leveille [14] modified Haugen's procedure by adding a third recovery tube to correct for quenching effects. Schultz and Natelson [15] used Amberlite CG-50 resin, adjusted the pH to 9.8—10.0 for the thiochrome reaction and subsequent extraction, and incorporated a urine blank containing no potassium ferricyanide in an attempt to eliminate non-specific fluorescence.

This study describes a procedure that eliminates urinary compounds which inhibit the thiochrome reaction. The utilization of HPLC further eliminates non-specific fluorescence.

EXPERIMENTAL

Reagents

Distilled, deionized water was used to prepare all of the reagent solutions. All compounds, unless noted otherwise, were reagent grade and purchased from commercial sources.

Isobutanol was Spectra AR grade from Mallinckrodt (St. Louis, Mo., U.S.A.) Thiochrome was purchased from Pfaltz & Bauer (Stamford, Conn., U.S.A.) and was dissolved in isobutanol to a final concentration of 0.1 μg/ml.

Thiochrome Decalso (Permutit-T), 50—80 mesh cation-exchange resin, was washed with water, 0.5 *M* acetic acid, 3.4 *M* potassium chloride, and finally with water until the final wash did not yield a precipitate with 2% silver nitrate. The resin was dried at 90° for 24 h and prepared as a water slurry when the columns were prepared.

Alkaline potassium ferricyanide was prepared immediately prior to use by diluting 4.0 ml of 1% potassium ferricyanide to 100 ml with 3.8 *M* NaOH; it was stored refrigerated in a brown bottle.

Thiamine hydrochloride (M.W. 337.3) was dried at 90° for 24 h before preparing standard solutions in 0.1 *M* HCl. The working thiamine solutions were 0.15, 0.30, 0.60, and 1.20 μg/ml (1 mg of thiamine hydrochloride equals 0.79 mg of thiamine). An intermediate thiamine standard solution of 10 μg/ml in 0.1 *M* HCl was prepared also. All thiamine solutions were stored refrigerated in brown bottles. The 0.5 *M* sodium acetate buffer was prepared by adding 20 ml of 2.5 *M* sodium acetate solution to 35.5 ml of 1.0 *M* HCl and diluting to 100 ml with water; the pH, if necessary, was adjusted to 4.2.

Methanol was glass-distilled over magnesium turnings and subsequently degassed and filtered through a sintered-glass funnel. Diethyl ether was similarly prepared except no magnesium was used when distilling. The HPLC mobile phase was methanol—diethyl ether (22:88 v/v) and was prepared daily.

Apparatus

Bio-Rad 10 × 0.7 cm I.D. glass-barrel Econo-Columns were used for the ion-exchange columns. The columns were filled to 9 cm with resin as an aqueous slurry and lightly tapped while settling.

An Altex 250 × 3.2 mm I.D. stainless-steel column containing stainless-steel frits and packed with LiChrosorb 5 -μm particles was used for HPLC. The column was initially washed by pumping methanol for 1 h. An Altex six-port sample injection valve equipped with a 25-μl external sample loop was used. A Milton-Roy controlled-volume mini-pump and a No. 1309 LDC fluoro-monitor equipped with a 360/400 + filter kit were employed. A Model No. 161 Linear Corp. chart recorder was used.

A Buchi Rotavapor >R< with water-aspired vacuum was used for all sample concentrating. An International Equipment Co. centrifuge, Model HN-S, was used in all sample preparations.

Samples

The urine samples were 24-h collections from healthy males and either 24-h or 2 × 12-h collections of pediatric patients (Cleveland Clinic Hospital), some of whom were receiving thiamine supplements. In all cases the urine was made acidic (0.1 *M*) with concentrated hydrochloric acid and stored frozen (—5°).

Rat urine samples from a control group of a study involving induced thiamine deficiency were used for recovery experiments only. These urines were made acidic (0.1 *M*) and stored frozen.

Procedure

HPLC method. Human urine (6.0 ml) in duplicate, or 6.0 ml of working thiamine solutions, or 6.0 ml of 0.1 *M* HCl (blank) was added to 2.0 ml of the sodium acetate buffer and 0.20 ml of 3.8 *M* NaOH. For recovery samples, an aliquot of the intermediate thiamine standard solution was added to the urine mixture. For rat urines, 2.0 ml of urine plus 4.0 ml of 0.1 *M* HCl or 4.0 ml of the working thiamine standard solutions (recovery samples) was used. In all cases, the pH was between 4.5 and 5.0. The samples were mixed and centrifuged.

The samples were added to the ion-exchange columns and eluted; the eluates and subsequent 2 × 8.0 ml water washings were discarded. Then 2 × 5.0 ml of dilute acid potassium chloride (0.5 *M* KCl — 0.1 *M* HCl) was eluted and discarded. Thiamine was then eluted with 15.0 ml of 3.4 *M* KCl — 0.1 *M* HCl. Alkaline potassium ferricyanide (5.0 ml) was added to the thiamine eluate and allowed to react for 20 min. The reaction was stopped by adding 15 ml of isobutanol and swirling for 15 sec to reduce excess ferricyanide. The samples were then extracted by shaking for 1 min. The isobutanol layer was then washed with 20 ml of water. Sodium sulfate was added and the isobutanol concentrated (35°—40°) on the Rotavapor to about 1—2 ml. The samples were

adjusted to a final volume of 3.0 ml with isobutanol and then centrifuged.

For HPLC, the mobile phase was pumped at 0.85 ml/min until a steady baseline was achieved. About 0.5 ml of the sample was loaded on the sample loop and then injected onto the column and the "zero time" marked. The peak height method was used for quantitation. In some cases, the thiochrome peak was collected to compare its fluorescent spectrum with that of authentic thiochrome.

For precision studies, a 24-h urine from a healthy male was collected and made 0.1 *M* with hydrochloric acid. This sample was analyzed ten times for within-day precision. For day-to-day precision, 15-ml aliquots were frozen and duplicates run daily for five successive days.

The ion-exchange resin was regenerated by adding 3 × 8.0 ml of 3.4 *M* KCl—0.1 *M* HCl solution followed by water washes until the eluate showed no precipitation with silver nitrate. The resin was used three times before repacking new columns. The used resin was washed three times with 0.0001 *M* NaOH and then treated as described previously and reused. The HPLC column was washed by pumping methanol for 30 min and inverted to remove any contaminants trapped on the upper frit.

Conventional method. After the samples were placed on the column, 2 × 8.0 ml water washes followed by 3 × 8.0 ml hot water washes were discarded. The thiamine was then eluted with hot 15.0 ml of 3.4 *M* KCl—0.1 *M* HCl and treated as described in the literature [6] using a 20-min reaction time. The samples were quantitatively determined on an Aminco-Bowman SPF spectrofluorimeter and, in some cases, fluorescent spectra were recorded and compared with that of authentic thiochrome.

Modified conventional method. This procedure was identical to the conventional method except that, after the hot water washes, 2 × 5.0 ml of the dilute acid potassium chloride was added to the columns and discarded. The

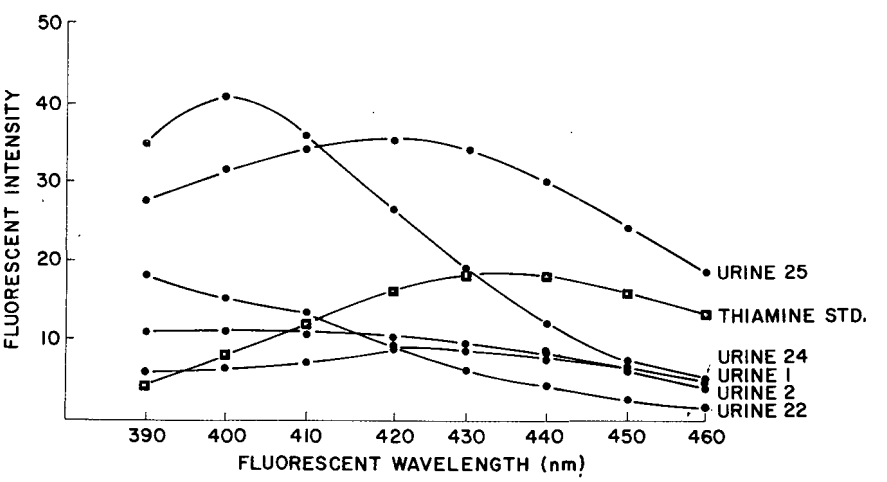

Fig. 1. Fluorescent spectra of urines and thiamine standard analyzed by the conventional method after subtracting respective blanks. Aminco-Bowman SPF; excitation wavelength, 365 nm.

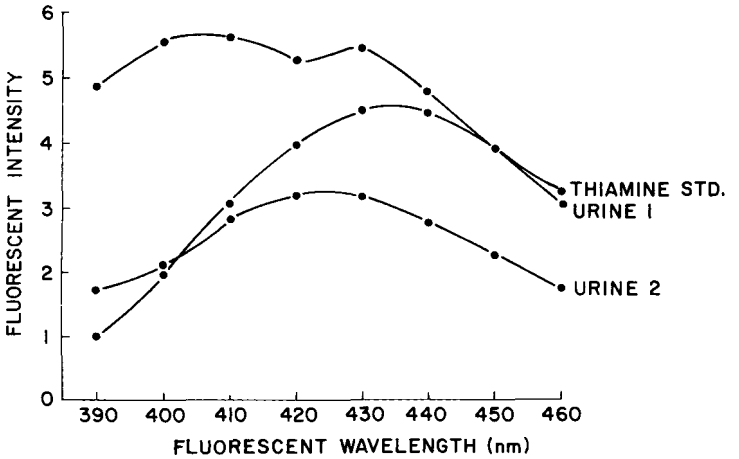

Fig. 2. Fluorescent spectra of urines and thiamine standard analyzed by the modified conventional method after subtracting respective blanks. Aminco-Bowman SPF; excitation wavelength, 365 nm.

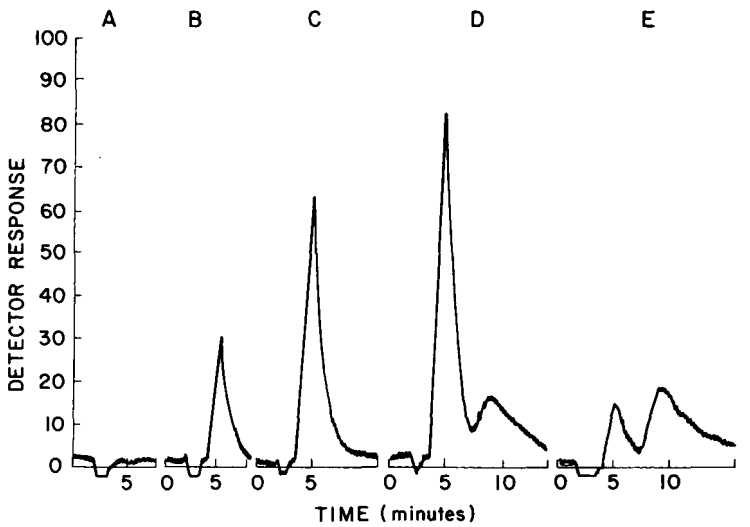

Fig. 3. HPLC chromatogram of: (A) reagent blank; (B) 0.30 μg/ml thiamine·HCl standard; (C) 0.60 μg/ml thiamine·HCl standard; (D) urine No. 7_1; (E) urine No. 24_1. Flow-rate, 0.85 ml/min; chart speed 8 in./h; range = 16; mode A on fluoro-monitor.

thiamine was then eluted with hot acid potassium chloride and treated as described under the conventional method.

RESULTS AND DISCUSSION

Initial attempts to assay for urinary thiamine by the conventional method [6] were unsuccessful, mainly because of low recoveries. Complete reduction of ferricyanide occurred in some cases and precipitation formed in almost

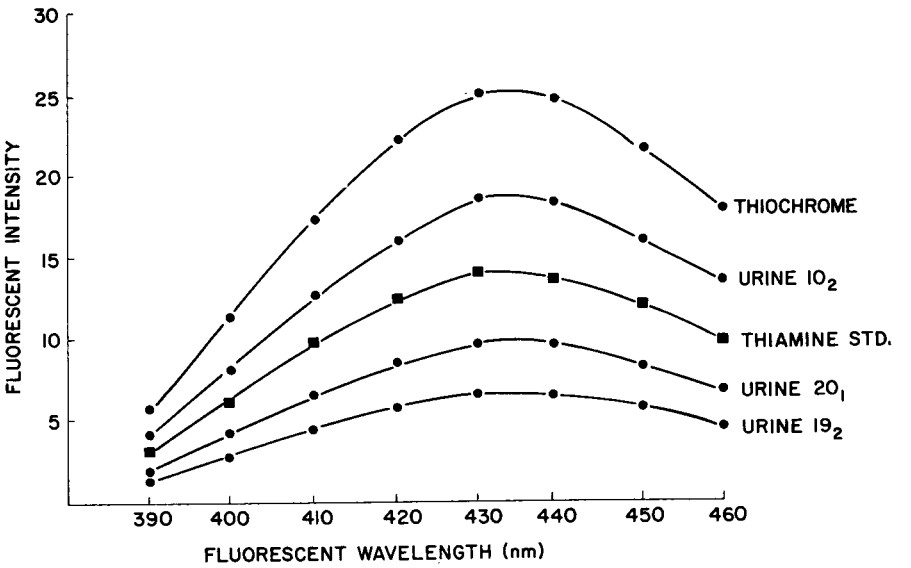

Fig. 4. Fluorescent spectra of samples eluted from the HPLC column at retention time corresponding to thiochrome; read against HPLC mobile phase except for thiochrome (0.10 μg/ml in isobutanol) which was read against an isobutanol blank. Aminco-Bowman SPF; excitation wavelength, 365 nm.

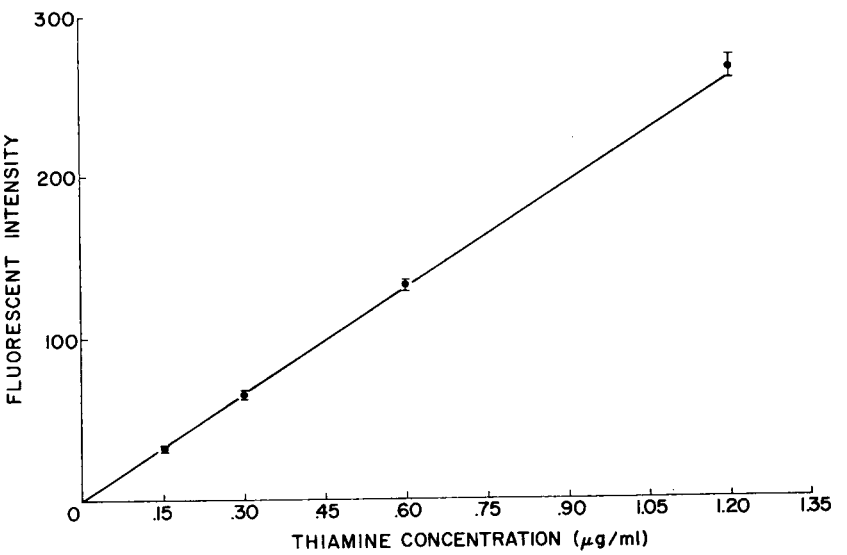

Fig. 5. Thiamine standard curve from a HPLC analysis.

all samples as the thiochrome reaction proceeded. The fluorescent spectra of some urines analyzed by the conventional method are shown in Fig. 1. Obviously, some nonspecific fluorescence was interfering with the assay.

It was found that 0.5 *M* KCl—0.1 *M* HCl solution did not elute thiamine

from the column, but did elute those compounds that reduced ferricyanide and which caused lowered recoveries. A modified conventional method was employed incorporating this solution prior to thiamine elution with hot 3.4 *M* KCl—0.1 *M* HCl solution. Recoveries proved quantitative, but the fluorescent spectra still indicated urinary interference as shown in the spectra of two urines in Fig. 2. It was concluded that the modified conventional method removed urinary inhibition but did not isolate thiochrome sufficiently from non-specific fluorescence.

Excellent results have been achieved using HPLC to analyze for nucleotides [16—19] which are cyclic nitrogenous compounds like thiochrome. Therefore, the HPLC method was set up in an attempt to resolve the thiochrome from the other non-specific fluorescence. A typical HPLC chromatogram is shown in Fig. 3; the urine samples clearly indicate that thiochrome is not the sole

TABLE I

RECOVERY OF THIAMINE ADDED TO URINE BY HPLC METHOD

Type of urine	Concentration of added thiamine·HCl (μg/ml)	Recovery (%)
Human urine		
1	0.40	104
2	0.50	98
3	0.60	102
4	0.80	109
5	0.80	95
6	1.20	108
7	1.20	100
8	1.20	100
9	2.00	88
		Mean 100.4
		S.D. 6.5
Rat urine		
1	0.04	100
2	0.13	103
3	0.16	96
4	0.16	103
5	0.16	100
6	0.30	95
7	0.40	110
8	0.40	98
9	0.50	99
10	0.50	90
11	0.60	102
12	0.67	98
		Mean 99.5
		S.D. 4.8
Average mean 99.9%		
Average S.D. 5.3%		

species being eluted. One prominent non-thiochrome peak was seen in 16 of the 25 human urines tested; some urines had one or two additional non-thiochrome peaks being eluted. The thiochrome peaks were collected and their fluorescent spectra recorded. These spectra are shown in Fig. 4 and, in all cases, confirmed its authenticity as thiochrome. It was evident that the HPLC eliminated the non-specific fluorescence found in the other two methods. A typical standard curve is plotted in Fig. 5 demonstrating the linearity of the HPLC method to 1.20 μg/ml. The actual urinary thiamine concentrations were obtained by the factor method.

The recovery data, demonstrated in Table I, cover a 50 fold range of added thiamine (0.04 to 2.0 μg/ml). The average recovery, 99.9±5.3%, demonstrates

TABLE II

PRECISION STUDIES OF HPLC METHOD

Type of precision	Concentration thiamine·HCl (μg/ml)
Within-day	
1	0.88
2	0.83
3	0.83
4	0.86
5	0.82
6	0.83
7	0.84
8	0.84
9	0.80
10	0.82
Mean	0.84
C.V.	2.5%
Day-to-day	
Day 1	
1	0.86
2	0.83
Day 2	
1	0.86
2	0.84
Day 3	
1	0.84
2	0.86
Day 4	
1	0.85
2	0.84
Day 5	
1	0.85
2	0.85
Mean	0.85
C.V.	1.2%

TABLE III

EFFECT OF POTASSIUM FERRICYANIDE CONCENTRATION ON THIOCHROME REACTION

Potassium ferricyanide concentration (%)	Fluorescence(%)*	
	Thiamine·HCl standard**	Urines***
0.5	99	99
1.0	100	100
2.0	97	99
5.0	84	74

*1.0% potassium ferricyanide used as the base comparison.
**Average of two determinations.
***Average of four human urines.

TABLE IV

EFFECT OF REACTION TIME ON THE THIOCHROME REACTION

Reaction time (min)	Fluorescence(%)*
5	87
10	96
15	100
20	100
30	96

*Average of two determinations; 20-min reaction time used as the base comparison.

that urinary inhibition was eliminated. The precision data for both the within-day and day-to-day runs, expressed as the coefficient of variation (C.V.), are displayed in Table II; values of 2.5% and 1.2% were obtained, respectively. The sensitivity of the HPLC method was 0.03 μg/ml using three times the height of baseline fluctuation as the minimum detectable peak height.

Two aspects of the thiochrome reaction, ferricyanide concentration and reaction time, were further investigated. Stock solutions of 0.5—5.0% potassium ferricyanide were diluted with 3.8 *M* NaOH and reacted with a thiamine standard solution diluted with 3.4 *M* KCl—0.1 *M* HCl. Four human urines were also analyzed, using the HPLC—ion-exchange separatory procedure, using the same ferricyanide solutions. For both urine and thiamine standards, as shown in Table III, only at the 5% concentration was there significant interference. The reaction time was tested by diluting a thiamine standard solution with 3.4 *M* KCl—0.1 *M* HCl, reacting with alkaline ferricyanide, and stopping the reaction after 5, 10, 15, 20, and 30 min. No significant differences were found from 10—30 min reaction time as shown in Table IV.

The urinary values obtained by the three methods are summarized in Table V. The values obtained by the conventional method are higher or lower than

TABLE V

COMPARISON OF URINARY THIAMINE LEVELS OBTAINED BY THE THREE METHODS

Urine sample No.	Concentration of thiamine (μg/ml) HPLC method*	Conventional method	Modified conventional method
22	0.09	0.38	0.40
5	0.12	0.09	0.13
23	0.15	0.14	0.17
2	0.20	0.28	0.33
11	0.27	0.38	0.38
1	0.29	0.41	0.52
4	0.35	0.38	0.41
17	0.41	0.49	0.49
18	0.45	0.38	0.49
24	0.52	0.88	1.01
7	0.60	0.87	0.88
13	0.84	IV**	IV
12	0.85	0.79	0.92
14	1.07	IV	IV
3	1.72	1.67	1.96
25	7.01	11.63	12.01
15	10.24	9.16	10.27
21	10.82	10.43	11.69
19	11.49	9.48	11.22
20	14.45	13.90	14.85
16	17.23	17.06	17.22
6	19.35	18.33	19.28
9	21.71	21.80	21.80
10	113.21	104.28	115.34

*Average of duplicates.
**Insufficient volume.

the HPLC method values depending on which factor is greatest: the urinary inhibition (lowering the thiamine readings) or the non-specific fluorescence (elevating the thiamine readings). In the HPLC method, both factors have been eliminated. Since the modified conventional method removes inhibition, but not non-specific fluorescence, it was expected that these values would be higher than the HPLC values. Such was the case, with the exceptions only occurring in those patients receiving thiamine supplements where non-specific fluorescence becomes less significant.

CONCLUSIONS

A sensitive, reproducible, and specific method for urinary thiamine has been established using ion-exchange chromatography, thiamine oxidation to fluorescent thiochrome with potassium ferricyanide, isobutanol extraction, and HPLC. Urinary inhibition, non-specific fluorescence, and the need for

urine blanks have been eliminated. This procedure is being employed in an attempt to further define effective thiamine deficiency in humans.

ACKNOWLEDGEMENTS

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Journal of Chromatography, 146 (1978) 55—65
Biomedical Applications

CHROMBIO. 168

MISE EN ÉVIDENCE ET ÉVALUATION DES "MOYENNES MOLÉCULES" DE LA TAILLE DE LA VITAMINE B_{12} PRÉSENTES DANS LES LIQUIDES BIOLOGIQUES DE SUJETS NORMAUX ET DE PATIENTS URÉMIQUES

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SUMMARY

Determination of "middle molecules" presenting vitamin B_{12} molecular size in normal and uremic body fluids

Uremic solutes with the molecular size of vitamin B_{12} are assumed to be toxic. An analytical method is proposed to detect and separate these solutes in body fluids using two combined techniques: gel filtration on Sephadex G-15 and ion-exchange chromatography on DEAE-Sephadex A-25. The vitamin B_{12} molecular size has been localized by ultrafiltration through membranes with a defined cut-off. Normal and uremic body fluids (urine, plasma, hemodialysis fluid) have been separated into 9 ultraviolet-absorbing peaks (a to i) by high-speed gel filtration. Peaks b and c present the molecular size of vitamin B_{12}, 10—15 Å molecular diameter in pH 7 aqueous solution. Peak b, which correlates with uremic neuropathy, is separated into 6 sub-peaks (b_1 to b_6) by ion-exchange chromatography, sub-peak $b_{4.2}$ is the only one to correlate with uremic neuropathy. The coefficient of variation in the integrated area of a single peak is 16%. This method gives the chromatographic profile of the vitamin B_{12} molecular size content from 500 µl of uremic plasma or 100 µl of normal urine within one hour.

INTRODUCTION

L'un des problèmes posé par le traitement de l'insuffisance rénale chronique par hémodialyse concerne l'accumulation dans le plasma des patients urémiques de solutés dits "moyennes molécules" d'une taille comparable à celle de la vitamine B_{12} [1] et dont certains seraient responsables de la polynévrite des urémiques. De nombreux auteurs ont utilisé la chromatographie d'exclusion stérique (CES) pour essayer de mettre en évidence ces solutés [2—8]. Jusqu'à présent, devant la diversité des conditions analytiques utilisées, il est impossible de relier entre eux les résultats obtenus.

Ce travail décrit l'analyse des liquides biologiques par CES haute performance et discute l'influence de différents facteurs qui interviennent sur le fractionnement de manière à proposer un système d'analyse reproductible. Les fractions qui contiennent des solutés de la taille de la vitamine B_{12} sont ensuite analysées par chromatographie d'échange d'ions (CEI) haute performance. Cette association de la CES et de la CEI a permis en particulier de mettre en évidence, parmi ces solutés, celui dont une étude ultérieure a montré qu'il est lié à la polynévrite des urémiques.

MATÉRIEL ET MÉTHODES

Préparation des échantillons

Les échantillons sont constitués par le plasma et l'urine de 24 h de sujets normaux (n = 20) et le plasma et les hémodialysats de patients urémiques dialysés (n = 53).

Avant d'être chromatographiés les plasmas sont ultrafiltrés à travers une membrane RP AN-69 (Rhone-Poulenc Industries, Vitry sur Seine, France) pour éliminer les solutés de poids moléculaire supérieur à 20,000 daltons. Les urines et les hémodialysats sont chromatographiés tels quels.

Les ultrafiltrations sur membrane, destinées à chiffrer le poids moléculaire apparent des solutés, sont réalisées sur module Amicon (diamètre 43 mm) ou Millipore (diamètre 13 mm) sous pression d'azote de 3 bars à 2°.

Les membranes suivantes ont donc été utilisées: RP AN-69 et RP à base d'acétate de cellulose (taux de rejet, T_R: 99% pour la vitamine B_{12}) (Rhone-Poulenc Industries); Pellicon PSAC-1000 (T_R: 90% pour la vitamine B_{12}) (Millipore, Bedford, Mass., É.U.).

T_R est donné par la formule: $T_R = 100\ (1-C_p/C_r)$ où C_p est la concentration en soluté du perméat et C_r celle du rétentat.

Chromatographie d'exclusion stérique (CES)

Le fractionnement est réalisé sur colonne de verre 75 × 0.635 cm (Technicon, Tarrytown, N.Y., É.U.)remplie de Séphadex G-15 (Pharmacia, Le Chesnay, France) dans les conditions suivantes: injection dans le flux en haut de colonne par l'intermédiaire d'un injecteur á septum (Touzart et Matignon, Paris, France), avec une seringue pointe 3 (Hamilton), de 5—500 μl d'échantillon à analyser; élution par une solution aqueuse 0.0024 *M* de sulfate de sodium (Normapur; Prolabo, Paris, France) contenant 0.01% d'une solution à 25% (p/v) de Brij 35 (Touzart et Matignon); réglage du débit d'élution à 36 ml/h par une pompe à piston (Dosapro; Milton Roy No. 296 33) couplée à un amortisseur de pulsations (Technicon); détection des éluats en continu dans l'ultraviolet à 254 nm par un détecteur Varian (densité optique pleine échelle 0.04) ou à 206 nm par un détecteur Schoeffel SF 770 (densité optique pleine échelle 0.1); enregistrement des chromatogrammes sur Servotrace PE 1-10 (Sefram, Paris, France) (vitesse d'enregistrement 30 cm/h).

Chromatographie d'échange d'ions (CEI)

Le fractionnement est effectué sur des colonnes de verre 8 × 0.635 cm remplies de DEAE-Séphadex A-25 (Pharmacia) dans les conditions suivantes:

injection comme en CES dans le flux, par seringue à travers un septum, de 10—500 µl de l'échantillon à analyser; élution à un débit de 30 ml/h par un gradient de sulfate de sodium 0.01 *M* à 0.50 *M* à raison d'une augmentation régulière de 3.5% par min de solution 0.50 *M* dans le mélange; le gradient est délivré par un préparateur automatique de gradient Mixograd (Gilson) relié à une pompe péristaltique Minipuls HP-4 (Gilson); détection des éluats en continu dans l'ultraviolet à 254 nm avec un détecteur Varian (densité optique pleine échelle 0.08) et à 206 nm avec un détecteur Schoeffel SF-770 (densité optique pleine échelle 1.0); enregistrement des chromatogrammes sur Varian A-25 (vitesse d'enregistrement 20 cm/h).

Association CES + CEI

Les éluats de la colonne de CES correspondant aux pics à analyser sont dans un premier temps collectés sur la colonne de CEI (Fig. 1); dans un deuxième temps la colonne de CEI est reliée au préparateur automatique de gradient. On analyse soit 500 µl de plasma, soit 100 µl d'urine.

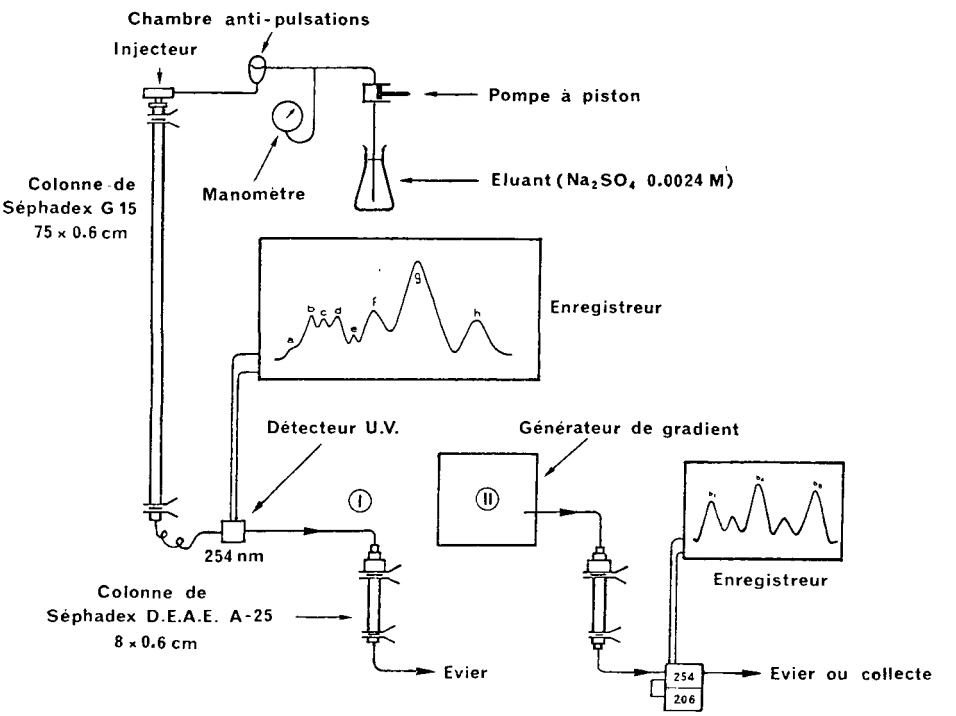

Fig. 1. CES + CEI. I = Fixation des pics b, c ou d sur DEAE-Séphadex A-25 après fractionnement sur Séphadex G-15. II = Élution des sous-pics par un gradient de concentration de sulfate de sodium (0.01 à 0.50 *M*).

RESULTATS

Chromatographie d'exclusion stérique

Etalonnage de la colonne. Cet étalonnage est destiné à établir la relation

entre le volume d'élution d'un soluté et son poids moléculaire (PM). En CES le volume d'élution (V_e) d'un soluté est lié au volume mort de la colonne (V_0), au volume interne des perles de gel (V_i) et au coefficient de partition (K_d) de ce soluté entre la phase fixe (gel) et la phase mobile (éluant) par la relation: $V_e = V_0 + K_d\ V_i$. Le volume mort est déterminé par une solution d'albumine humaine à 1 g/l: V_0 = 7.2 ml dans nos conditions opératoires. Le volume interne est déterminé à partir du volume d'élution de l'urée, de la créatinine, du chlorure de sodium et de l'eau: $V_0 + V_i$ = 13.8 ml dans nos conditions opératoires.

La Fig. 2A représente le taux de rejet, T_R, mesuré pour plusieurs solutés

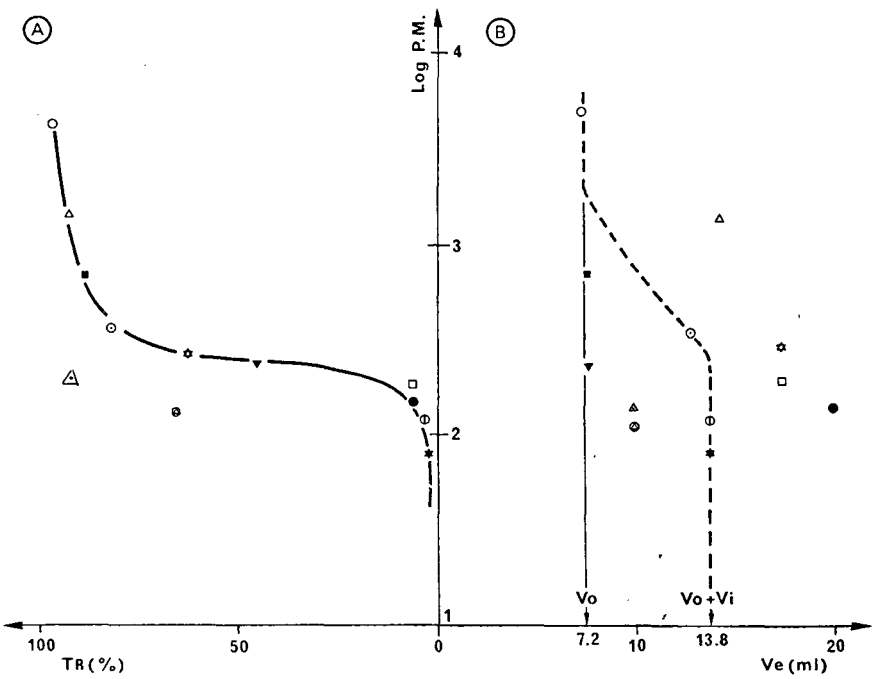

Fig. 2. Comparaison entre: (A) le taux de rejet (T_R) mesuré par ultrafiltration sur membrane RP et (B) le volume d'élution (V_e) obtenu par CES sur Séphadex G-15. Résultats obtenus avec des solutions aqueuses à 1 g/l. ○, Inuline (5200); △, vitamine B_{12} (1355); ▪, calcéine (666); ⊙, glutathion (307); ☆, xanthosine (284); ▼, acides citrique: iso-citrique, cisaconitique (194); □, acide hippurique (180); ●, acide urique (163); ⊿, Na_2HPO_4 (142); ⊘, Na_2SO_4 (142); ⊙, créatinine (113); ★, urée, chlorure de sodium (60,58,5). — — —, Courbe théorique (perméation pure).

par ultrafiltration sur membrane RP à base d'acétate de cellulose. On observe pour la plupart des solutés que T_R est lié au PM, la seule exception concerne les ions sulfate et phosphate pour lesquels il suggère un PM plus élevé.

Par contre les V_e associés à ces mêmes solutés par chromatographie sur Séphadex G-15 sont, pour un grand nombre d'entre eux, indépendants du PM comme le montre la Fig. 2B. On observe pour plusieurs solutés soit un V_e supérieur à $V_0 + V_i$ ($K_d > 1$: pour l'acide urique, l'acide hippurique, la xanthosine) soit un V_e supérieur à celui attendu d'après les caractéristiques du Sé-

phadex G-15 (vitamine B_{12}) ce qui dans les deux cas indique des interactions avec le gel [6, 7, 9, 10]. Ainsi la vitamine B_{12} ne peut pas être utilisée comme étalon. Le comportement des acides citrique, iso-citrique et cis-aconitique est plus intéressant. La Fig. 2B montre qu'ils sont élués comme des solutés de PM plus élevé que le leur. De plus l'expérience démontre que leur volume d'élution dépend du pH de l'élution, il augmente quand le pH diminue jusqu'à une valeur compatible avec leur PM à pH 3. Ce comportement peut être interprété en tenant compte de la nature chimique du Séphadex G-15. La matrice du gel contient en effet des groupements acides carboxyliques [11] qui lui confèrent des propriétés d'échangeur de cations. Des substances chargées négativement seront exclues du gel si la force ionique de l'éluant est trop faible (inférieure à 0.01 *M*). Ainsi il suffira de diminuer le pH ou d'augmenter la force ionique de l'éluant [11, 12] pour éliminer ces effets secondaires. La Fig. 2B renseigne également sur le comportement particulier des sulfates et des phosphates [11, 13, 14], qui sont élués comme des solutés de PM plus élevés.

De tout ceci il résulte qu'il est impossible d'effectuer l'étalonnage universel d'une colonne de CES. Cependant par ultrafiltration sur membrane RP ou Millipore on associe à chacun des pics repérés par CES un T_R qui permet d'attribuer un PM apparent aux solutés correspondant à chacun de ces pics.

Liquides biologiques des sujets normaux. La Fig. 3 représente les chromatogrammes, obtenus par CES, de l'urine et du plasma de sujets normaux. A 254 nm 9 pics détectés dans l'urine sont repérés de a à i dans l'ordre des

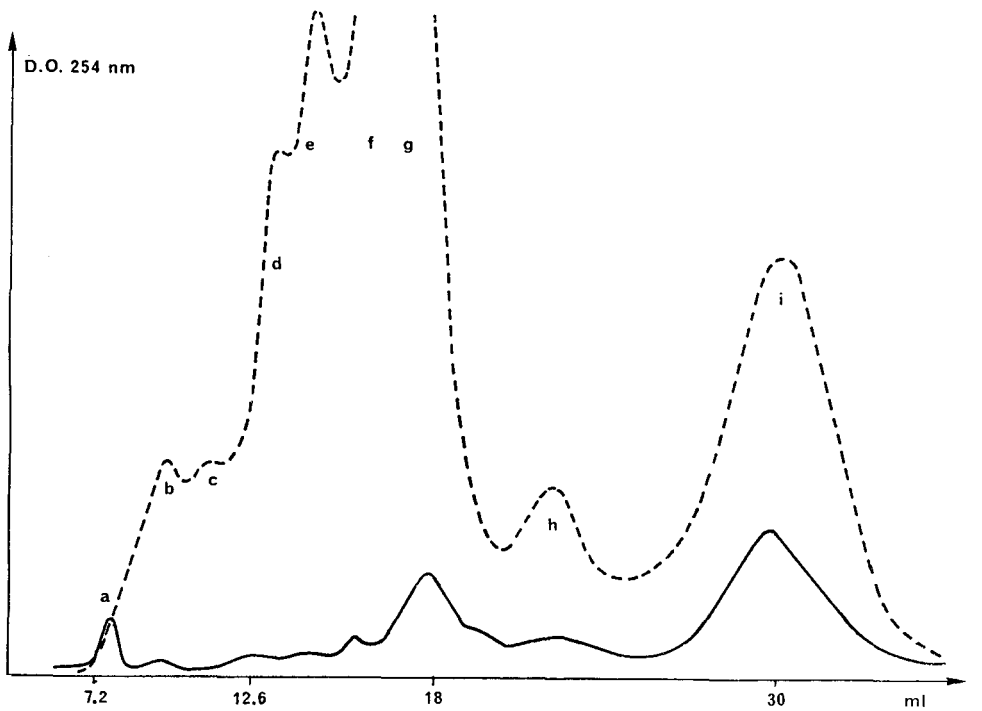

Fig. 3. Chromatographie de l'urine et du plasma de sujet normal sur Séphadex G-15. — — — 5 µl urine; ——, 25 µl plasma.

volumes d'élution croissants. Tous les pics de l'urine sauf le pic c sont présents dans le plasma à des concentrations beaucoup plus faibles. A 206 nm le même nombre de pics est repéré avec des intensités plus fortes.

Liquides biologiques de sujets urémiques. Tous les pics de l'urine (Fig. 4)

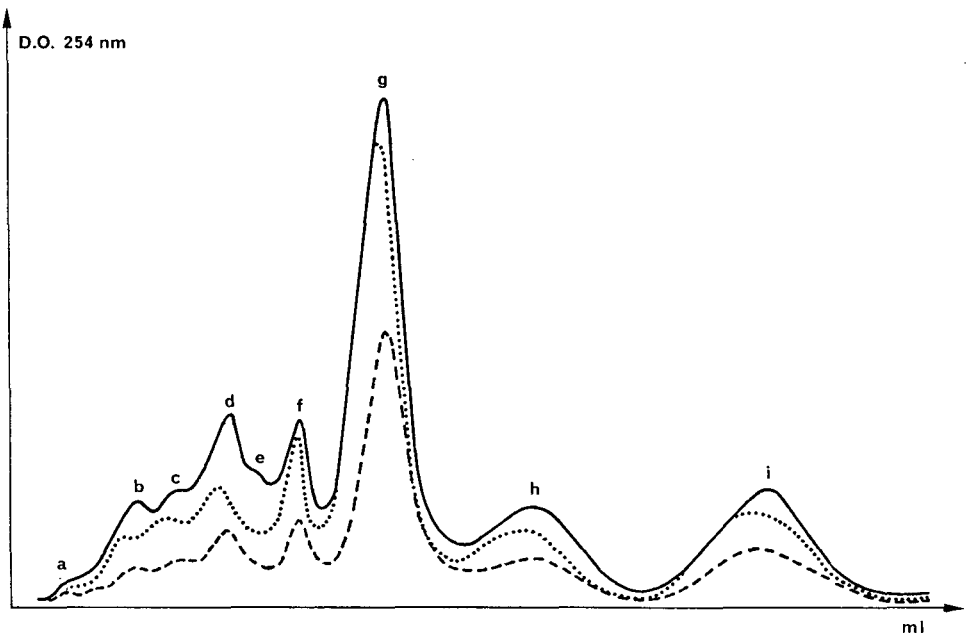

Fig. 4. Chromatographie du plasma d'un urémique avant et après dialyse et du bain de dialyse sur Séphadex G-15. ——, 25 µl plasma avant dialyse; — — —, 25 µl plasma après dialyse; ....., 50 µl bain de dialyse.

se retrouvent dans le plasma des sujets urémiques. Tous ces pics contiennent des solutés dialysables. L'ultrafiltration sur membrane RP et Millipore montre que seuls les pics b et c correspondent à des solutés qui se comportent comme la vitamine B_{12} (Tableau I). Ces solutés, bien que se comportant de la même façon vis à vis des membranes, ont des volumes d'élution différents sur Séphadex G-15. L'influence du caractère échangeur d'ions du Séphadex est ici retrouvée. Ainsi le montre la Fig. 5: les volumes d'élution des pics b et c augmentent avec la force ionique de l'éluant; les solutés correspondants contiennent donc des substances chargées négativement en milieu aqueux à pH 7. Le choix du sulfate de sodium 0.0024 *M* comme solution d'élution repose sur la meilleure séparation des pics b et c dans les conditions analytiques. Il faudra toutefois noter qu'à pH 7 plusieurs polyacides comme l'acide citrique sont élués avec le pic b.

Chromatographie d'échange d'ions

La séparation des solutés qui composent le pic b a été l'objet d'un intérêt particulier. Le chromatogramme obtenu à partir du pic b isolé par CES préparative d'un bain de dialyse de sujet urémique et représenté Fig. 6 a permis de détecter:

à 254 nm: 5 sous-pics b_1, (b_2 + b_3), $b_{4.2}$, b_5 et b_6

TABLEAU I

TAUX DE REJET MESURÉS POUR LES PICS a À i SUR DEUX TYPES DE MEMBRANES, RP ET MILLIPORE

Ces valeurs sont calculées à partir des pics obtenus en CES avec du plasma de patients urémiques.

Solutés correspondant aux pics	T_R (%) Sur membrane RP à base d'acétate de cellulose	Sur membrane Millipore PSAC-1000
a	100	100
b	90—95	65—70
c	90—95	65—70
d	80—84	40—45
e—i	50—55	0—10

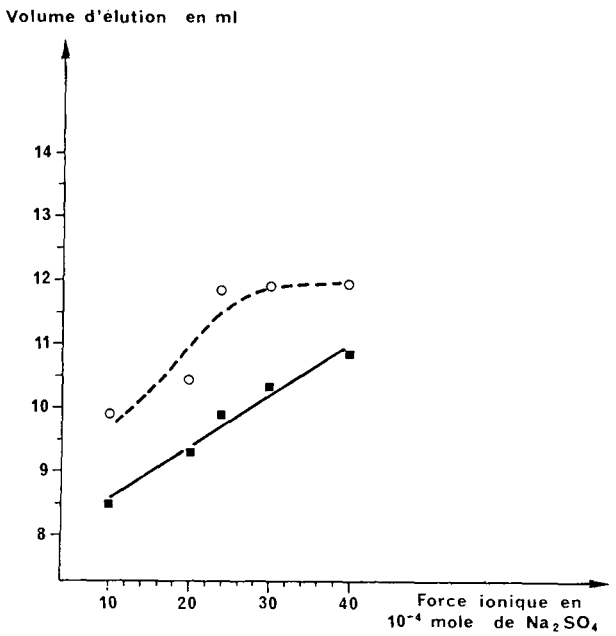

Fig. 5. Variation du volume d'élution avec la force ionique de l'éluant en CES sur Séphadex G-15. ——, Pic b; — — —, pic c.

à 206 nm: 4 sous-pics b_1, (b_2 + b_3), $b_{4.1}$ et b_5.

Les sous-pics $b_{4.1}$ et $b_{4.2}$ sont pratiquement confondus, le sommet du sous-pic $b_{4.1}$ correspond à un léger épaulement du sous-pic $b_{4.2}$ à 254 nm.

·Association CES + CEI

En CES l'injection de 500 µl de plasma, au lieu de 25 µl, ou de 100 µl d'urine, au lieu de 5 µl, est rendue nécessaire par la perte de sensibilité provoquée en CEI par le gradient d'élution. Il a été vérifié que cette injection 20 fois plus forte en CES ne modifiait pas la séparation des pics b, c et d. Tous

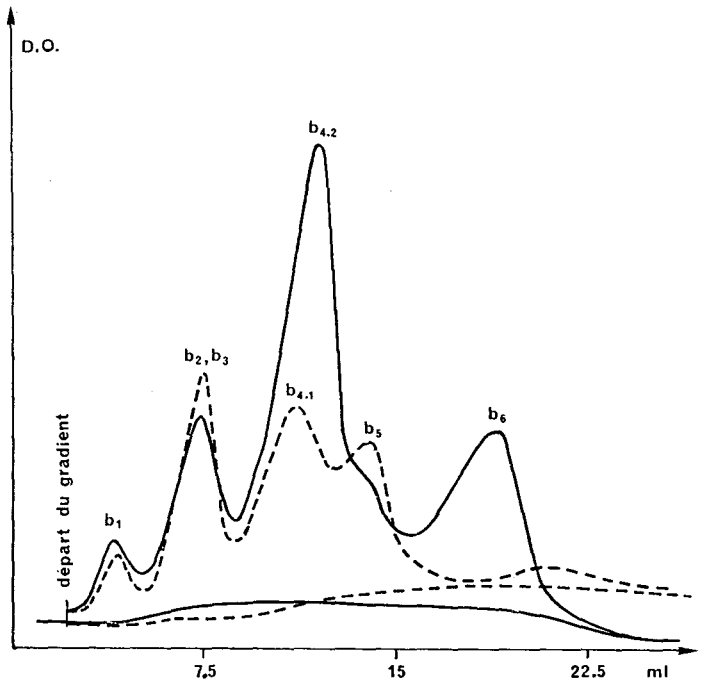

Fig. 6. Fractionnement du pic b par chromatographie sur DEAE-Séphadex A-25. ——, 254 nm; — — —, 206 nm.

les sous-pics b_1 à b_6 sont effectivement repérés après injection de 500 μl de plasma et collecte du pic b. Chez le sujet normal tous ces sous-pics sont présents dans l'urine alors que seuls les sous-pics (b_2 + b_3) sont notablement repérés dans le plasma.

Reproductibilité de la méthode

L'analyse par chromatographie liquide (CES + CEI) permet de déterminer la concentration des solutés détectés par la mesure des surfaces de ces pics.

Les vérifications suivantes ont permis de constater: que le même pic est toujours repéré au même volume d'élution, sur 10 injections l'erreur sur ce volume ayant été de ± 1.2%; qu'à un même volume d'un même échantillon déposé sur la colonne correspond pour chaque pic la même surface. Ainsi pour 13 injections d'un même plasma durant 18 mois, avec changement de la colonne de CES tous les mois, la surface du pic b a été de 404 ± 64 cm^2/ml soit une reproductibilité de 16%. Cette reproductibilité est suffisante pour classer les malades urémiques suivant l'importance des pics détectés. Il faut d'ailleurs noter que sur une même colonne de CES cette reproductibilité est de 10% ce qui signifie qu'une partie de la dispersion est liée, comme il était prévisible, au changement de colonne. Il semble néanmoins important d'intégrer dans la reproductibilité des résultats le changement de colonne. Il est en effet impossible de calibrer entre elles les colonnes sur un échantillon universel car la réponse de la colonne dépend de la nature du soluté déposé sur la colonne.

DISCUSSION

(1) Le volume d'élution d'un soluté sur Séphadex G-15 ne dépend pas uniquement de son PM mais aussi de sa nature chimique surtout si ce soluté est ionisable [11]. Par contre, en ultrafiltration sur membrane, les taux de rejet mesurés sont fonction des PM des solutés, la seule exception concernant les ions sulfates et phosphates. Ces anions, comme en CES, se comportent comme des solutés de PM plus élevés. Ce comportement particulier est dû à l'hydratation qui donne à ces anions un diamètre plus important que celui des ions libres [11]. Nous avons confirmé cette interprétation en observant la variation du taux de rejet mesuré pour l'ion phosphate avec le changement de pH. Ce taux de rejet passe de 90% à pH 7 (forme Na_2HPO_4) à 18% à pH 3 (forme NaH_2PO_4). La diminution de taille que suggère cette variation va bien dans le sens d'une diminution de l'hydratation puisque celle-ci est fonction de la charge des molécules.

Pour tenir compte de toutes ces observations, il semble préférable de dire que les pics b et c contiennent des solutés qui à pH 7 en milieu aqueux ont une taille comparable à celle de la vitamine B_{12} (diamètre moléculaire libre 14.6 Å) [15] plutôt que d'attribuer à ces solutés le PM de la vitamine B_{12}.

(2) Le système de CES a été comparé à celui décrit par Fürst et collaborateurs [6, 7]. Ces auteurs utilisent pour l'ultrafiltration préliminaire des plasmas une membrane Amicon Centriflo qui retient les solutés de PM supérieurs à 50,000 daltons et du Séphadex G-15 pour la séparation chromatographique ultérieure mais dans des conditions analytiques différentes des nôtres. Cette comparaison a conduit aux remarques suivantes: la préparation des échantillons de plasmas par ultrafiltration à travers la membrane RP AN-69 est préférable car il existe dans l'ultrafiltrat obtenu avec la membrane Centriflo des solutés qui sont élués avec le pic b (pic 8 de Fürst), solutés qui sont absents de l'ultrafiltrat sur RP AN-69. De ce fait ces auteurs ne peuvent détecter que les solutés que nous repérons dans les pics c et d (pic 7 de Fürst); l'ordre d'élution est le même dans les deux systèmes mais nos conditions permettent une analyse plus rapide (1 h 20 au lieu de 4 h) et montrent plus nettement les différences qui existent entre les échantillons analysés.

(3) La détection dans l'ultraviolet à 206 nm permet de détecter en milieu biologique le maximum de solutés [16]. Cependant, en CES, la détection à 254 nm est suffisante pour repérer les pics a à i. En CEI il est en revanche indispensable d'effectuer la détection avec deux longueurs d'ondes 206 et 254 nm pour repérer le maximum de solutés. De plus le rapport d'absorption 254/206 permet de mieux différencier les pics (différence entre $b_{4.1}$ et $b_{4.2}$).

(4) La séparation des produits d'origine biologique par CEI sur des supports à base de polystyrène ne permettait pas d'obtenir des résultats satisfaisants [17]. Ainsi les solutés correspondant aux pics b et c après fixation sur résine Dowex 1-X8 n'ont pas pu être élués alors que Takita et al. [18] utilisent une résine comparable (Amberlite) pour fractionner les polypeptides urinaires. Les supports à base de polydextran introduits par Porath et Lindner [19] ont été largement utilisés avec succès pour fractionner des produits biologiques. Malheureusement ces supports non rigides et gonflables ont une résistance limitée à la pression. Ils ne peuvent donc pas être utilisés dans de véritables conditions

de chromatographie haute performance où des débits d'élution élevés sont rendus nécessaires. Un compromis a donc été choisi entre la rapidité d'analyse et la résolution.

(5) La technique associant CES + CEI et décrite ici, est applicable à l'analyse de tous les pics a à i avec des modifications sur les conditions de CEI. Elle a été utilisée pour rechercher plus particulièrement parmi les solutés de la taille de la vitamine B_{12} celui correspondant au pic $b_{4.2}$ qui est responsable de la polynévrite des urémiques [20]. Nous considérons qu'il est indispensable de procéder à ce type de fractionnement, afin de visualiser le maximum de solutés contenus dans les liquides biologiques, avant d'entreprendre l'identification des métabolites présents dans l'urine ou dans les prélèvements d'hémodialysats de patients urémiques. La chromatographie en phase gazeuse associée à la spectrométrie de masse [21—26] est une technique qui a été largement utilisée avec efficacité dans ce domaine mais qui laisse dans l'ombre des produits, soit très polaires, soit de PM élevés non transformables en dérivés volatils et donc non chromatographiables en phase gazeuse [21]. Nous avons précisément observé cette situation au cours de l'étude des solutés qui constituent le pic b. Nous y avons identifié par cette technique les acides citrique, iso-citrique, cisaconitique, tratrique et un produit non identifé alors que le soluté responsable de la polynévrite des urémiques (pic $b_{4.2}$) ne peut pas être transformé en dérivé volatil.

CONCLUSION

En associant la chromatographie liquide haute performance d'exclusion stérique et d'échange d'ions il est possible d'analyser en 1 h les solutés de la taille de la vitamine B_{12} contenus dans 100 μl d'urine de sujet normal ou dans 500 μl de plasma de patient urémique. Le système chromatographique décrit est utilisé pour suivre, chez les patients urémiques dialysés, la concentration plasmatique du soluté repéré comme sous-pic $b_{4.2}$ et qui est responsable de la polynévrite des urémiques.

RESUME

On décrit l'analyse chromatographique des solutés urémiques dont certains de la taille de la vitamine B_{12} seraient toxiques. Les liquides biologiques de sujets normaux et urémiques (500 μl de plasma ou 100 μl d'urine) sont fractionnés par chromatographie d'exclusion stérique sur Séphadex G-15 en 9 pics (a à i) qui absorbent à 254 nm. Par ultrafiltration sur membrane on attribue aux solutés élués dans les pics b et c la taille de la vitamine B_{12}. Le pic b est fractionné par chromatographie d'échange d'ions sur DEAE-Séphadex A-25 en 6 sous-pics (b_1 à b_6); le sous pic $b_{4.2}$ est lié à la polynévrite des urémiques. La méthode proposée est rapide (1 h) et reproducible (coefficient de variation de la surface des pics = 16%).

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MONOACYLCADAVERINES IN THE BLOOD OF SCHIZOPHRENIC PATIENTS

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SUMMARY

Concentrations of cadaverine, monoacetylcadaverine and monopropionylcadaverine in the blood of schizophrenic and nonschizophrenic subjects were measured. Two groups, one from the U.S.A. the other from Japan, were tested. Monoacetylcadaverine and monopropionylcadaverine were found elevated in the blood of some schizophrenic patients in comparison with those in controls in each group. Their increase could be caused by a reduced monoamine oxidase activity or by an increased acylation in schizophrenic patients.

INTRODUCTION

Monoacetylcadaverine and monopropionylcadaverine were identified in the urine of schizophrenic patients [1]. Their connection with the mental illness, however, was excluded in view of current opinion on the exogenous origin of cadaverine.

It was demonstrated that cadaverine in mammals originates from bacterial decomposition of food in intestines [2] or from tissue putrefaction [3]. Moreover, it was shown that the urine levels of monoacetylcadaverine and monopropionylcadaverine could be substantially reduced by administering broad spectrum antibiotics, indicating that the suppression of intestinal bacterial flora resulted in less cadaverine formed exogenously and consequently in less cadaverine catabolites [1].

Some recent findings indicate that the actual role of cadaverine might be different from that of an exogenous contaminant. Cadaverine is physiolog-

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ically present in the mammalian brain and blood [4, 5]. There is also an uptake system for cadaverine in mammalian brain which is inhibited by cyanide and some polyamines [6]. Neither the blood nor the brain concentrations of cadaverine in mice are lowered by the absence of bacterial flora in the intestine [7]. Biosynthesis of cadaverine in the rat kidney was recently reported [8]. Thus, even though there is no doubt that cadaverine is produced by bacteria, the brain concentration of cadaverine is maintained through mechanisms which are independent of bacterial decarboxylation.

Since monoacetylcadaverine and monopropionylcadaverine were originally identified in the urine of schizophrenic patients [1], a comparison between their concentrations in the blood of schizophrenic and nonschizophrenic subjects was made in this study.

METHOD

Subjects

The measurements were performed on blood samples obtained from two groups of subjects. The first group consisted of patients and controls from the United States (N=37), the second group originated from Japan (N=36). In both groups, the schizophrenic patients were hospitalized at inpatient units. They were admitted in a psychotic episode if the presumptive diagnosis was schizophrenia. They manifested delusions, hallucinations and/or a thought disorder as well as inappropriate affect. The patients were free of known organic disease. Their diagnoses were made using criteria by Feighner et al. [9]. Chronic or acute pattern of the illness and schizophrenia subtypes were not distinguished in this comparison. Most of the schizophrenic subjects (with the exception of two naive patients from the second group) were taking phenothiazine medication.

The control subjects included laboratory personnel and neurological patients hospitalized for stroke. Table I gives the composition and age of both groups.

TABLE I

COMPOSITION OF THE TWO GROUPS OF ASSAYED SUBJECTS

		Males	Females	Age (mean ± S.E.)
Group I	Controls	10	4	59.8 ± 3.9
U.S.A.	Schizophrenics	14	9	39.6 ± 2.6
Group II	Controls	15	0	41.0 ± 3.8
Japan	Schizophrenics	21	0	23.5 ± 1.3

Samples

Cadaverine, monoacetylcadaverine and monopropionylcadaverine were measured in samples of the whole blood from the first group and in the blood plasma from the second group. The sample of the venous blood from the first group was transferred into a glass vial containing an equal volume of

0.2 *M* perchloric acid, and the vial was immediately sealed. In the second group, the blood was collected into tubes containing ethylenediaminetetraacetic acid disodium salt (EDTA), 1 mg/ml of blood, centrifuged (150 *g* for 10 min), the plasma was transferred into a glass vial containing an equal volume of the perchloric acid, and the vial was sealed. With each sample in both groups, a second vial was simultaneously filled with the perchloric acid, sealed, and processed as its blank.

Analytical procedures

A thin-layer chromatography—mass spectrometry (TLC—MS) method was used for determinations. Dansyl derivatives formed in the whole blood homogenate (first group) or plasma (second group) with perchloric acid were separated by TLC, eluted, and quantified by MS. The molecular ions of interest were identified by peak matching against ions corresponding to the internal standards and were quantified by the integrated ion technique.

Dansylation and TLC

The sample was weighed and homogenized in five volumes of 0.2 *M* perchloric acid. The whole homogenate was then submitted to dansylation (reaction with 1-dimethylaminonaphthalene-5-sulfonyl chloride), for six hours at room temperature [10]. Dansyl derivatives of amines were extracted into toluene and separated by TLC on silica gel-coated plates (Merck G). The fraction which co-chromatographed with the dansylated compound in question was scraped off, eluted, and separated with the second chromatography system. The TLC fractions were eluted again and their contents measured by high-resolution MS. The solvent systems for chromatography are shown in Table II.

Quantitative MS

A modified version of the integrated ion current technique [11, 12] with the peak matching circuit [13] and the internal standard of a dansylated compound in question [14, 15] was used. A known quantity of an internal standard was added to each eluted chromatographic fraction and the dried mixture was introduced via the probe into the mass spectrometer (AEI MS-902). The list of internal standards and their molecular ions is shown in Table III. The sample was evaporated over 30—45 sec by heating it to 350° into the source maintained at 220°, and ionized with the electron beam energy 70 eV. The molecular ions corresponding to the dansylated compound in question (Table III) and to its internal standard were recorded using the peak matching circuit of the spectrometer at a resolving power between 2000 and 8000. Their molecular ratio was preset with an accuracy of 2 ppm. If the mass ratio between the two matched peak maxima differed at any time during the evaporation of the sample by more than 40 ppm (due to drift of the instrument, sample contamination, or electrical interference), the sample was disregarded. The evaluation of the accuracy of the peak matching and rejection of samples was done by computer (Xerox Sigma-2). The lower values for the number of reported measurements in Table IV compared with Table I were caused by the rejected samples.

TABLE II

SOLVENT SYSTEMS FOR TLC SEPARATION OF DANSYLATED CADAVERINE, MONOACETYLCADAVERINE AND MONOPROPIONYLCADAVERINE

Compound	First chromatography			Second chromatography		
	Solvent	Runs	Eluent	Solvent	Runs	Eluent
Bis-Dns-cadaverine	Heptane—acetone (1:1)	1	Ethyl acetate	Chloroform—triethylamine (10:1)	1	Ethyl acetate
Dns-acetylcadaverine Dns-propionylcadaverine	Heptane—acetone (1:1)	1	Methanol	Benzene—methanol (14:1)	3	Ethyl acetate—acetone (1:1)

TABLE III

MS INTERNAL STANDARDS FOR QUANTITATIVE DETERMINATION OF DANSYLATED CADAVERINE, MONOACETYLCADAVERINE AND MONOPROPIONYLCADAVERINE

Compound	Composition	*m/e*	Standard	Composition	*m/e*	Ratio
Bis-Dns-cadaverine	$C_{29}H_{36}N_4O_4S_2$	568.2178	Bis-Dns-hexamethylenediamine	$C_{30}H_{38}N_4O_4S_2$	582.2344	1.024666
Dns-acetylcadaverine	$C_{19}H_{27}N_3O_3S$	377.1773	Dns-acetylhexamethylenediamine	$C_{20}H_{29}N_3O_3S$	391.1930	1.037159
Dns-propionylcadaverine	$C_{20}H_{29}N_3O_3S$	391.1930	Dns-propionylhexamethylenediamine	$C_{21}H_{31}N_3O_3S$	405.2086	1.035828

TABLE IV

CONCENTRATIONS OF CADAVERINE, MONOACETYLCADAVERINE AND MONOPROPIONYL CADAVERINE IN BLOOD SAMPLES FROM TWO GROUPS OF CONTROL SUBJECTS AND SCHIZO PHRENIC PATIENTS

Values $\times 10^{-12}$ mole/g of wet weight, mean ± standard error; the numbers of subjects are given i parentheses.

Compound	Group	Control subjects	Schizophrenic patients	t*	P**
Cadaverine	I	14.13 ± 2.78 (10)	15.53 ± 1.74 (18)	0.45101	>0.5
	II	7.59 ± 1.32 (14)	16.70 ± 2.73 (21)	2.58470	<0.05
Monoacetylcadaverine	I	3.28 ± 0.37 (13)	33.30 ± 7.93 (23)	2.82916	<0.01
	II	0.48 ± 0.10 (13)	4.14 ± 0.91 (21)	3.14762	<0.01
Monopropionylcadaverine	I	1.70 ± 0.67 (14)	15.75 ± 5.66 (17)	2.23594	<0.05
	II	1.09 ± 0.16 (15)	2.64 ± 0.31 (20)	4.07265	<0.001

*t = t values of t-test.
**P = Level of significance, using two-tailed t-test.

The quantity of the substance of interest in a sample was calculated from the ratio between the intensity of the ion of interest and that generated by an internal standard substance. The calibration functions calculated by linear regression for 12 calibration samples containing known picomole quantities of each compound had the correlation coefficients 0.9922, 0.9914 and 0.9865, for cadaverine, monoacetylcadaverine and monopropionylcadaverine, respectively. The reported concentrations were measured as quantities more than three times higher than their blanks. They were not corrected for losses during extraction and TLC.

RESULTS

As shown in Table IV, the concentrations of the measured compounds were lower in the samples of blood plasma from the second group of subjects than in the whole blood of the first group. With the exception of cadaverine in schizophrenic patients, all other concentrations in the second group were lower than in the first group.

There was no significant difference between the mean concentrations of cadaverine in the blood of controls and schizophrenic patients in the first group. In the plasma samples from the second group, there was a significant increase of cadaverine in schizophrenic patients against controls.

Monoacetylcadaverine and monopropionylcadaverine concentrations in both the blood and plasma of schizophrenics showed a larger variance than those of the controls (Fig. 1). While several values were within the region of control concentrations, the others were almost one order of magnitude higher. The mean values were significantly higher in schizophrenics than in controls for both monoacetylcadaverine and monopropionylcadaverine in both the groups (Table IV).

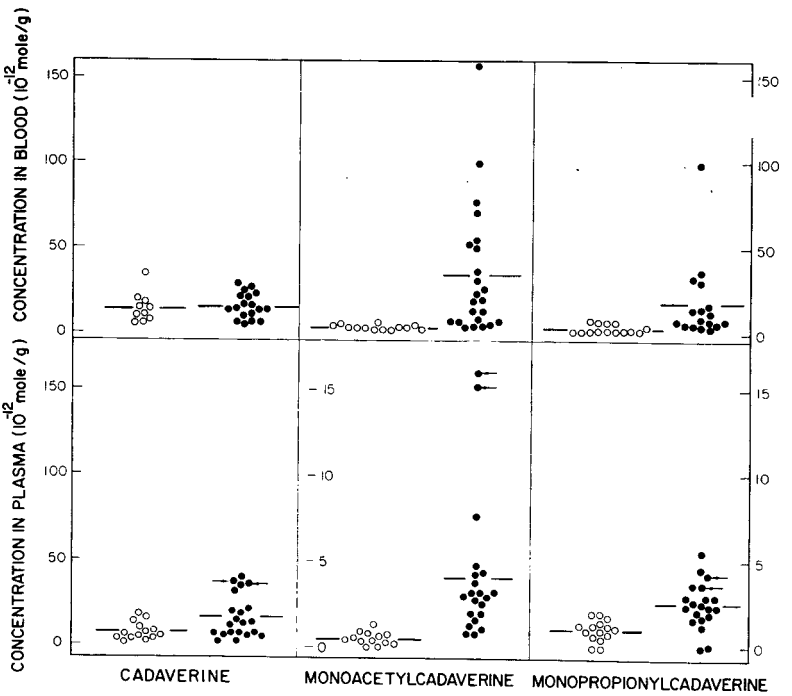

Fig.1. Concentrations of cadaverine, monoacetylcadaverine and monopropionylcadaverine in the blood samples from the two groups of control subjects and schizophrenic patients. Group I in the upper, group II in the lower part of the diagram. Note the expanded concentration scale for monoacetylcadaverine and monopropionylcadaverine in group II. ○, Controls; schizophrenic patients; mean concentrations are indicated by the horizontal lines. The arrows in group II are pointing to values obtained on two newly admitted patients having no medication.

DISCUSSION

This report is based on a relatively small number of subjects and the conclusion about an increase in the blood concentrations of monoacetylcadaverine and monopropionylcadaverine in some schizophrenic patients should not be generalized. Further analysis of elevated monoacylcadaverines in the blood of schizophrenic patients will require an extensive and detailed study.

The lower values of all measured compounds in the plasma of the second group are puzzling. They are not caused by relatively higher concentrations in the blood cells or by the different processing of the samples. Table V shows values of monoacylcadaverines found in test samples processed as the whole blood or plasma in the same manner as were the samples from the first and second group, respectively. The fact that there are different concentration ratios between cadaverine and monoacetylcadaverine or monopropionylcadaverine in the two groups, and that there is a significantly higher mean cadaverine concentration in the plasma of schizophrenics than in controls from the second group while there is no difference in the blood samples of the first group, seems to indicate that the observed differences between the groups might have some biological importance.

TABLE V

CONCENTRATIONS OF MONOACYLCADAVERINES IN IDENTICAL BLOOD SAMPLES PROCESSED AS WHOLE BLOOD OR BLOOD PLASMA

Values $\times 10^{-12}$ mole/g of weight, mean ± standard error; the numbers of blood samples are given in parentheses.

	Monoacetylcadaverine	Monopropionylcadaverine
Whole blood (Group I)	4.00 ± 0.42 (9)	3.97 ± 1.19 (9)
Blood plasma (Group II)	3.20 ± 0.30 (9)	3.98 ± 0.96 (9)

There is a genetic polymorphism in man for metabolism of some drugs (niazid, sulphadimidine) [16—18]. The two major phenotypes, rapid and slow inactivators, differ in the rate of their hepatic acetylation [19]. In Caucasian and Negro populations the slow allele is approximately three times more frequent than the rapid allele, but among Japanese exactly the reverse proportion was found [20, 21]. In a way similar to niazid and sulphadimidine, the observed differences in concentrations of monoacylcadaverines between the first and second group in this study might reflect genetic variations in the acylation of cadaverine, and the distribution of acylator phenotypes for cadaverine in American and Japanese populations, respectively. The use of independent controls for hypothetical slow and fast allele in each population could possibly increase the differences, and their significance, found between the schizophrenic and control subjects, assuming that either group, or both, have genetically controlled bimodal distribution of monoacylcadaverine concentration in the blood.

The elevated blood levels of monoacylcadaverines in schizophrenic patients do not seem to be pharmacologically induced. In contrast, it is possible that phenothiazines are decreasing the elevated concentrations of monoacylcadaverines in the blood of schizophrenics because the concentrations found in samples from the two schizophrenic patients without medication (Fig. 1, arrows) were among the highest in that group.

Both the role and origin of cadaverine, monoacetylcadaverine and monopropionylcadaverine in human blood are unknown. Cadaverine concentrations in the blood and brain have been reported to fluctuate during sleep in mice and during hibernation in molluscs [4, 22].

It has been demonstrated that 1,4-diaminobutane is preferentially acetylated by the rat brain tissue [23]. Because of the low substrate specificity of enzymes metabolizing diamines [2] it seems reasonable to consider the possibility that a similar mechanism might metabolize both 1,4-diaminobutane and 1,5-diaminopentane (cadaverine) in humans. The increase of monoacetylcadaverine and monopropionylcadaverine in blood could be caused by a higher rate of acylation or by a lowered catabolism of monoacylcadaverines.

It is probable that monoacetylcadaverine and monopropionylcadaverine are catabolized by monoamine oxidases, since their four-carbon analogue (monoacetylputrescine) is a substrate for monoamine oxidase in the rat [24]. In order to test this assumption, we have measured brain concentrations

of monoacylcadaverines in mice treated with monoamine oxidase inhibitors. The results in Table VI indicate that monoacetylcadaverine and monopropionylcadaverine are probably the substrates for monoamine oxidases in the mouse.

TABLE VI

THE EFFECT OF MONOAMINE OXIDASE INHIBITORS ON CONCENTRATIONS OF MONOACETYLCADAVERINE AND MONOPROPIONYLCADAVERINE IN THE MOUSE BRAIN

Values × 10^{-12} mole/g of wet weight, mean ± standard error; the numbers of subjects are given in parentheses. The mice were injected for 7 days with 25 mg/kg/day of Nialamide or Pargyline intraperitoneally. Controls were injected with saline.

	Controls	Nialamide	Pargyline
Monoacetylcadaverine	2.7 ± 0.7 (8)	19.8 ± 7.8 (7)*	12.5 ± 2.8 (8)**
Monopropionylcadaverine	3.4 ± 1.0 (8)	17.7 ± 4.4 (6)**	30.4 ± 4.9 (8)***

Levels of significance using two-tailed *t*-test:
*$P<0.05$.
**$P<0.01$.
***$P<0.001$.

The observed higher values of monoacetylcadaverine and monopropionylcadaverine in the blood of some schizophrenic patients could therefore be caused by an inefficient monoamine oxidizing system. Several studies have indicated that an altered activity of blood platelet monoamine oxidase is an accompaniment to mental illness [25—31]. The investigators were also searching for alterations of monoamine oxidase activity in the brain corresponding to those found in blood platelets. Disappointingly, no changes were found in brain monoamine oxidase activity of mental patients as evaluated post mortem [32, 33]. The attempts to search for an endogenous substrate for monoamine oxidase displaying concomitant changes with lowering of blood platelet monoamine oxidase activity were also unsuccessful [34]. Monoacylcadaverines in the blood and their changes might be a promising step in that direction. Irrespective of whether the blood concentrations of monoacylcadaverines are actually dependent on, or a reflection of, brain concentrations of the same compounds they might be sensitive indicators of the functional activity of monoamine oxidizing systems. Ultimately, it would be interesting to see whether by using monoacylcadaverines as substrates, changes in brain monoamine oxidase activity could be detected in mental patients.

The potential use of blood levels of acylcadaverines as biological markers for schizophrenia could be twofold:

(i) Monoacetylcadaverine and monopropionylcadaverine could be simply blood metabolites having no connection with the physiology of the central nervous system or with etiopathogenesis of mental illness. Their concentrations may or may not depend on the overall monoamine oxidase activity and/or on rate of acylation of cadaverine in tissues of the body. Yet, if a significantly high correlation is found between their blood levels and some forms of mental illness, they could be successfully used as an epiphenomenon for a more "objective" form of clinical diagnosis.

(ii) Monoacetylcadaverine and monopropionylcadaverine could be metabolites of cadaverine, preferentially formed in the brain, their blood levels reflecting an equilibrium with the corresponding concentrations in the brain tissue. Their increase in the blood of schizophrenic patients would be caused by a lowered activity of a monoamine oxidase isoenzyme specific for monoacylcadaverines in the brain, or by an increased rate of acylation which might be genetically controlled. This type of enzymatic anomaly could be directly connected with the etiology of some forms of mental illness.

The two types of conditions described above delineate the extremes of a range of different possibilities for connections between the elevated blood levels of monoacylcadaverines and schizophrenia. The actual relationship will be probably somewhere between those two extremes.

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Biomedical Applications

CHROMBIO. 171

ELECTROPHORETIC BIMORPHISM OF SERUM ALBUMIN IN THE PRESENCE OF INDOCYANINE GREEN

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SUMMARY

The presence of indocyanine green during extended traditional electrophoresis and immunoelectrophoresis of serum is associated with bimorphism of albumin. This occurs over a range of dye—albumin molar ratios an order or more greater than was obtained in similar phenomena described previously. The bimorphism seems not to be dose dependent beyond a certain point, and the two albumins so separated show tinctorial differences. The phenomenon has been observed to apparently the same degree in all normal sera tested, and may represent a means of distinguishing ligand-loaded and ligand-light serum albumin.

INTRODUCTION

Serum albumin generally migrates as a single entity during traditional electrophoresis, but in certain circumstances may separate into two or more components. Two fundamentally different types of such electrophoretic polymorphism are recognized [1] and, in both, demonstration depends to some extent upon the conditions of analysis. The primary variety is based upon hereditary differences in amino acid make-up, includes numerous both unusually fast and unusually slow variants [2] and has been appropriately designated "alloalbuminemia" [3]. The other, apparently secondary, form appears to be attributable to either (a) various ligands including non-esterified fatty acids (NEFAs) [4—9], dyes [10] and penicillin and cephalotin [11]; or (b) partial enzymatic degradation as may occur in acute pancreatitis with hyperamylasemia [12].

In previous reports of the varieties of polymorphism of albumin induced by ligands, the quantity of the abnormal or new component present has varied over a relatively narrow range directly with the concentration of ligand, to the extent that complete conversion to the new form has usually occurred with a ligand—albumin ratio of little more than first order proportions. By contrast,

the anomalous fast albumin component described in this communication is not evident below an indocyanine green (ICG)—albumin molar ratio of 32, and even at a dye—albumin ratio of over 1400:1 has not progressed further than approximately a 50% proportion. The mechanism of this phenomenon has not yet been elucidated, but the relatively high concentrations of inducing agent required for full development suggests that the manifestation may reflect alteration of the electrophoretic medium rather than any straightforward dye-binding artifact.

MATERIALS AND METHODS

Blood was taken into plain tubes from healthy male and female human subjects, and allowed to clot before separation of serum by centrifugation. Sera were analyzed immediately after separation, or after periods of up to 5 days at 4°, or after freezing at −70° and thawing at 37°. These various forms of storage were found not to affect the results of the experiments. The electrophoretic and immunoelectrophoretic analyses were carried out with the serum, undiluted, and at dilutions of 1:2, 1:4, 1:8, 1:16 and 1:32, with immediately-prior additions of either an aliquot of electrophoresis buffer, or of a solution of ICG (K & K Labs., Plainview, N.Y., U.S.A.; M.W. = 774.99) in electrophoresis buffer at a series of concentrations to give dye—albumin molar ratios as shown in Table I (approximated for albumin in serum at G = 4.0%). Exclusion experiments comparing ICG and Ponceau S (C.I. No. 27195; M.W. = 760.598) were carried out by mixing aliquots of different dilutions of serum with one or two aliquots, alternately, of both dyes dissolved in electrophoresis buffer at concentrations of approximately 0.023 *M*, a short time before electrophoretic analysis.

Cellulose acetate electrophoresis was carried out after the technique of

TABLE I

APPROXIMATE RATIOS OF MOLES OF ICG PER MOLE OF ALBUMIN

Calculated in mixtures of dilutions of ICG (vertical) and of serum (horizontal). Thus ICG at 0.9% and serum at 1/32 gives a dye—albumin ratio of approximately 640:1. ND = not done.

ICG solution G(%)	Serum dilution					
	Undiluted	1/2	1/4	1/8	1/16	1/32
0.0028	ND	ND	ND	0.5	1	2
0.0056	ND	ND	ND	1	2	4
0.0112	ND	ND	ND	2	4	8
0.0225	0.5	1	2	4	8	16
0.045	1	2	4	8	16	32
0.09	2	4	8	16	32	64
0.225	5	10	20	40	80	160
0.45	10	20	40	80	160	320
0.90	20	40	80	160	320	640
1.80	40	80	160	320	640	1280
2.0	44	89	178	355	711	1422

Kohn [13] using materials by Helena Labs., Beaumont, Texas, U.S.A., including a "Super-Zee" semi-automatic wire-bridge type serum sample applicator which delivers approximately 0.2 μl of serum sample onto the electrophoresis strip in the form of a narrow band. Specimens were electrophoresed for 15 min at constant 5 mA per strip (6—7 V/cm) in an aqueous barbital buffer of pH 8.6 comprising 0.015 *M* diethyl barbituric acid with 0.075 *M* sodium diethyl barbiturate. Completed preparations were then stained for proteins by immersion in an aqueous solution of Ponceau S at G 0.9/l and sulphosalicylic and trichloracetic acids each at G 13.4/l and afterwards cleared in a solution of glacial acetic acid 20% in methanol.

Micro-immunoelectrophoresis was carried out after the technique of Scheidegger [14], and simple agar electrophoresis was effected by a similar process but omitting the antiserum stage, the preparations being fixed and stained immediately after completion of electrophoresis. In both types of preparation the medium used was "Ionagar No. 2" (Oxoid, London, Great Britain), 1% in aqueous barbital buffer comprising 0.01 *M* diethyl barbituric acid and 0.05 *M* sodium diethyl barbiturate to give a pH 8.5 ± 0.1, with 0.05% (w/v) sodium azide as preservative. Sample volumes of 3 μl of previously mixed aliquots of serum-dilution and either plain buffer or dye-dilution in buffer were subjected to electrophoresis for 60—180 min at constant 10 mA per slide (approximately 20 V/cm). Immunoelectrophoretic preparations were developed with a rabbit antisera either polyvalent to whole human plasma and serum, or monovalent to albumin (Canadian Hoechst, Toronto, Canada) with a diffusion period of 16—18 h at room temperature in a moist chamber. Each slide was then gently overlaid with a 15 cm long strip of 7.5 cm wide Whatman 31ET chromatography paper to allow absorptive removal of fluid and unprecipitated proteins, and afterwards washed for 48 h in changes of stirred normal saline. Completed preparations were simultaneously fixed and stained for protein in a solution of 0.1% (w/v) each of water-soluble Nigrosin (C.I. No. 50420) and Ponceau S for 16 h, and cleared in changes of 2% aqueous acetic acid. All chemicals used were of analytical grade.

RESULTS

The presence of ICG during electrophoresis resulted in anodal extension of the albumin region to the point of bimorphism, and anodal extension of the precipitin arc in immunoelectrophoresis. In runs of ordinary length, these results were very much as have been previously reported [16]. The very extended runs used here, however, directly increased the effect so that bimorphism was electrophoretically much more obvious, and the precipitin arc in immunoelectrophoresis took on a wavilinear* conformation.

*The word "wavilinear" is coined here upon an analogy to curvilinear, to describe a wave form quality of a precipitin arc linear upon a straight axis. The dictionary definition of the word curvilinear is inadequate in this respect since it distinguishes from the conformation here designated wavilinear neither the lesser type of double arc formed as an "S" by two opposite convexities, nor a simple "single-humped" arc. The word was coined by Wendy Somerville, R.T., and the necessary etymological research was carried out by Dr. C. Wood, Department of English, McMaster University, who certified its contemporary absence from the English language, and that its inclusion would fill a need not presently catered for otherwise.

Cellulose acetate electrophoresis

The presence of ICG in sufficient concentration produced two effects. The first was lengthening of the albumin band due to a new fast component which began to appear at a dye—albumin molar ratio of approximately 30:1 and, at a dye—albumin molar ratio of greater than approximately 45:1, what appeared to be a slightly slower than normal small new component in addition. The second alteration was a slight foreshortening of the gamma globulin spread, combined with the greater lateral diffusion of this component previously reported as dye induced diffusion alteration (DIDA) [17]. At the greatest concentrations, some ICG ran free anodally to the electrophoretic spread of the serum proteins, well ahead of the albumin area. This dye disappeared during processing of the cellulose acetate strip but ICG was evident in processed preparations as a dense green band extending from the origin anodally and fading out into the alpha-2 to alpha-1 regions, presumably reflecting especially firm binding by lipoproteins or other proteins [16, 18].

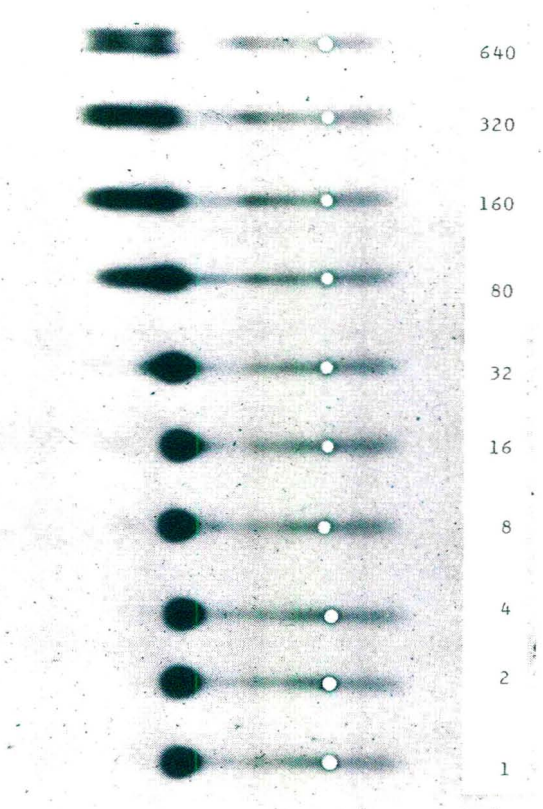

Fig. 1. Simple agar gel electrophoresis of aliquots of normal human serum at a dilution of 1:16 and ICG at the concentrations noted in Table I giving ICG—albumin molar ratios as indicated. Anodes left, cathodes right. The very beginning of the new fast albumin is visible at a molar ratio of 32:1, and is very obvious at all ratios higher than this. At ratios of 160:1, and above, the proportions of the two albumins appear to remain the same.

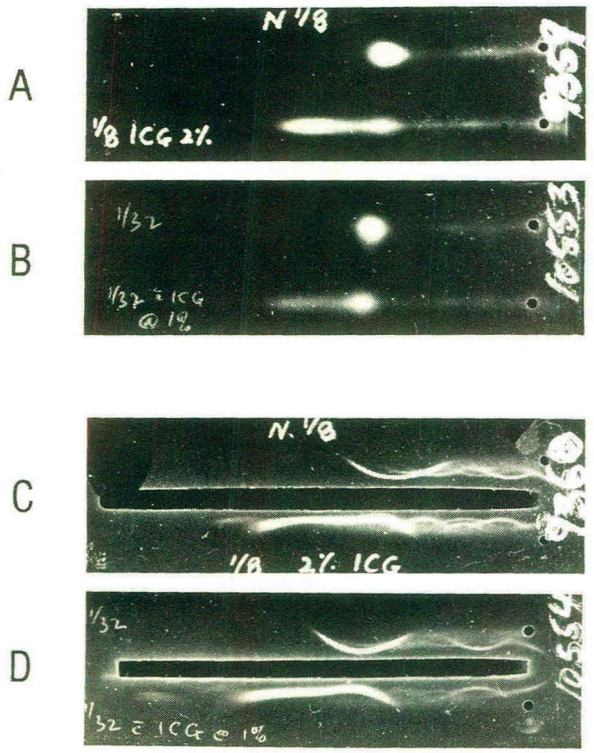

Fig. 2. Anodes left, cathodes right (preparation numbers on right). In each of all four preparations the upper well contains a 1/8 (A and C) or 1/32 (B and D) dilution of native normal human serum while the lower well contains an identical dilution with ICG in a final concentration of 1% (B and D) or 2% (A and C). A and B, simple agar gel electrophoresis. In A the ICG—albumin molar ratio is approximately 355:1 while in B the ratio is approximately 700:1. The electrophoretically fast albumin component is very obvious and, in spite of the molar differences, appears proportionately very much the same in both specimens. Variations in density between the pairs of patterns relates to differences in uptake of stain from the staining solution due to the different final concentrations of albumin in the particular dilutions. C and D, immunoelectrophoretic parallels of A and B respectively, developed with polyvalent rabbit antiserum to whole human serum and illustrating a precipitin line of apparently total identity between the two albumin components. These preparations also show that the ICG—albumin ratio differences between the two specimens do not appear to have affected the quantity or degree of separation of the fast and the slow albumin components. Some other serum proteins are represented by the adjacent faint precipitin arcs developed with this polyvalent antiserum.

Agar gel electrophoresis

In short runs the presence of ICG was associated with short anodal extension of the albumin spot. Extended runs provided greater resolution so that clear bimorphism of the albumin was evident. This bimorphism was first faintly detectable at a dye—albumin molar ratio of approximately 32:1. With further increasing dye—albumin molar ratios the distance between the fast and normal mobility albumin did not increase, but the proportion of fast to slow albumin did increase up to approximately 50:50 proportion at a dye—albumin molar ratio of 160:1. Beyond this ratio no further increase was evident, so that even

at a dye—albumin molar ratio of over 1400:1 the proportion of fast to slow albumin was still no more than approximately 50:50. Dye—albumin ratios greater than this were not explored because (a) solubility of ICG in a buffer at room temperature is little more than 2%, and (b) use of serum at dilutions greater than 1:32 provided albumin spots too faint to be interpreted with certainty. Only minimal cathodal extension of the albumin spot was evident at any dye concentration and however long the electrophoretic run. It became evident at a dye—albumin molar ratio of approximately 40:1, but was undetectable in preparations using high dilutions of serum. An example of the agar gel electrophoretic bimorphism is illustrated in Fig. 1.

Examination of completed agar gel electrophoretic preparations stained in aqueous acetic acid with the Nigrosin—Ponceau S mixture showed that up to an albumin concentration of approximately 0.5% the staining of the fast albumin was different from that of the slow. The slow albumin stained reddish purple with exactly the same tinge as that of albumin in native serum which had been subjected to electrophoretic analysis without ICG prestaining. In contrast, the fast albumin resulting from prestaining with ICG stained almost black, clearly selecting out the Nigrosin from the poststaining mixture. This observation suggested the exclusion experiments referred to previously and indeed it was found that addition of Ponceau S to serum at a final concentration of approximately 0.023 *M* negated almost completely the induction of bimorphism by ICG, whether the Ponceau S was added before or after the ICG at approximately the same concentration. In preparations with albumin at less than 0.5% concentration, the poststaining colours were too faint to be made out with certainty.

Immunoelectrophoresis

Immunoelectrophoretic preparations at all concentrations and at all analytical durations showed no evidence of immunological distinction between the two albumin moieties, nor any difference in apparent precipitin activity. The precipitin arc in immunoelectrophoresis thus took on a wavilinear conformation without any suggestion of crossing or spur formation between its two elements. However, the faster element was less prominent than the slower at the lesser dye—albumin molar concentrations, presumably reflecting less extensive diffusion of the new fast albumin as a result of its relatively lesser proportion at the lower dye concentrations. A typical immunoelectrophoretic result is illustrated in Fig. 2.

DISCUSSION

These results indicate that in the presence of a wide range of concentrations of ICG, two forms of serum albumin can be distinguished by features of electrophoretic mobility at pH 8.4 and relative affinity for Nigrosin and Ponceau S in dilute aqueous acetic acid.

The immunologically indistinguishable reactivity of the two ICG-induced electrophoretic components indicates that both are indeed albumin, and that the new fast moiety is not some other serum protein which has undergone an exaggerated dye-induced electrophoretic mobility alteration (DIMA) as can

occur with alpha-1 lipoprotein and other serum proteins [15—17, 19]. The evidence of immunological identity probably also precludes major but not minor [20] conformational change as a basis for the electrophoretic heterogeneity. It would, however, be consistent with occultation of some of the dye-avid basic groups of arginine and lysine [21] by partial polymerization, or partial saturation of binding sites by natural ligands [22—26] as has been indicated by work with other dyes under other conditions [27—31].

Previously described forms of electrophoretic heterogeneity of serum albumin appear to be distinct from the ICG-associated variety described here. The primary genetic types show close demographic limitations based upon heredity, whereas the ICG phenomenon was present equally in all sera tested from the ethnically very heterogeneous group of normal subjects. Heterogeneity associated with the presence of NEFAs [5—8, 32] which was probably the basis for observations of electrophoretic polymorphism described by Aronsson and Gronwall [33] and McLoughlin [34], or other substances [10, 11], is characterized by progression to complete conversion to the new form across a relatively narrow range of molar ratios commensurate with the recognized [22] number of binding sites on albumin. In sharp contrast, the effect of ICG begins only at a molar ratio at which the effects of other substances are complete, and does not progress beyond a 50% proportion at molar ratios approaching two orders greater. At such levels the dye—albumin molar ratio substantially exceeds that of the buffer molecules, suggesting that the ICG effect may represent no ordinary binding phenomenon but more a modification of the electrophoretic medium. The ICG effect also differs from the transient bisalbuminemia associated with hyperamylasemia in pancreatitis [35, 36] and likely due to enzymatic degradation in vivo [12] in that the dye phenomenon occurs with all normal sera from healthy subjects, is unaffected by incubation, and is not associated with evident antigenic modification.

The observations described here suggest the existence of a new form of dye-induced electrophoretic mobility alteration (DIMA) [15], a phenomenon previously recognized in four different varieties [17]. The phenomenon with ICG and albumin appears, immunoelectrophoretically, to be DIMA-2 (increased electrophoretic spread), but differs from DIMA-2 in that separation of two electrophoretically distinct albumins occurs. Since both of these show immunological identity the dye effect cannot be classified as DIMA-3, which represents separation of different proteins previously appearing as one [19], and thus may represent a fifth form of the phenomenon, i.e. DIMA-5. Definition of this will have to await more extensive characterization of the two ICG—albumins.

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MICRODETERMINATION OF CAFFEINE IN BLOOD BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY*

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SUMMARY

A micromethod for the quantitative analysis of caffeine present in small quantities (100 μl) of whole blood is described. It is based on the gas chromatographic—mass spectrometric analysis of chloroform extracts of biological samples. The method is relatively simple, rapid, specific and sensitive; as little as 20 ng of caffeine can be measured.

INTRODUCTION

Caffeine, a plant alkaloid, possesses several pharmacological actions of therapeutic significance such as its ability to stimulate the central nervous system, cardiac muscle and skeletal muscle, to relax smooth muscle, and to produce diuresis. For these reasons, it is formulated in many pharmaceutical preparations. Moreover, caffeine is found in several widely consumed beverages including coffee, tea, cocoa, maté and various cola-flavored "soft drinks". Of potential significance are reports that it is mutagenic in bacteria [1, 2] and clastogenic in cultured human lymphocytes [3—5], and that it inhibits cyclic 3′:5′-nucleotide phosphodiesterase [6, 7], post-replication DNA repair [8, 9], and mitosis [10] in various mammalian cells.

A great deal of investigative effort has been directed toward determining the physiological disposition of caffeine [11]. However, these investigations were often handicapped in that a rapid, simple, specific and sensitive method for measuring this drug in biological samples was not available. Of those available prior to 1968 (listed and referenced in ref. 12), the methods of choice were

*A description of this investigation has appeared in a thesis submitted by R.L. Merriman in 1976 to the Department of Pharmacology, University of Minnesota, Minneapolis, Minn., in partial fulfillment of the requirements for the Doctor of Philosophy degree.
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based on spectrophotometric analysis. These methods lacked sufficient sensitivity and specificity for estimating pharmacologically reasonable caffeine concentrations in biological material.

A significantly improved method for the determination of caffeine was described by Grab and Reinstein [12]. This method was based on the gas chromatographic separation and quantitation of caffeine extracted from biological samples with chloroform. The minimum amount of caffeine that could be quantitated accurately was about 500 ng. Ingestion of pharmacological amounts of caffeine, ca. 100 mg, produces blood caffeine concentrations that can be accurately monitored for several hours by this method provided that blood samples greater than 1—2 ml can be repeatedly obtained.

The present report describes a gas chromatographic—mass spectrometric (GC—MS) micromethod that is even more rapid, specific and sensitive; as little as 20 ng of caffeine can be measured. Therefore, accurate estimates of caffeine concentrations in 100 μl or less of biological samples can easily be made following the ingestion of pharmacological amounts of caffeine*

EXPERIMENTAL

Apparatus

A Finnigan Model 3200 gas chromatograph—mass spectrometer (Finnigan, Sunnyvale, Calif., U.S.A.) was used. The column was a silanized U-shaped Pyrex glass tube, 0.25 in. O.D. (2 mm I.D.) and 5 ft. long, packed with 3% Dexsil 300 on 80—100 mesh Chromosorb Q (Supelco, Bellefonte, Pa.,U.S.A.). Prior to its initial use, it was conditioned overnight at 350° with the carrier gas flowing. Helium (14 SCCM) was used as the carrier gas. Operating temperatures were: column, 210°; injection port, 260°; separator, 255°; transfer line, 245°. Electron energy was 68 eV and the emission current was 102 μA. Responses were recorded by a Sargent-Welch Model DSRG two-pen recorder (Sargent-Welch, Skokie, Ill., U.S.A.).

Chemicals

Caffeine, the emulsifying agent mannide monooleate, and glutethimide were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.), Sigma (St. Louis, Mo., U.S.A.), and Ciba-Geigy (Summit, N.J., U.S.A.), respectively. Chloroform (ACS certified) and acetone (ACS certified) were obtained from Fisher (Fair Lawn, N.J., U.S.A.).

A caffeine-containing oil—water emulsion was prepared for subcutaneous injection by sonicating a mixture of caffeine in 0.9% NaCl solution (12.5 ml; 20 mg caffeine/ml), mannide monooleate (5 ml), and light mineral oil (7.5 ml).

Glutethimide was dissolved in chloroform (1.35 μg/ml). It was chosen for use as the internal standard for the GC—MS assay of caffeine because (1) both it and caffeine are readily extracted from aqueous solutions by chloroform,

*Subsequent to the completion of the present investigation but prior to its submission for publication, a report describing the use of GC—MS to quantitate caffeine content was called to our attention [13]. Deuterated caffeine was used as the internal standard in these studies. The lower limit of sensitivity was not reported.

(2) its retention time is very similar to that of caffeine (Fig. 1), and (3) it shows a convenient fragmentation pattern for use in GC—MS.

Preparation of biological samples

Caffeine was subcutaneously injected, 200 mg/kg (20 ml/kg), into the inguinal region of 80—90-g male Holtzman rats. Blood samples, 0.1 ml, were drawn from the tail vein into heparinized pipettes at various times after caffeine injection and analyzed for caffeine content as described below.

Analytical procedure

Blood samples 0.1 ml, or caffeine in 0.9% solution, 0.1 ml, were added to test tubes containing 0.2 ml of 0.9% NaCl solution and 0.5 ml of the glutethimide in chloroform solution. The mixtures were mixed for 60 sec and the phases were separated by low-speed centrifugation. Preliminary experiments established that virtually all (>95%) of the caffeine and glutethimide partitioned into the chloroform phase. The water layer was removed by aspiration and the bulk of the remaining chloroform phase was transferred with a Pasteur pipette to clean test tubes in order to remove it from precipitated blood constituents present at the chloroform—water interface. The samples were then evaporated nearly to dryness, dissolved in 0.5 ml of acetone and assayed for caffeine content. Caffeine concentrations, relative to glutethimide concentrations, were determined by monitoring the molecular ion of caffeine (*m/e* 194) and the *M*—28 ion of glutethimide (*m/e* 189) in a gas chromatograph—mass spectrometer. A standard curve establishing the relationship between peak-height ratios of caffeine/glutethimide vs. caffeine concentrations was constructed (Fig. 2) and was used in estimating caffeine concentrations in biological samples.

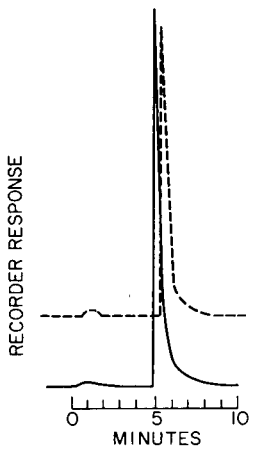
Data analysis

Caffeine/glutethimide peak-height ratios were determined in triplicate for each caffeine concentration to establish a standard curve. The equation describing this relationship was determined by weighted linear regression analysis [14]. The reciprocals of the variances were used as the weights for each datum point. All calculations were conducted with the aid of a Control Data Corporation 6400 Series computer.

RESULTS AND DISCUSSION

The major fragment ion of glutethimide was the *M*—28 ion (*m/e* 189); the molecular ion of caffeine (*m/e* 194) was also the base peak (data not presented). These observations are in agreement with those of others [15, 16].

Retention times for caffeine and glutethimide were approximately 5—6 min (Fig. 1), and sharp peaks with minimal tailing were observed for both agents. The relationship between caffeine/glutethimide peak height ratios and caffeine concentrations is shown in Fig. 2. A linear (r = 0.998) and reproducible relationship was observed over a wide range (1 μM to at least 1 mM) of caffeine concentrations. Values for the slope and y intercept of the regression line defining this relationship were 0.093 and —0.027, respectively. In



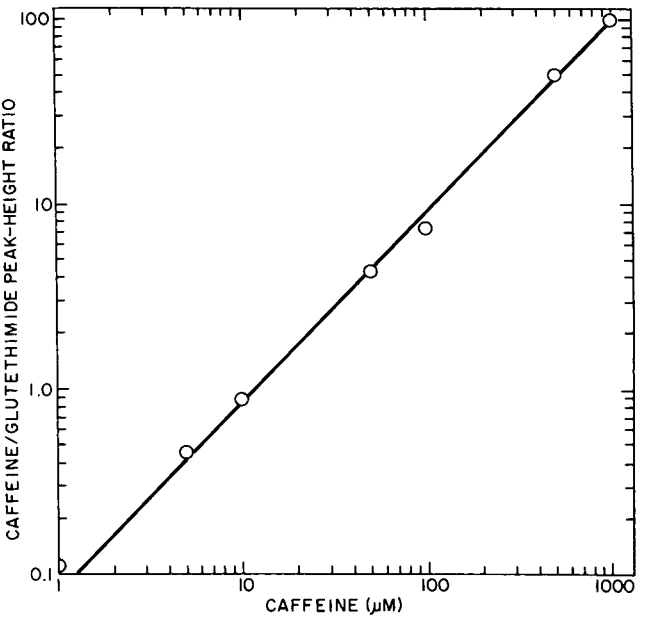


Fig. 1. Gas chromatogram of caffeine (— — —) and glutethimide (————). Recorder responses were obtained by monitoring the appearance of the molecular ion of caffeine (*m/e* 194) and the *M*—28 ion of glutethimide (*m/e* 189) as described in Experimental.

Fig. 2. Standard curve describing the relationship between caffeine/glutethimide peak height ratios and caffeine concentration. Peak height ratios were obtained by GC—MS as described in Experimental.

subsequent experiments, blood caffeine concentrations were estimated from the relationship:

C = (R — intercept)/slope = $(R + 0.027)/0.093$

where C represents the caffeine concentration in μmoles/l and R represents the caffeine/glutethimide peak height ratio.

The lowest caffeine concentration that could be measured with accuracy was about 1 μM since the relationship between caffeine/glutethimide peak height ratios and caffeine concentrations deviated from linearity at lower caffeine concentrations in that the amount of caffeine present was overestimated (data not presented); the reason for this phenomenon was not established. The sample volume was 100 μl. Thus, as little as 20 ng of caffeine could be measured with accuracy.

The maximum plasma caffeine concentration after ingestion of coffee containing 100 mg of the drug is about 100 to 200 ng/0.1 ml [12]. This amount of caffeine would easily be measured by the present method but not by previously available methods unless relatively large sample volumes were obtained. Because the increased sensitivity of the present method permits the use of smaller sample sizes, caffeine concentrations could be determined in blood drawn from the finger tip, ear lobe, or umbilical cord and repeated sampling in a single small animal would be possible.

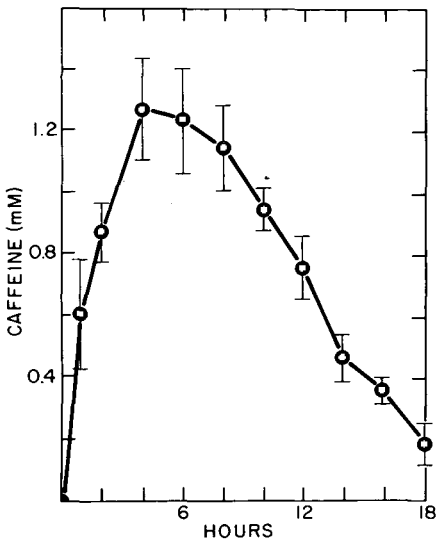


Fig. 3. Blood caffeine concentrations in rats following the subcutaneous injection of caffeine. Three male Holtzman rats were injected with caffeine, 200 mg/kg, and blood caffeine concentrations were determined as described in Experimental. Each point represents the mean ± S.E. of the values obtained.

The present method is also more specific than any method previously employed. Specificity is promoted by (a) differential extraction into chloroform from many potentially troublesome endogenous compounds, (b) differential separation of the extract on the gas chromatographic column, and (c) monitoring of specific mass fragments. Consequently, the probability of an interfering substance or blank error is small.

The method is simple and rapid in that the retention times of caffeine and glutethimide on the gas chromatographic column are only 5—6 min and extraction of caffeine and glutethimide into chloroform is quantitatively and rapidly effected.

Application of the method to a problem of current interest in our laboratory provided data illustrating its utility (Fig. 3). In this experiment, a sustained-release preparation of caffeine was injected subcutaneously into each of three rats and 100-μl blood samples, taken at various times thereafter, were analyzed for caffeine content. Eleven blood samples were taken from each of the three rats. Blood samples taken just prior to caffeine injection showed no interfering peaks. This experiment did not challenge the expected lower limit of sensitivity of the method; the lowest caffeine concentration measured was about 0.2 mM (approximately 4 μg of caffeine). However, the experiment does illustrate that, because of the sensitivity of the method, only small sample volumes are required and therefore multiple blood samples can be taken from small animals, e.g., rats, so that half-lives, etc., could be determined in single animals.

ACKNOWLEDGEMENTS

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PARAFFIN OIL PNEUMONIA

ANALYSIS OF SATURATED HYDROCARBONS IN DIFFERENT HUMAN TISSUES

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SUMMARY

Temperature-programmed gas chromatographic analysis on columns packed with Apiezon L as stationary phase is shown to be the best method for the qualitative and quantitative analysis of simple and complex hydrocarbon mixtures when compared with all the other applicable techniques (thin-layer chromatography, column chromatography, ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy, mass spectrometry) described in this paper. Using the method in a patient with mineral oil pneumonia it could be demonstrated that he expectorated a maximum of 79.5 mg liquid paraffin daily and also transported equally complex saturated hydrocarbons in a concentration of 1.3 mg% in plasma and of 1.6 mg% in the cellular blood components. In an additional experiment the direct determination of liquid paraffin resorbed from the gastrointestinal tract was possible in a patient with a left chyle fistula in the neck. After a dose of 50 g liquid paraffin administered as a laxative, 246 ml chyle was collected within the following 14 h which yielded a total of 4.5 mg liquid paraffin. Its composition was identical with the administered laxative. Assuming a daily lymph volume of 1.5 l, the resorbed amount would correspond to a resorption rate of 0.5 $^{0}/_{00}$ liquid paraffin. The importance of these results as well as the diagnostic consequences arising from the described analytical technique are discussed in detail.

INTRODUCTION

Since the first description by Laughlen [1] in 1925, more than 400 cases of mineral oil (synonyms: liquid paraffin, liquid petrolatum) pneumonia have

been reported in the literature up to 1953 [2] and continue to be described [3]. There is strong evidence, however, that paraffin oil pneumonia occurs much more frequently. Many cases either remain unknown, especially those with only slight pulmonary affection, which are often free of complaints, or are misinterpreted (even in the presence of severe pulmonary involvement [2]) as some other pulmonary disease, such as chronic pneumonia, sarcoidosis, tuberculosis, bronchiectasis, fungus disease or neoplasm (paraffinoma) [4]. Consequently, exogenous lipid pneumonia is commonly an accidental discovery by the pathologist [5]. Intra vitam diagnosis without surgical intervention has only been described exceptionally. Because none of the clinical features is strongly characteristic, the suspicion of mineral oil pneumonia or pulmonary paraffinoma is in most cases only raised by a medical history of paraffin uptake, often unrestricted, and usually in the form of laxatives, oily nose drops or throat sprays. The discovery of lipophages [4,6] in the sputum is not specific, and they are often absent [7]. Histochemical staining methods are only applicable if mineral oil is present in the form of readily visible droplets and none of the various dyes used for lipids is strongly specific for mineral oil [8].

Other methods for the identification of mineral oil have been infrared spectrometry [9—11], nuclear magnetic resonance (NMR) spectrometry [12,13], mass spectrometry [14, 15], column chromatography [16] and thin-layer chromatography (TLC) [7,8]. Though these techniques are virtually specific, they are not valid for reliable quantitative analysis or composition studies of mineral oil preparations ingested for medical purposes and extracted from human or animal tissues under special conditions. Gas—liquid chromatographic (GLC) analysis of liquid paraffin (which is a very complex mixture composed of some hundred saturated hydrocarbons of varying chain length, including straight-chain and branched-chain alkanes, cyclo-alkanes and polycycloalkanes) is the only method to fulfill these criteria tolerably.

In the presented new case of chronic paraffin oil pneumonia, a report is given for the first time about qualitative and even quantitative studies of mineral oil in sputum, plasma and erythrocytes, supplemented by all other methods known so far. The results are completed by our findings in human chyle which was collected after ingestion of a single 50-g dose of liquid paraffin for the treatment of constipation from a man with a surgically induced fistula in the thoracic duct just in front of the angulus venosus sinister.

EXPERIMENTAL

Mineral oil pneumonia was found in a 19-year-old male. The diagnosis was mainly based on a typical 5-year history of gargling with Presido®★, a 99.9% pure mineral oil (total consumption by the patient 5—6 l) and on the histological appearance of an open lung biopsy specimen. Details about the clinical, radiographic and histological findings in this patient will be reported in a separate paper together with new therapeutic approaches [17].

During hospitalization the patient was instructed in breathing exercises and in intermittent exaggerated coughing (although there was no spontaneous

★Paraffin. perliquid. 1000.0, *p*-aminobenzoic acid ethyl ester 0.04. Ol. citri 1.0.

cough) in order to obtain as much expectoration as possible. Prior to this regimen the aspiration of mineral oil (Presido) had been stopped. He continued these activities subsequently as an outpatient.

Saturated hydrocarbon standards were purchased from WGA (Düsseldorf, G.F.R.). All chemicals used were of analytical grade. Solvents freshly redistilled before use were free of hydrocarbon contaminants as tested with all the analytical methods mentioned below.

Extraction and isolation of mineral oil

The volume of each sputum sample (10—150 ml/day) was measured. The sputum was sonified twice for half a minute with maximum energy (Branson-Sonifier) followed by lyophilization. The lyophilisate was extracted once with 150 ml hexane—benzene 1:1 (v/v) for 30 min under reflux conditions. The suspension was filtered and the residue discharged. As proven by eight preceding experiments, more than 99% of the mineral oil content of the lyophilized sputum samples were obtained by this extraction procedure so that further extractions were not necessary. The extract was taken to dryness, redissolved in a small volume of hexane and completely transferred into small PTFE-lined screw cap vials. The solvent was evaporated in a stream of nitrogen. A 100-μl volume of an *n*-undecane (500—600 μg) internal standard, dissolved in hexane, was added and the vial was screwed up, ready for quantitative GLC analysis. Some of the crude sputum extracts were dissolved in 20 ml hexane and divided into two parts. One part was used for further quantitative GLC analysis, and the other pooled for additional analytical experiments.

As demonstrated by TLC on silica gel H coated analytical plates (Merck, Darmstadt, G.F.R.) carried out in the solvent system dichlorethane—methanol (98:2, v/v), sprayed with 50% sulfuric acid after development and charred at 150° for 30 min, the sputum extract contained (besides mineral oil) some lipid contaminants. For further detailed identification studies, these contaminating neutral lipid components were separated from the supposed hydrocarbon fraction by column chromatography. Separation was performed on a 20 × 2.5 cm O.D. glass column packed with 10 g Florisil® (magnesium silicate; C. Roth, Karlsruhe, G.F.R.) slurried in hexane. After application of the crude pooled sputum extract dissolved in hexane on top of the column, a thin-layer chromatographically pure hydrocarbon fraction was eluted from the column with a 100 ml portion of hexane. Recovery rates for this analytical procedure, tested with 100—1500 mg samples of Presido, were 98 ± 2%. The hexane eluate was evaporated to dryness. The residue was used for further investigations. A 500 mg sample of Presido, the gargle preparation that our patient had chronically aspirated, was suspended by sonication in distilled water, lyophilized, extracted and purified in the same manner as reported for the pooled sputum sample in order to get sufficient material for comparison purposes.

For examination of saturated hydrocarbon blood levels, a 100 ml sample of anticoagulated blood (ACD) was obtained from our patient and processed immediately. After centrifugation the plasma was decanted and preserved. The cellular residue was thoroughly washed three times with cold isotonic saline. The supernatant was discharged. Plasma and washed red cells, including leucocytes and platelets, were then analyzed following the extraction and isolation procedures as described above.

Since it has been assumed that mineral oil is absorbed from the gastrointestinal tract, we collected all the lymph discharged from a fistula of the ductus lymphaticus which had been introduced in a male patient in the course of a neck dissection operation. Immediately after ingestion of a 50 g dose of liquid paraffin for the treatment of constipation, we collected the patient's chyle over a 14-h period, divided into three successive fractions. The fractions amounted to 46 ml (4 h), 58 ml (4 h) and 142 ml (6 h), respectively. Each chyle fraction was finally subjected to the complete analytical program for mineral oil determination.

Identification and quantitation of mineral oil

A comparative thin-layer chromatogram of the crude unpurified sputum extract and of the purified remedy Presido was developed with hexane on a silica gel H plate. Spots became visible after spraying with 50% sulfuric acid and charring.

Ultraviolet spectra of the purified sputum extract as well as of the purified Presido, recorded with a Beckman Spectralphotometer, Model DB, did not show any ultraviolet absorption, a finding which is typical for saturated hydrocarbons which are free of contaminants.

Infrared spectra of purified sputum extract and of the purified mineral oil "remedy" were recorded between NaCl-plates with a Perkin-Elmer spectrometer, Model IR 225.

^{1}H·NMR spectra of the two purified samples were registered with the JNM-MH-100 spectrometer (JEOL) at room temperature in hexadeuterobenzene (C_6D_6) as solvent with tetramethylsilane (TMS) as internal reference.

Mass spectra of the identical samples used for infrared spectrometry and NMR analysis were recorded with the MAT III spectrometer (Varian).

GLC analysis

A Hewlett-Packard gas chromatograph, Model 5830A, equipped with a dual flame ionization detector (FID) and an integrator was used for all analyses. For quick routine analysis the chromatograph was fitted with two 50 cm × 1/8 in. O.D. stainless-steel columns packed with 3% SE-30 on Chromosorb Q, 100—120 mesh. All packings were self-prepared. The columns were filled with packing material under vacuum and gentle vibration. Oven temperature was kept constant for 1 min at 90° and then programmed from 90° to 253° at a rate of 30°/min. The maximum temperature of 253° was maintained for a further 9 min before cooling. Recorded analysis time for the crude mineral oil extract derived from sputum, plasma, blood cells and chyle was 13 min. Injection and detector temperature was 280°. The sample size was 0.1—1.0 µl. An 11-ml nitrogen flow was used as carrier gas. Trying a series of column lengths (1/2 m; 2 m; 3.6 m) filled with different packings of low (Apiezon L; SE-30; OV-101) and intermediate (OV-17; OV-210) polarity, we selected SE-30 coated packings in a 50 cm × 1/8 in. O.D. column for the rapid analysis. This non-polar stationary phase, which is resistant to a temperature of 300°, gave the desired separation of the combined peak for saturated hydrocarbons of varying chain lengths (including straight and branched-chain alkanes, cycloalkanes and polycycloalkanes) from *n*-undecane, and cholesterol peaks when programmed over a

wide temperature range. In order to save time when working with our analytical program for a great number of samples, it was advisable to sacrifice as much column efficiency as possible without reasonable variation of reproducibility.

In order to establish the GLC recovery of liquid paraffin we used six standard mixtures (a–f) composed of *n*-undecane (0.5 mg) as internal standard, purified Presido (a = 5 mg; b = 10 mg; c = 20 mg; d = 30 mg; e = 40 mg; f = 50 mg) and cholesterol (a = 0.05 mg; b = 0.1 mg; c = 0.2 mg; d = 0.3 mg; e = 0.4 mg; f = 0.5 mg), each standard mixture running ten times. Though we used an internal standard it became necessary to correct our results following GLC analysis, expressed as mean value of a triplicate determination by a factor of 1.165. This factor was the result of an overall decreased recovery of liquid paraffin amounting to 16.5% ± 2.4% S.D. However, the decreased recovery rate was unreal and the results had to be adjusted by a standard factor, because *n*-undecane did not fulfill the attributes of an ideal internal standard, which besides others should have nearly the same retention time as the sample.

The individual reproducibility of wt.%, expressed as maximum percent deviation of the mean value of ten chromatograms, was 0.2% and 4.6%, respectively. All quantitative results given here for liquid paraffin are mean values of a triplicate determination, corrected by a factor of 1.165.

The following conditions were required for a column to give the best efficiency in composition studies of the complex hydrocarbon mixtures extracted from the different investigated tissues: 2.2 m × 1/4 in. O.D. coiled glass column packed with 12% Apiezon L on Gas-Chrom Q, 100—120 mesh; oven temperature 70—300°, programmed at a rate of 4°/min; injection and detector temperature 300°; carrier gas: nitrogen, flow-rate 28 ml/min (Fig. 5).

RESULTS

According to some earlier reports [7,8] TLC of crude lipid extracts using hexane as solvent system represents a simple and rapid technique for the detection of saturated hydrocarbons. As shown in Fig. 1A this technique has been found effective for the identification of liquid paraffin present in sputum extract and also in all the other human tissues under consideration (plasma, blood cells, chyle). TLC can even be used for the semi-quantitative estimation of saturated hydrocarbons (Fig. 1B), provided that these hydrocarbon mixtures are of approximately adequate composition in comparison to the reference. This limitation is worth mentioning because *n*-alkanes are almost completely resistant to treatment with sulfuric acid. Other paraffin components can be sufficiently charred, but spot size and intensity are not strongly proportional when equal quantities of differently defined branched-chain or cyclic hydrocarbon standards are compared.

For more detailed identification the crude lipid extracts (sputum, plasma, blood cells, chyle) were purified by column chromatography. The isolated purified non-polar lipid samples were used for several spectrometric analyses. The spectra in Figs. 2—4 are representative of all purified extracts (sputum, plasma, blood cells, chyle), and not only for the pooled purified sputum sample. The infrared spectra in Fig. 2 show identical absorption peaks at 1380 cm^{-1} (7.27 μ), 1460 cm^{-1} (6.83 μ), 2860 cm^{-1} and 2925 cm^{-1} for the liquid paraf-

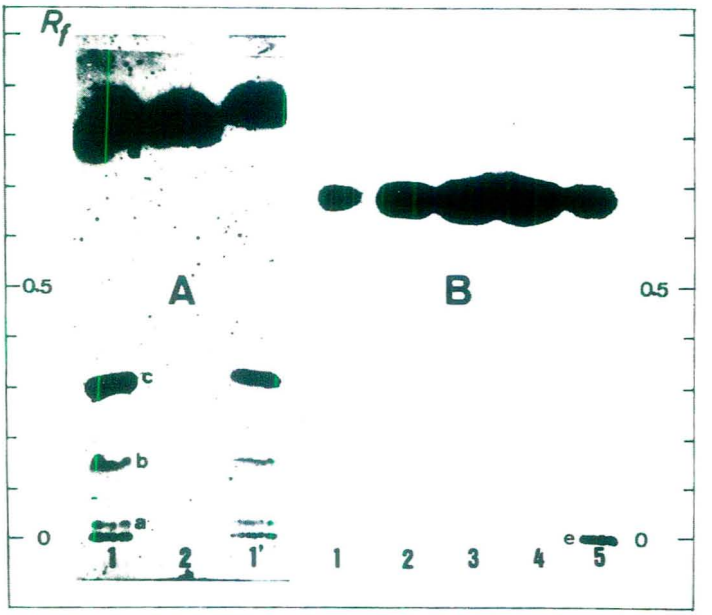

Fig. 1. (A) Thin-layer chromatogram of lipids extracted from sputum in a case of paraffin oil pneumonia (spots in 1 and 1') compared with a standard of pure paraffin hydrocarbons (spot in 2) identical with Presido: a = monoglycerides; b = free fatty acids; c = cholesterol; d = cholesteryl esters and saturated hydrocarbons
(B) Semi-quantitative thin-layer chromatograms of purified saturated paraffin hydrocarbons (Presido) compared with the crude lipid extract from sputum: 1 = 50 μg; 2 = 100 μg; 3 = 150 μg; 4 = 200 μg (all Presido); 5 = sputum extract (hexane—benzene 1:1), e = lipid contaminants. For analytical conditions see Experimental.

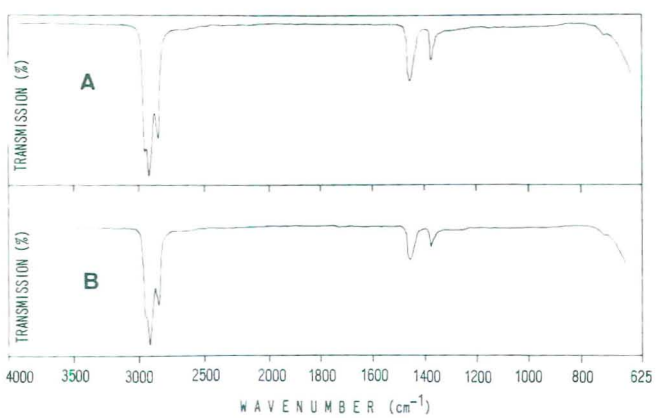

Fig. 2. Infrared spectra of purified Presido (A) and of purified hydrocarbons extracted from sputum (B). For details see Experimental.

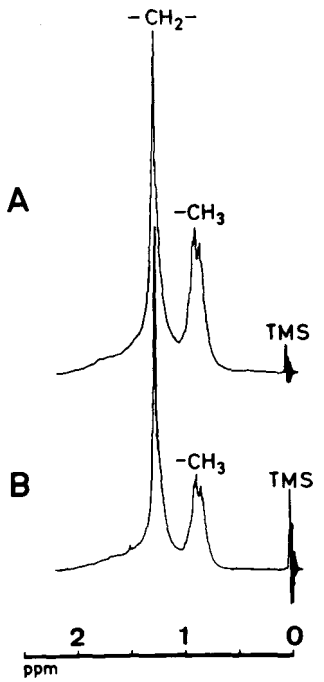

Fig. 3. ^{1}H·NMR spectra of the same samples as recorded in Fig. 2. For details see Experimental.

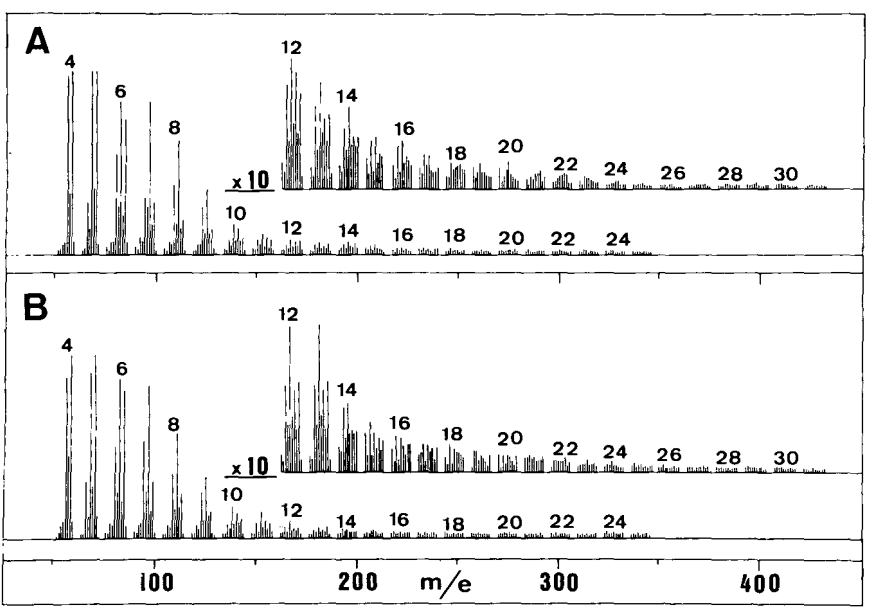

Fig. 4. Mass spectra recorded from (A) purified Presido and (B) purified sputum extract. For details see Experimental.

fin standard (A) and for sputum hydrocarbons (B), thus proving the extracts to be liquid paraffin.

Figs. 3 and 4 show the ^{1}H·NMR spectroscopic and mass spectrometric results of the analyses of a liquid paraffin standard (A) and sputum hydrocarbons (B). Comparison reveals that the spectra are completely identical in each case, except for some insignificant differences in intensity which are of no consequence in interpretation because there is a mixture of compounds. The ^{1}H·NMR spectra show two signals at 1.27 and 0.87 ppm which are typical for CH_2 and terminal CH_3 groupings of a long-chain paraffin [12]. The mass spectra (highest observed mole peak at 436) demonstrate the characteristic fragmentation pattern: a stepwise split-off of CH_2-groups of linear (C_nH_{2n+2}) or cyclic (C_nH_{2n}) hydrocarbons [12]. In accordance with the iodine number being zero, the NMR spectra as well as the infrared spectra of the investigated extracts rule out the presence of unsaturated hydrocarbon compounds.

Due to abnormal paraffins, the resolution of the purified tissue extracts on GLC (Fig. 5) was poor, so that only a few *n*-alkanes, identified by means of pure standards, became recognizable from the typical unresolved broad peak. Chromatograms recorded from pooled sputum extract as well as from many individual sputum samples showed quite the same pattern as did the liquid

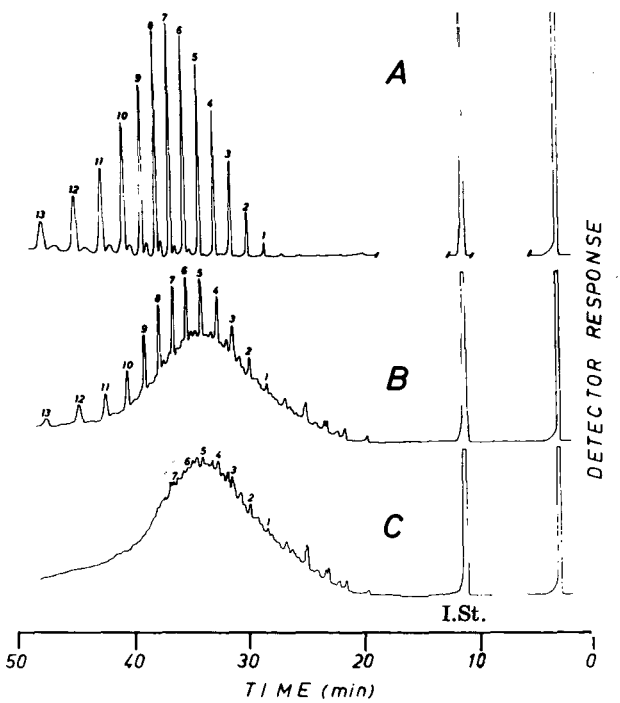

Fig. 5. Gas chromatograms for detailed composition studies of: mixtures of *n*-alkanes used as tissue embedding medium in histology (A); Presido mixed with a small amount of the mixture of *n*-alkanes (B) and purified sputum extract (C) obtained from the patient with paraffin oil pneumonia. I.St. = internal standard. Numbers above the peaks in A, B and C indicate their identification in the normal alkane series (1 = *n*-eicosane and 13 = dotriacontane, respectively). For analytical details see Experimental.

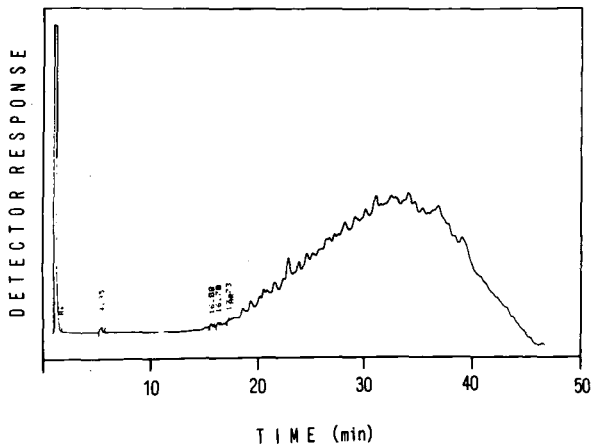

Fig. 6. Gas chromatograms of the purified, saturated hydrocarbon fraction extracted from plasma in the young man suffering from mineral oil pneumonia. For analytical details see Experimental.

paraffin Presido. A GLC pattern quite similar to the sputum extracts could be recorded from the non-polar lipids derived from the patient's plasma (Fig. 6) and blood cells and from the chyle extract (Fig. 7).

Quantitative GLC analyses (Fig. 8) yielded a daily expectoration rate of liquid paraffin within the range of 4.1 mg—79.5 mg measured over a period of 233 days. Paraffin plasma levels were 1.3 mg%. The corresponding value for the thoroughly washed cellular blood residue was 1.6 mg%. The total mineral oil content of the analyzed 246 ml chyle volume, collected within a 14-h period

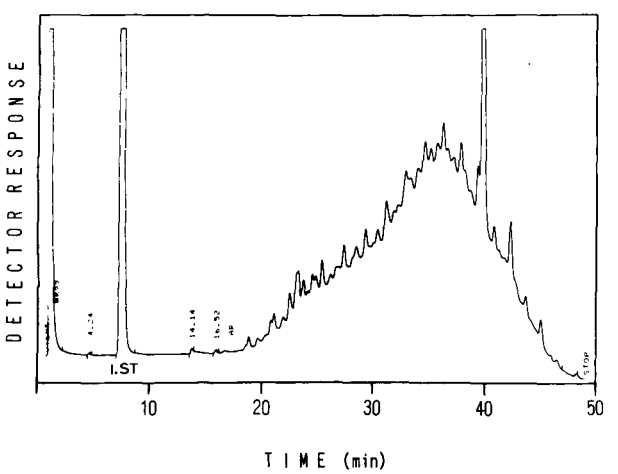

Fig. 7. Fractogram of the purified, saturated hydrocarbon mixture derived from the chyle of a man with a fistula of the thoracic duct. Chyle was collected within 14 h of the ingestion of a 50 g dose of liquid paraffin. I.St = internal standard. For analytical details see Experimental.

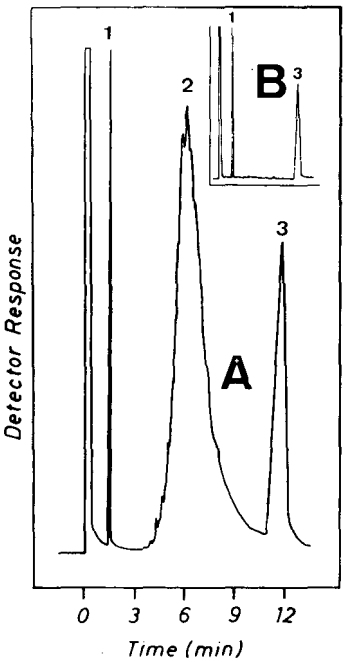

Fig. 8. Quantitative rapid GLC record of a complex mixture of saturated hydrocarbons (2) containing sample extracted from sputum (A) with *n*-undecane as internal standard (1) and cholesterol (3). A sputum sample of a healthy control person is shown in (B). For analytical details see Experimental.

after the ingestion of mineral oil, amounted to 4.5 mg. This value is composed of 0.9 mg, 2.2 mg and 1.4 mg moieties, respectively, when enumerated in the actual collection order of lymph fractions. Since we are quite sure that only a small volume of the chyle formed during the 14-h period could be collected from the observed patient for analytical purposes, we assume that the real quantity of mineral oil absorbed by the human intestinal tract after ingestion is much greater than was measured here.

DISCUSSION

Our results show that GLC is a very suitable method for the qualitative and quantitative demonstration of saturated hydrocarbon mixtures in various human tissues and secretions. It is the only one of the numerous analytical methods for the demonstration of hydrocarbons discussed in this paper to permit, to a limited degree, a characterization and differentiation of simple and complex hydrocarbon mixtures.

The saturated hydrocarbons of vegetable and animal origin consist mainly of *n*-alkanes, with the exception of some fish liver oils which contain considerable amounts of branched-chain alkanes [8]. In contrast, the saturated hydrocarbons found in mineral oil (a defined refinery product obtained from petroleum) contain considerable amounts of very heterogenous cycloalkanes and

polycycloalkanes. It is this very complex portion that distinguishes mineral oil from other saturated hydrocarbon mixtures. Its presence in biological material, which can be unequivocally proven only by GLC, permits the definite conclusion that liquid paraffin has been incorporated. This incorporation can be due to the administration of paraffin oil preparations for medical purposes but also to the ingestion of food since these preparations are frequently used as additives in the food processing industry [18]. The chemical composition of the liquid saturated paraffins which are ingested by humans in rather considerable quantities (an annual per capita consumption of 47.5 g has been estimated for the U.S.A. in 1964 [18]) is by no means completely identical but subject to wide fluctuations, depending on the composition of the crude petroleum which varies from oil field to oil field [19].

This is demonstrated by Figs. 6 and 7 in which small, but distinct and reproducible differences can be recognized in the composition of the liquid paraffin used as a laxative and that of the gargling preparation, Presido. Furthermore, GLC has the advantage that the crude lipid extracts obtained do not have to be purified for the demonstration of hydrocarbons, whereas this is essential for all the other methods described.

The demonstration of complex paraffin in the sputum permits the rapid and definite diagnosis of paraffin oil pneumonia or pulmonary paraffinoma without resorting to lung biopsy. The possible demonstration of lipophages in the sputum [4,6] is no definite proof of the existence of mineral oil pneumonia or pulmonary paraffinoma because the lipophages may be missing. This was the case with our patient, although his expectoration of mineral oil was up to 79.5 mg daily. Likewise, the detection of pulmonary mineral oil deposits in lung biopsy material by the pathologist can at most lead to the tentative diagnosis of exogenous lipid pneumonia since there is no strongly specific dye to permit the definite differentiation of mineral oil from other lipids [8].

Since GLC also yields reproducible quantitative results, it can be helpful in testing various forms of treatment in cases of mineral oil pneumonia. As far as equipment is concerned, an integrator system permitting peak summation is necessary for a quantitative analysis. The demonstration of typically composed complex hydrocarbons in the plasma and blood cells of our patient with mineral oil pneumonia, more than a year after the long-term use of mineral oil, seems to indicate that small quantities of paraffin are being removed from the involved lung segments, probably via the lymphatic channels of the lung. It is also conceivable that the liquid paraffin, which is always deposited in the mesenteric lymph nodes, the spleen and the liver after prolonged oral ingestion of paraffin oil [9] finds its way from these organs into the bloodstream, or that the resorbed liquid paraffin is directly carried into all organs with the bloodstream. The latter assumption is supported both by the postulation of previous authors [20—24] that ingested paraffin oil is resorbed to a considerable extent from the gastrointestinal tract via the lymphatic channels, and by the results of our own first exact measurements in man. Assuming that the daily human lymph volume amounts to an average of 1.5 l (range: 0.5 l—3.0 l) [25], the linear conversion to a total volume of 1.5 l would result in a resorption rate of approximately 0.5‰ of liquid paraffin. This does not take into account that considerably more liquid paraffin can probably be resorbed with

a high-fat diet because emulsifying conditions are better [21]. Although the estimated resorption rate of 0.5‰ in humans is still far below that of 2% measured by Ebert and coworkers [26] with tritiated mineral oil in rates, it is of some importance because there is no evidence that complex saturated hydrocarbons can be metabolized by the human or animal organism. Contrary to medium- or short-chain-*n*-alkanes, which can be oxidized to fatty acids by the animal organism [23,27,28], they are deposited unchanged into the human reticuloendothelial system and sometimes suggest follicular lipidosis of the spleen to the attentive pathologist [10]. That such exogenous lipidosis is not always without clinical importance is demonstrated by the case study of Nochomovitz et al. [9] of a 54-year-old man who died from severe malnutrition which probably resulted from the unrestricted use of paraffin oil as a laxative for many years.

The use of liquid paraffin oil in medicine should be restricted to a few clearly indicated fields, e.g. treatment of intoxications resulting from certain organic solvents.

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CHROMBIO. 156

DETERMINATION OF TRIMETHOPRIM AND SULFAMETHOXAZOLE (CO-TRIMOXAZOLE) IN BODY FLUIDS OF MAN BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of trimethoprim, sulfamethoxazole and its metabolite and a series of structurally related sulfonamides is described. The half-life time of elimination of sulfamethoxazole and its metabolite N_4-acetylsulfamethoxazole is 9 h for both compounds. The renal excretion rate of sulfamethoxazole depends strongly on the urinary pH. The renal excretion rate of the metabolite N_4-acetylsulfamethoxazole is not dependent on the urinary pH.

INTRODUCTION

Sulfamethoxazole in combination with trimethoprim (Co-Trimoxazole®) is nowadays accepted and used as a highly effective antibacterial formulation. Both compounds interfere with folate synthesis in bacteria and many papers have appeared devoted to the clinical applications and its chemotherapeutic activity [1—5].

However in papers dealing with the various aspects of the pharmacokinetic parameters of absorption, metabolism and tissue concentration of the two components of co-trimoxazole, the determinations were carried out with rather intricate or non-specific methods such as microbiological [6], spectrophotometric [7,8], spectrofluorimetric [9] and spectrodensitometric [10] assays or pyrolysis gas chromatographic—mass spectrometric analysis [11].

The use of high-performance liquid chromatography (HPLC) for pure sulfonamides has been described recently [12,13] and some determinations of these compounds in biological fluids have been accomplished [14,15].

The measurement of plasma concentration and renal elimination of sulfon-

amides, especially in patients with kidney insufficiency [16—18] can be of advantage in treatment, as the renal excretion of sulfamethoxazole and of its metabolite N_4-acetylsulfamethoxazole account for 50—100% of the total elimination [19,20].

This has led to the development of an HPLC determination of trimethoprim and sulfamethoxazole in the body fluids, plasma, serum and urine of man. The method described in this communication covers the therapeutic range of concentrations in serum and urine for trimethoprim from 0.1 μg/ml up to 10 μg/ml and for sulfamethoxazole from 1 μg/ml to 200 μg/ml.

The differences in pharmacokinetic behaviour of sulfamethoxazole and its metabolite N_4-acetylsulfamethoxazole under different urinary pH conditions have been investigated.

MATERIALS AND METHODS

Apparatus

A Spectra Physics 3500 B high-performance liquid chromatograph was used, equipped with a spectrophotometric detector (model 770). The detector was connected to a 1 mV recorder (BD7, Kipp & Zonen, The Netherlands). A stainless steel column, 15 cm × 4.6 mm I.D. packed with LiChrosorb RP 8, particle size 5 μm, was obtained from Chrompack (Middelburg, The Netherlands). An injection loop of 100 μl was used. Detection of sulfonamides only was effected at 260 nm, the detection limit is 0.5 μg/ml. Detection for sulfonamides and trimethoprim simultaneously was effected at 225 nm. The detection limit of trimethoprim is 0.75 μg/ml.

Solvents

Sulfonamides. The solvent is a mixture of phosphate buffer and methanol of pH 6.7. Therefore 390 ml of 0.067 *M* KH_2PO_4 were mixed with 10 ml of 0.067 *M* Na_2HPO_4 and 80 ml of methanol. The solvent flow-rate is 1.6 ml/min at a pressure of 115 atm (Fig. 1, Table I).

Sulfamethoxazole + trimethoprim. The solvent is a mixture of 390 ml 0.067 *M* KH_2PO_4, 10 ml of 0.067 *M* Na_2HPO_4 and 80 ml of ethanol. The solvent flow-rate is 1.2 ml/min at a pressure of 85 atm (Fig. 2 and Table II).

Drugs

Sulfamethoxazole, N_4-acetylsulfamethoxazole and trimethoprim were obtained from Hoffmann-La Roche (Mijdrecht, The Netherlands) by the courtesy of Dr. J. Kuitert. Sulfapyridine, sulfadiazine, sulfathiazole, sulfadoxine were obtained from the St. Radboud Hospital Pharmacy. Sulfadimidine and sulfamethoxypyridine were obtained from the Department of Pharmacology, State University of Groningen, The Netherlands by courtesy of Dr. D.K.F. Meyer. According to the HPLC chromatogram all compounds were 100% pure.

Subjects and patients

Ten subjects, all employees of the Department of Clinical Pharmacy participated in this study. Sulfamethoxazole was administered in doses of 800, 400, 200, and 100 mg (powder in a gelatine capsule). The drug was taken orally in

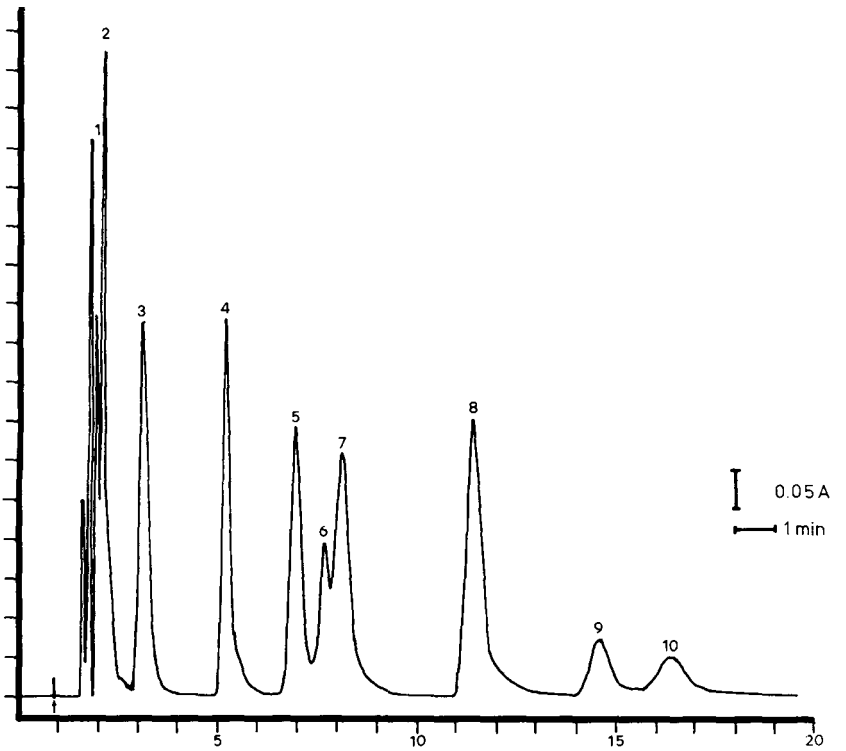

Fig. 1. HPLC chromatogram of sulfamethoxazole and related sulfonamides. The column used was LiChrosorb RP 8, and the solvent a phosphate buffer of pH 6.7 + 20% methanol. Flow-rate, 1.6 ml/min. 1 = Sulfacetamide; 2 = sulfanilamide; 3 = sulfadiazine; 4 = sulfamethoxazole; 5 = sulfathiazole; 6 = sulfadoxine; 7 = sulfapyridine; 8 = N_4-acetylsulfamethoxazole; 9 = sulfamethoxypyridine; 10 = sulfadimidine.

TABLE I

RETENTION TIMES OF SULFAMETHOXAZOLE AND RELATED SULFONAMIDES RELATIVE TO THE UNRETAINED COMPONENT (K′)

Column, LiChrosorb RP 8; solvent, phosphate buffer (pH 6.7) + 20% methanol.

Compound	Relative retention time K′
Sulfacetamide	1.53
Sulfanilamide	1.87
Sulfadiazine	3.00
Sulfamethoxazole	5.60
Sulfathiazole	8.20
Sulfadoxine	9.00
Sulfapyridine	9.73
N_4-Acetylsulfamethoxazole	14.40
Sulfamethoxypyridazine	18.40
Sulfadimidine	20.73

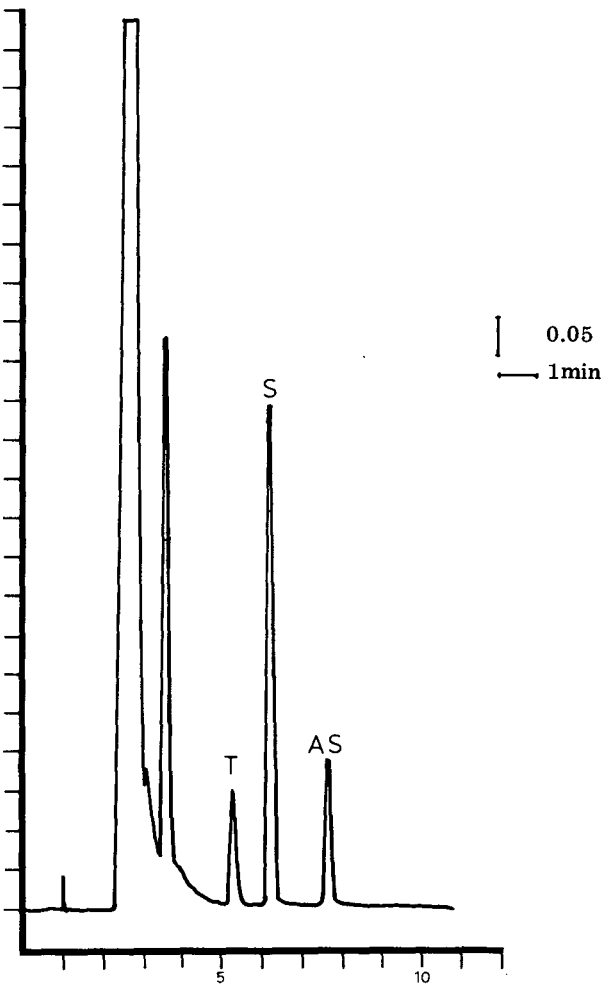

Fig. 2. HPLC chromatogram of sulfamethoxazole (S), its metabolite N_4-acetylsulfamethoxazole (AS) and trimethoprim (T) in plasma of a patient receiving co-trimoxazole as treatment. The column used was LiChrosorb RP 8 and the solvent a phosphate buffer of pH 6.7 + 20% ethanol. Flow-rate, 1.2 ml/min.

TABLE II

RETENTION TIMES OF SULFAMETHOXAZOLE, ITS METABOLITE AND TRIMETHOPRIM RELATIVE TO THE UNRETAINED COMPONENT (K′)

Column, LiChrosorb RP 8; solvent, phosphate buffer (pH 6.7) + 20% ethanol.

Compound	Relative retention time K′
Trimethoprim	3.50
Sulfamethoxazole	4.33
N_4-Acetylsulfamethoxazole	5.50

the morning, 1.5 h after a standard breakfast. Blood samples of 0.2 ml were collected at scheduled intervals by fingertip puncture (Microlance no. 433, Becton Dickinson). Spontaneously voided urine was collected during 56 h. An alkaline urine pH was reached by the daily intake of 10 g of sodium bicarbonate (pH 7—8). An acidic urine pH was reached by the daily intake of 8 g of ammonium chloride (pH 5—6).

Blood samples from patients receiving co-trimoxazole as treatment were obtained from the Intensive Care Unit (Dr. R. van Dalen) of the St. Radboud Hospital.

Sample preparation

Human serum is 10 times diluted with distilled water, and to 0.2 ml of the diluted serum a 0.8 ml volume of perchloric acid (0.33 *M*) is added. The solution is mixed thoroughly on a Vortex mixer and subsequently allowed to stand for 10 min. After centrifugation at 2600 *g* for 5 min, 100 μl of the supernatant is injected onto the column.

A calibration curve is made by adding known concentrations of sulfamethoxazole to blank human serum. Once a calibration curve has been made, it is desirable to check 3 points of this curve with each series of determinations in order to confirm whether the column is still stable.

The human urine sample is prepared by addition of 0.5 ml of perchloric acid (0.33 *M*) to 10 μl of urine. The solution is mixed and 100 μl is injected onto the column.

Recovery

Recovery of sulfamethoxazole added to human serum in the concentration range of 1—200 μg/ml was found to be 88 ± 4%. For trimethoprim the recovery was found to be 83 ± 2% in the concentration range of 0.5—16 μg/ml.

If, for the estimation of sulfamethoxazole, the serum is not diluted, the recovery after deproteinization is somewhat less, 80 ± 7%.

The recovery of sulfamethoxazole and trimethoprim added to urine is 100 ± 2%.

RESULTS

Sulfamethoxazole is well separated from its metabolite N_4-acetylsulfamethoxazole, other related sulfonamides and trimethoprim, with which it may occur frequently in chemotherapeutic combination (as can be seen in Figs. 1 and 2 and Tables I and II).

A dose of 100—800 mg of sulfamethoxazole results in plasma concentrations of sulfamethoxazole and N_4-acetylsulfamethoxazole that can easily be measured (0.5 μg/ml—200 μg/ml). The urine concentrations of both compounds after a dose of 100—800 mg range from 10—500 μg/ml.

The pharmacokinetics of sulfamethoxazole in man under acidic and alkaline urinary pH conditions reveal a difference in the excretion pattern of sulfamethoxazole which is shown in Fig. 3 and Table III. The most striking observation is that sulfamethoxazole is hardly excreted unchanged with acidic urine, while

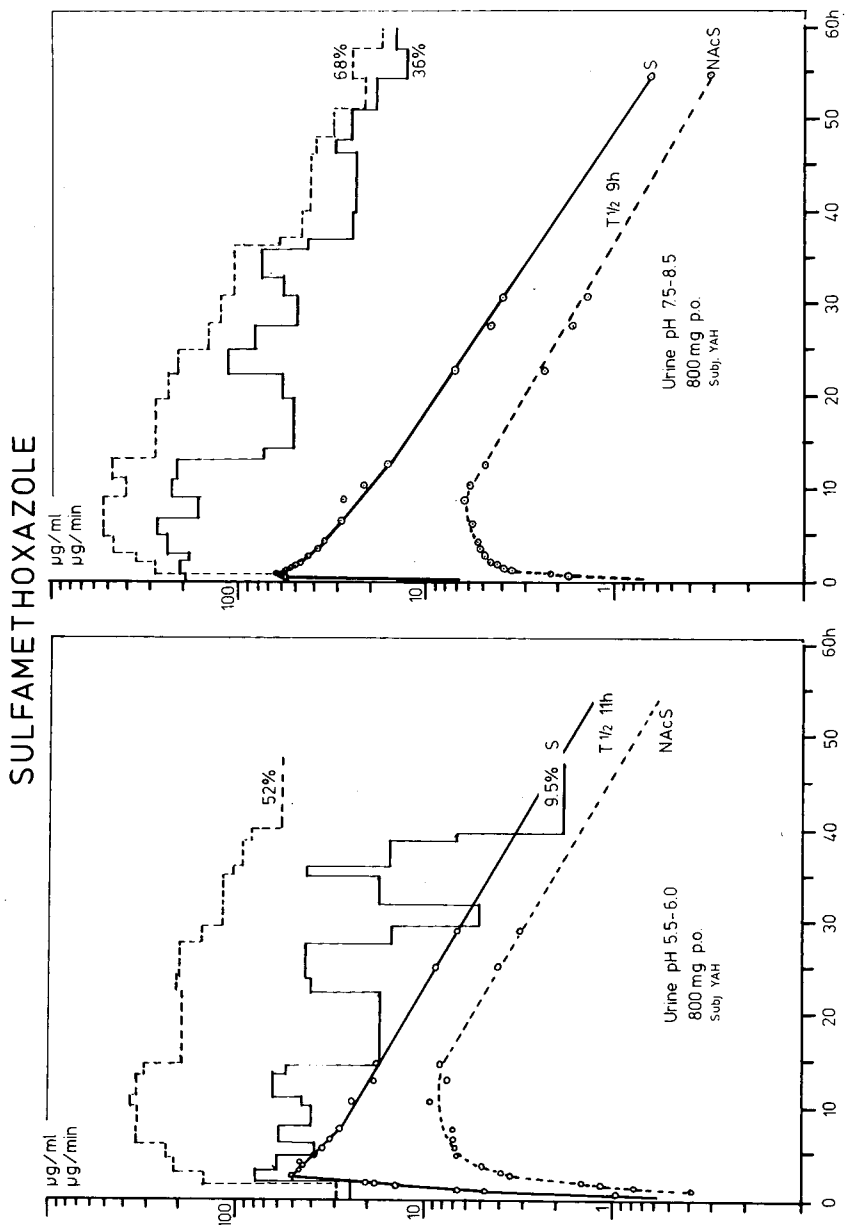

Fig. 3. Plasma concentration–time profile of sulfamethoxazole (S) and its metabolite N_4-acetylsulfamethoxazole (NAcS) and the renal excretion rate of both compounds in a volunteer who took 800 mg sulfamethoxazole orally. The experiment was performed under different urinary pH values. Note that with acidic urine (pH 5.5–6.0) only 9.5% of sulfamethoxazole is excreted unchanged, while this value is 36% when the urine is maintained alkaline (pH 7.5–8.5). Due to the enhanced excretion under alkaline urine conditions the T½ of elimination is shorter (9 h) than under acidic urine conditions (11 h).

TABLE III

PHARMACOKINETIC PARAMETERS OF SULFAMETHOXAZOLE IN MAN

Subjects	Dose	pH Urine	$T_{1/2}$ (h)		Excreted in the urine (%)	
			Sulfameth.	N-Acetylsulf.	Sulfamethoxazole	N_4-acetylsulfamethoxazole
$n = 10$	200 mg (5) 400 mg (2) 800 mg (3)	7—8	9.6 ± 2.5	11.2 ± 2.4	35.0 ± 2.0	42.8 ± 12.9
$n = 5$	400 mg (2) 800 mg (3)	5—6	9.5 ± 3.5	9.0 ± 1.7	7.1 ± 3.2	44.0 ± 6.9

the percentage of the dose of N_4-acetylsulfamethoxazole followed by excretion in all volunteers is almost constant.

The renal clearance constant, the proportionality constant between the renal excretion rate (μg/min) and the plasma concentration (μg/ml) of sulfamethoxazole are strongly dependent on the urinary pH (Fig. 4), while the renal clearance constant of the metabolite N_4-acetylsulfamethoxazole is hardly affected by the urinary pH.

Fig. 5 shows an example of the plasma concentrations of sulfamethoxazole,

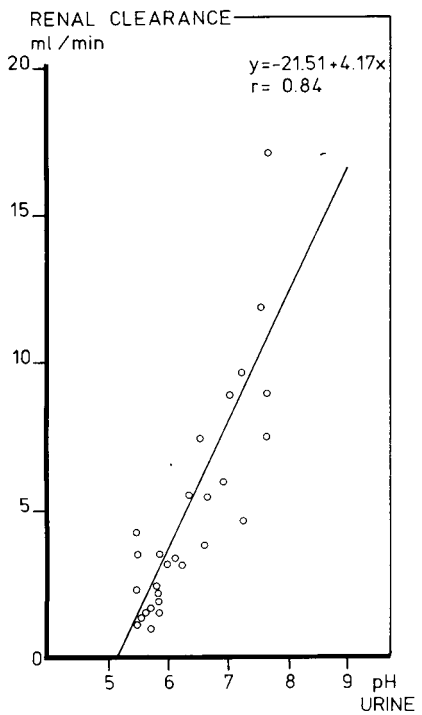

Fig. 4. Relationship between renal clearance (ml/min) of sulfamethoxazole and the urinary pH. Both variables were measured in each urine portion voided. Note that the renal clearance is increased by a factor 10 when the pH changes from pH 5 to pH 8. No obvious relationship could be observed ($r = 0.25$) for the metabolite N_4-acetylsulfamethoxazole.

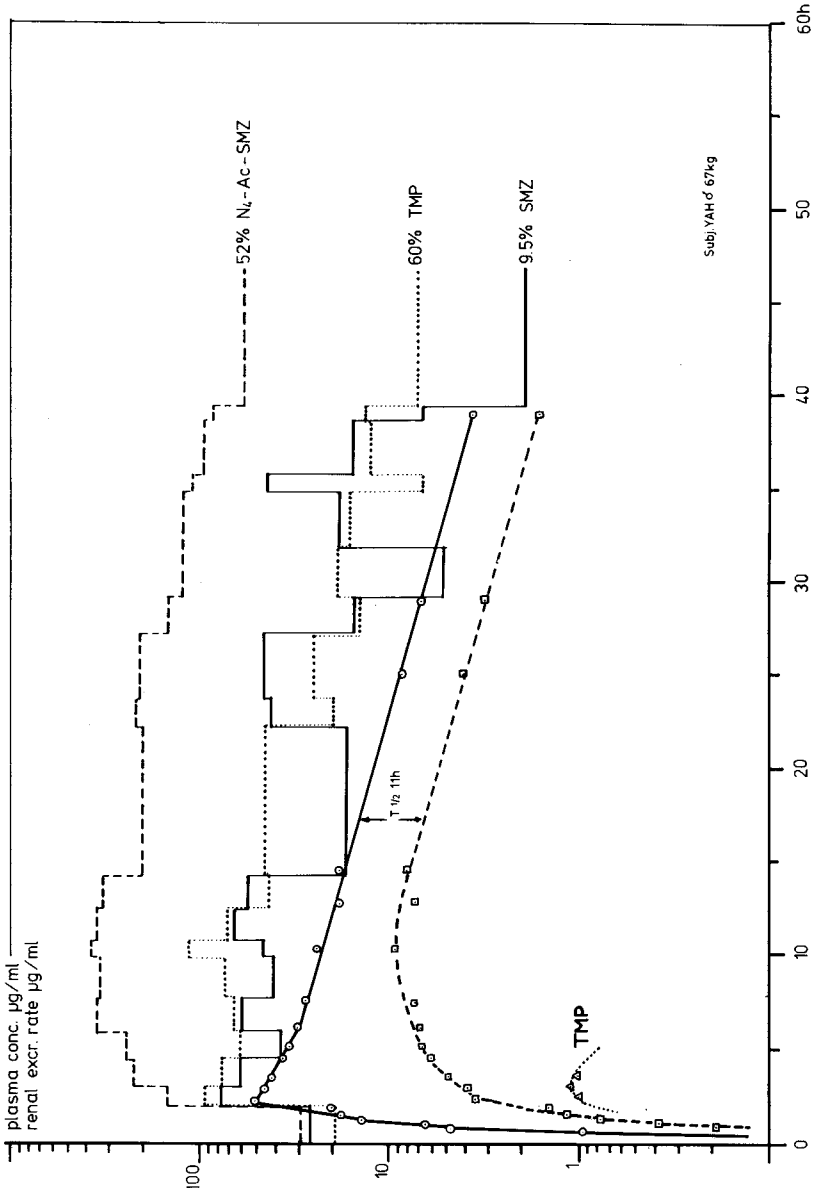

Fig. 5. Plasma concentration–time profile and renal excretion rate of sulfamethoxazole (SMZ), its metabolite N_4-acetylsulfamethoxazole (N_4 AcSMZ) and trimethoprim (TMP) in a volunteer who took 800 mg sulfamethoxazole and 160 mg of trimethoprim orally. The urinary pH is not influenced and appeared to be mainly acidic (pH 5—6). Trimethoprim concentrations could be measured in plasma only for a short period, its renal excretion accounts for 60% of the dose administered. Sulfamethoxazole is excreted 9.5% unchanged and 52% as the N_4-acetylsulfamethoxazole.

its metabolite N_4-acetylsulfamethoxazole and the co-medication trimethoprim. While the concentrations of both sulfa- compounds are relatively high and can be measured over a long period, trimethoprim can only be measured for a short time.

The renal excretion rate of trimethoprim is much higher than that of sulfamethoxazole. 60% of trimethoprim is excreted unchanged, while for sulfamethoxazole this value is 9.5% and 52% is excreted as metabolite. The pH of the urine in this experiment is not influenced.

DISCUSSION

The HPLC method for sulfonamides has the advantage over the Bratton and Marshall photometric method [7,8] that the compounds are measured specifically and selectively and that several sulfonamides ingested at the same time, can be simultaneously measured [13]. The pharmacokinetic parameters can be compared under exactly the same circumstances. As plasma and urine concentrations can be measured conveniently, the renal clearance constant can be calculated. It was shown that the renal excretion of sulfonamides is strongly dependent on the urinary pH [21—23]. The implications of this pharmacokinetic behaviour for clinical treatment are the subject of further research.

It may be deduced that in the case of an infection of the urinary tract, the urine should be kept alkaline in order to ensure that the concentration of sulfamethoxazole is as high as possible, because the metabolite is inactive. When trimethoprim is used in chemotherapy in combination with sulfamethoxazole, three compounds can be easily recognized in the HPLC chromatograms of plasma and urine samples (Fig. 1 and Table I).

The ease of the sample preparation and the avoidance of extraction procedures, the small sample volume required, the possibility of simultaneous determination of several sulfonamides and the short retention time (10—15 min) all make this method useful for the analysis of large series of samples for both research and routine applications.

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QUANTITATION OF ZIMELIDINE AND NORZIMELIDINE IN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitation of a new non-tricyclic antidepressant, zimelidine, and its pharmacologically active, N-demethylated metabolite, norzimelidine, in plasma. The method involves a single extraction of basified plasma with diethyl ether, concentration of the ethereal extract, chromatography on a high-performance liquid chromatograph and quantitation using a variable-wavelength UV detector.

The respective geometric isomers of zimelidine and norzimelidine are used as internal standards for quantitation. Resolution is effected using a 5-μm silica gel column with an aqueous methanolic solution of ammonium nitrate as the mobile phase. The minimum quantitated amount was 25 ng and the coefficient of variation for the method did not exceed 7% in the range 25 to 1000 ng/ml for both compounds. The method has been applied in monitoring the plasma concentration of zimelidine and norzimelidine in plasma from depressed patients and an example of this application is presented.

INTRODUCTION

Zimelidine (I) is a new anti-depressant drug which is presently at an early stage of clinical trial in several countries. Animal studies have revealed that its N-demethylated metabolite, norzimelidine (II), has similar pharmacological properties and potency to the parent drug (I). Consequently, in

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studies of the efficacy of this new drug in humans, it was necessary to monitor the plasma concentration of both zimelidine and norzimelidine.

A method for quantitating zimelidine and norzimelidine in plasma using high-performance liquid chromatography (HPLC) based on the method of Schill et al. [2] has been applied clinically [3]. This method effected resolution using ion-pair partition chromatography and employed a mobile phase which consisted of an organic solvent (methylene chloride–butanol) saturated with an aqueous solution of perchlorate salts. The whole system required precise thermostating to prevent variable retention times and emulsion formation which resulted in intolerable detector noise. This requirement could not be achieved reliably with the instrumentation available to us. The method reported here describes an alternative HPLC system which simultaneously resolves the geometric isomers of both zimelidine and norzimelidine and which is also compatible with a simple procedure for their extraction from plasma.

EXPERIMENTAL

Reagents and materials

The molecular structures of the quantitated compounds are presented in Fig. 1. The dihydrochloride monohydrates of I, II and III, and the oxalate of IV were gifts from Astra Chemicals (Sydney, Australia). The ammonium nitrate, sodium hydroxide and methanol were analytical grade substances (Ajax Chemicals, Sydney, Australia) and were used without further purification. The diethyl ether was B.P. anaesthetic grade and freshly distilled before use.

All glassware was cleaned with a chromic acid mixture and washed with distilled water. The glass evaporation tubes were silylated with Siliclad (Clay Adams, Parsippany, N.J., U.S.A.) washed with distilled water and dried.

Extraction from plasma

Plasma (1 ml) and an aqueous solution of internal standards (500 ng of III · 2HCl · H_2O and 500 ng of IV · oxalate per 100 μl) was made alkaline (0.5 ml, 5 *M* NaOH) and was shaken with diethyl ether (10 ml) on a vortex mixer for 2 min. After centrifugation at 1500 *g* for 2 min the diethyl ether phase was transferred to an evaporation tube — a 15-ml glass tube with a 100-μl capillary at the base. The extract was concentrated on a water-bath at 40° using an anti-bumping granule (BDH, Melbourne, Australia). When no diethyl ether remained the stoppered tube was immersed in an ice–water bath. This allowed the diethyl ether to condense and wash down the internal walls of the evaporation tube. The remaining diethyl ether was evaporated under a gentle stream of nitrogen and the residue redissolved in 60 μl of the mobile phase by shaking on a vortex mixer for 15 sec. All of this extract was injected onto the column using a loop-injection valve.

Chromatography

A Varian Aerograph Model 8500 high-performance liquid chromatograph equipped with a Spectra-Physics Model 770 variable-wavelength UV detector

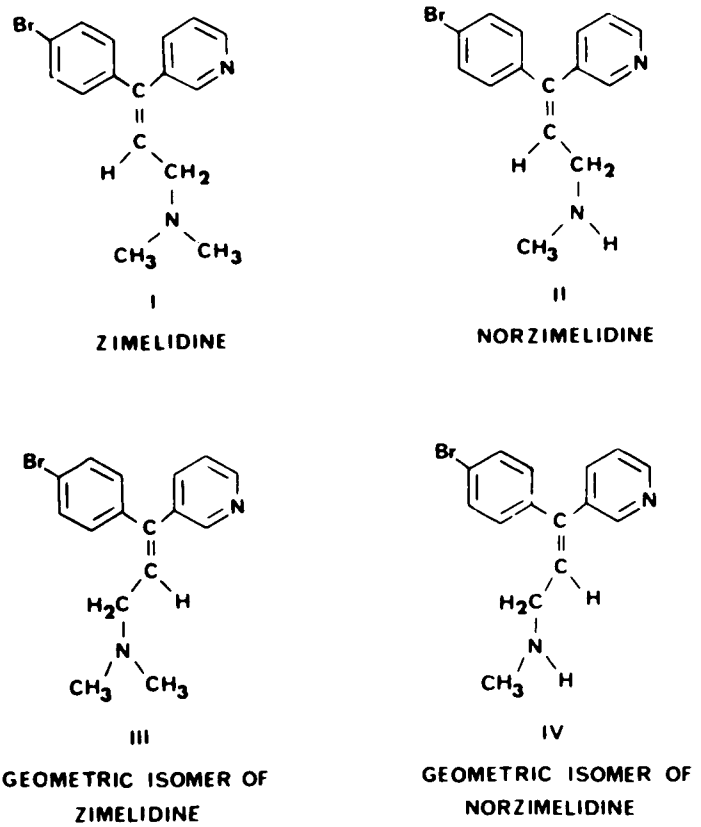

Fig. 1. Molecular structures of zimelidine, norzimelidine and their respective geometric isomers.

operated at 258 nm was used. Samples were injected using a Valco 7000-p.s.i. injection valve fitted with a 60-μl loop. The column was 120 × 4.5 mm I.D. stainless steel tubing packed with silica gel having an average particle diameter of 5 μm (Partisil 5). The column temperature was maintained at 30° with a thermostated water jacket. The mobile phase consisted of methanol and an aqueous solution of ammonium nitrate (0.1 *M*) in the ratio 100 : 5 and its flow-rate was 50 ml/h. Minor decreases in the ratio of aqueous ammonium nitrate to methanol were required occasionally to optimize the resolution and at these times another calibration curve was prepared.

Calibration and reproducibility

Known quantities of zimelidine and norzimelidine (25—1000 ng of each as the dihydrochloride monohydrate) were added to blank plasma which were then analysed. Calibration curves were constructed by plotting peak height ratios between zimelidine (I) and its geometric isomer (III), and norzimelidine (II) and its geometric isomer (IV) versus the respective amounts of added zimelidine and norzimelidine. The reproducibility of the assay was determined for both zimelidine and norzimelidine by adding a known amount of

each compound to a bulk plasma sample and then carrying out replicate analyses on this sample.

RESULTS AND DISCUSSION

Initial effort was directed toward the development of a method using gas chromatography with electron capture detection. Although this approach was satisfactory for the quantitation of zimelidine, no suitable internal standard could be found for the quantitation of norzimelidine. Irregular losses of norzimelidine occurred when ethereal extracts were concentrated by gentle evaporation. The molar response on electron capture of norzimelidine com-

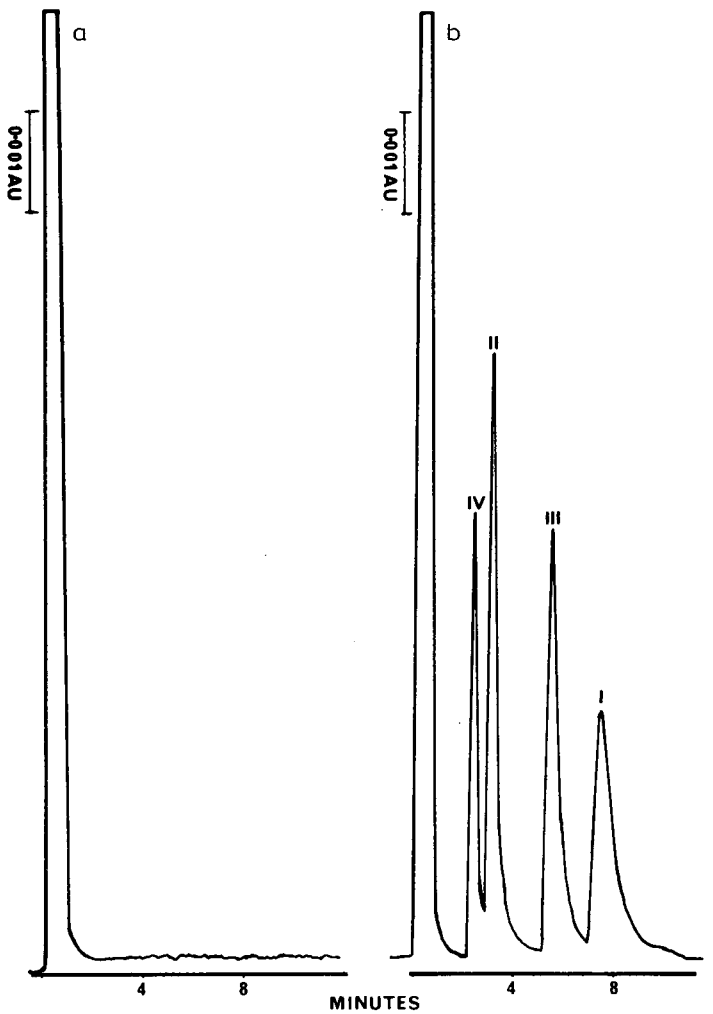

Fig. 2. Chromatograms of blank human plasma and human plasma containing 200 ng of zimelidine and 300 ng of norzimelidine. Chromatographic conditions are given in the text and compound identification in Fig. 1.

pared to zimelidine, chromatographed on a glass column packed with 3% OV-1 or 3% OV-17 on Gas-Chrom Q, was both erratic and low (< 10%). The geometric isomers were unsuitable as internal standards on these systems because neither compounds I and III, nor II and IV could be resolved. The possibility of resolving these geometric isomers on HPLC stimulated our interest in this approach.

Preliminary work using tritiated material [4] had shown that diethyl ether could extract 100% of zimelidine and norzimelidine from basified plasma. This factor, combined with the ease of concentration of diethyl ether, made it a most suitable solvent for extraction. Compounds II and IV were also completely extracted into diethyl ether from alkaline plasma. When diethyl ether extracts of blank plasma were chromatographed, there were no interfering endogenous compounds with the same retention time as compounds I to IV (Fig. 2a). In fact, all the detected endogenous compounds eluted before them. No other drugs which co-chromatograph with compounds I to IV have been observed in extracts from plasma of patients (Table I).

The chromatography system that has been developed is based on that reported by Jane [5]. Applications of the original systems to forensic problems have been reviewed more recently [6]. The mechanism that determines the separation with the use of a polar mobile phase and microparticulate silica-packing is not understood. Wheals [6] has suggested that processes such as ion exchange and hydrogen bonding may be contributing. The ability of this system to resolve the geometric isomers is illustrated in the chromatogram in Fig. 2b.

When the optimum composition for the mobile phase was being sought, it became evident that a balance had to be achieved between adequate resolution of norzimelidine and its geometric isomer, and the peak shape of zimelidine and its geometric isomer. This balance was influenced by the strength of the aqueous ammonium nitrate solution and the ratio of methanol to water in the mobile phase. The retention times of all four components were decreased by increasing either the ammonium nitrate concentration or the water-to-methanol ratio. The resolution of the four components illustrated in Fig. 2b was found to be adequate for accurate and reproducible quantitation.

The sensitivity of this method was adequate for monitoring clinically relevant concentrations of zimelidine and norzimelidine. The sensitivity could be

TABLE I

DRUGS WHICH DID NOT INTERFERE IN THE ANALYSIS OF ZIMELIDINE AND NORZIMELIDINE IN PLASMA

Chlorpromazine	Paracetamol
Danthron	Penicillin
Diazepam	Prednisone
Dioctyl sodium succinate	Sodium salicylate
Indomethacin	Theophylline
Meclozine	Thioridazine
Nitrazepam	

TABLE II

REPRODUCIBILITY DATA FOR THE QUANTITATION OF ZIMELIDINE AND NORZIMELIDINE

Compound	Plasma concentration (ng/ml)	No. of assays	Peak height ratio (mean ± S.D.)	Coefficient of variation (%)
I	1000	6	2.488 ± 0.059	2.4
II	1000	6	3.217 ± 0.052	1.6
I	500	6	1.147 ± 0.040	3.5
II	500	6	1.503 ± 0.077	5.1
I	300	6	0.728 ± 0.044	6.0
II	300	6	1.011 ± 0.067	6.6
I	100	6	0.232 ± 0.008	3.2
II	100	6	0.359 ± 0.011	3.0
I	25	6	0.075 ± 0.005	6.4
II	25	6	0.150 ± 0.007	4.3

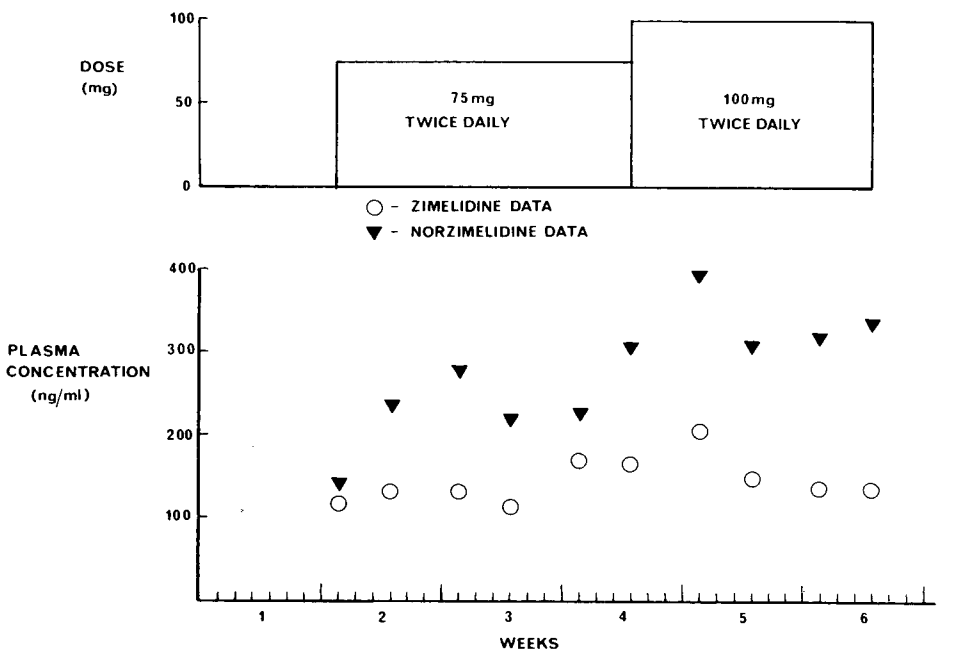

Fig. 3. Some typical plasma concentration—time data for a patient on oral zimelidine. Blood samples were collected twice weekly, 6 h after the 8 a.m. dose. The patient took placebo tablets during the first week.

extended easily for more sophisticated pharmacokinetic studies by increasing the volume of biological fluid assayed and by using a more sensitive, fixed-wavelength UV detector.

The reproducibility of the technique is demonstrated by the linearity of the calibration plots (see also the data in Table II). The slight positive devia-

tion of the norzimelidine calibration curve from the origin was caused by the presence of a trace amount of norzimelidine as an impurity in its internal standard (IV). The presence of this impurity has been observed previously [4]. The reproducibility data in Table II show that the coefficient of variation for quantitation of both components in plasma is less than 7% in the range 25 to 1000 ng/ml.

This method has been applied to analysis of over 300 plasma samples from patients. Typical plasma concentration—time profiles for zimelidine and norzimelidine for a patient on different dosages of zimelidine are illustrated in Fig. 3.

The high efficiency of microparticulate silica columns operated with polar mobile phases has permitted the resolution of geometric isomers so that they can be quantitated reproducibly. The similarity in physico-chemical properties between geometric isomers may make them an ideal internal standard for quantitation now that they can be rapidly resolved by HPLC.

ACKNOWLEDGEMENTS

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SIMPLE, RAPID AND MICRO HIGH-PRESSURE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF TOLBUTAMIDE AND CARBOXY TOLBUTAMIDE IN PLASMA

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SUMMARY

A rapid high-pressure liquid chromatographic (HPLC) assay is described for the quantitative analysis of tolbutamide and its major metabolite, carboxy tolbutamide, in plasma. An aliquot (25—100 μl) of plasma was prepared for chromatography by deproteinization as follows. One volume of plasma and 2.5 volumes of acetonitrile were vortex mixed for a few seconds and then centrifuged for approx. 1 min. A 50-μl sample of the clear supernatant was injected into the chromatograph. A μBondapak C_{18} reversed-phase column was used with a mobile phase of acetonitrile—0.05% phosphoric acid (45:55) at a flow-rate of 1.5 ml/min. The column effluent was monitored by a variable-wavelength UV detector set at 200 nm. Tolbutamide and its metabolite had retention times of 5.75 and 3.25 min, respectively. The procedure yields reproducible results with sensitivity adequate for routine clinical monitoring of plasma levels or for single-dose pharmacokinetic studies. A number of commonly used drugs do not interfere with the method. A single plasma sample can be analyzed in approx. 9 or 10 min.

INTRODUCTION

Tolbutamide is a sulfonylurea that is orally effective as a hypoglycemic agent and has found wide application for the treatment of diabetes mellitus of the maturity-onset type. It has been suggested that therapeutic plasma concentrations of tolbutamide lie in the range of 53—96 μg/ml [1]. The chronic nature of diabetes usually means that tolbutamide may need to be administered over long periods to a patient population comprised of middle-aged or elderly people in whom progressive physiological changes are occurring which could result in clinically important alterations in drug disposition or intrinsic responses. It is usually recommended that patients of advanced age begin ther-

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apy with about half the usual daily dose of tolbutamide because some such individuals are very responsive to sulfonylureas and may develop severe hypoglycemia after usual doses [2]. Therefore, it may be prudent to monitor the plasma level of tolbutamide occasionally in elderly patients who receive the drug over a prolonged period, to ensure the suitability of the established dosage regimen. Riegelman and Sadee [3] have advocated the monitoring of plasma levels of tolbutamide when the patient does not appear to have achieved the expected therapeutic response.

A number of methods based on absorption in the ultraviolet [4, 5] or visible [6—8] regions have been reported over the last twenty years for the quantitative determination of tolbutamide in biological fluids. However, those procedures suffer from disadvantages which include lack of adequate sensitivity and specificity, and laborious work-up procedures. Gas chromatographic procedures which have been described [8—12] overcome some of the disadvantages of the spectrophotometric methods, but the analysis time is greatly lengthened by the necessity to derivatize the tolbutamide molecule prior to gas chromatography. A report of an HPLC method for quantitative analysis of tolbutamide in 1-ml aliquots of plasma has recently appeared in the literature [13]. Sample preparation prior to chromatography required lengthy solvent extraction and evaporation steps.

The purpose of the present paper is to report a new HPLC method for the simultaneous quantitative analysis of tolbutamide and its carboxy metabolite in plasma. The method described involves an extremely simple sample preparation followed by HPLC analysis on a reversed-phase column with UV detection of the compounds in the column effluent. The assay is fast and sensitive, and only very small plasma samples are required.

EXPERIMENTAL

Reagents and standards

Tolbutamide and 1-butyl-3-(*p*-carboxyphenylsulfonyl)-urea (hereafter referred to as carboxy tolbutamide) were kindly supplied by Upjohn (Kalamazoo, Mich., U.S.A.). Standard solutions of these compounds were made up in methanol and stored at —20° when not in use. Glass-distilled methanol and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and phosphoric acid was obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.).

Other drug substances which were tested for potential interference of the assay had, in most cases, been donated by pharmaceutical manufacturing companies. Solutions of these compounds in methanol or distilled water were prepared and stored at —20° when not in use.

A 0.05% solution of phosphoric acid in distilled water (final pH 2.6) was prepared and passed through a 0.45 μm membrane filter for subsequent use in the preparation of the HPLC mobile phase.

HPLC instrumentation and conditions

A model M-6000A pump was used to deliver the mobile phase to a model U6K injection loop and a 30-cm μBondapak C_{18} reversed-phase column (par-

ticle size 10 μm), all of which had been supplied by Waters Assoc. (Milford, Mass., U.S.A.). A model LC 55 variable-wavelength HPLC UV-detector obtained from Perkin-Elmer (Oakbrook, Ill., U.S.A.) was used to monitor the column effluent. The output from the detector was connected to a 10-mV potentiometric 10-in. recorder (Houston Instruments, Austin, Tex., U.S.A.) via an attenuator (Perkin-Elmer) with time constant control which reduced the level of background noise.

The mobile phase for the chromatographic separation was prepared by mixing 45 parts of acetonitrile with 55 parts of 0.05% phosphoric acid solution. This mobile phase system was chosen because it provided good separation of tolbutamide from its major metabolite, endogenous compounds, and a large number of other drugs. The mobile phase was pumped through the HPLC system at a rate of 1.5 ml/min which resulted in a pump pressure of approx. 1500 p.s.i.g. The chromatography was carried out at ambient temperature. The wavelength of the UV HPLC detector was set at 200 nm and the recorder chart speed was 0.5 cm/min.

Preparation of plasma samples

Aliquots of 100 μl of plasma were pipetted into 13 × 100 mm screw-capped culture tubes followed by the addition of 250 μl of acetonitrile. After capping, each tube was vortexed for a few seconds and this was followed by centrifugation at 800 *g* for approx. 1 min. Most of the clear supernatant solution was poured into another culture tube and 50 μl of this solution was then injected into the chromatograph for analysis. It is worthwhile to note that it is not necessary to include the step where the supernatant is poured into another tube provided that, in obtaining the aliquot for injection, due care is exercised to avoid disturbing the precipitate.

Blank human plasma was spiked with known concentrations of tolbutamide and its carboxy metabolite. The spiking was achieved by placing a 5—10 μl aliquot of a methanolic solution of tolbutamide and carboxy tolbutamide in culture tubes. The methanol was allowed to evaporate before adding the 100-μl aliquots of blank human plasma to each tube. The samples were analyzed as described above. The peak height of each compound was measured and plotted against the concentration to provide standard curves.

Reproducibility studies

A 2-ml aliquot of blank human plasma was spiked such that the tolbutamide and carboxy tolbutamide concentrations in plasma were 50 and 25 μg/ml, respectively. Ten aliquots (100 μl each) of the resulting plasma sample were analyzed in replicate on the same day. In addition, 100-μl aliquots of the same plasma sample were assayed once a day for five days over a period of one week.

Drug interference study

Several drugs and drug metabolites were tested for potential interference of the assay by injecting stock solutions of the compounds into the chromatograph. Additionally, aliquots of plasma obtained from patients receiving other drug therapy were treated according to the sample preparation and HPLC procedures described above for the tolbutamide assay.

Preliminary study in a rabbit

Tolbutamide (40 mg in 1.6 ml of 95% ethanol) was infused over 1 min into the medial vein of one ear of a 4-kg albino rabbit. Blood samples (0.5 ml) were collected from the marginal vein of the contralateral ear prior to drug administration and at intervals for 5 h thereafter. Blood samples were placed in heparinized tubes which were centrifuged immediately to obtain plasma. The separated plasma samples were stored at −20° until the time of analysis.

RESULTS AND DISCUSSION

Tolbutamide is metabolized in humans by oxidation to carboxy tolbutamide and this metabolite accounts for the majority (about 75%) of the urinary recovery of a dose of the drug [2]. Although this metabolite is reported to be devoid of hypoglycemic activity [14] it may be important from a toxicological standpoint. In addition, in some pharmacokinetic studies it may be desirable to study the time course of formation and removal of the metabolite. Therefore, the tolbutamide assay described in this paper has been designed so that it also accommodates quantitation of the major metabolic transformation product of tolbutamide.

The UV absorption spectrum of tolbutamide dissolved in the HPLC mobile phase is shown in Fig. 1. The earlier reported UV methods for quantitative analysis of tolbutamide [4, 5] made use of the peak at approx. 228–230 nm. Under the conditions employed in the present work the absorbance at 200 nm was approx. 2.5 times greater than the absorbance at 230 nm. As a result the lower wavelength (200 nm) was chosen for the monitoring of the HPLC column effluent by the UV detector although 230 nm could also be used.

Chromatograms resulting from the acetonitrile treatment and HPLC of blank human plasma together with similarly treated plasma which had been previously spiked with tolbutamide and carboxy tolbutamide are shown in Fig. 2. Tolbutamide eluted from the HPLC system after 5.75 min and was well resolved from its more polar carboxy metabolite which had a retention time of 3.25 min. No interference in blank plasma was observed at the retention time of tolbutamide for this particular sample of plasma or for plasma collected from eight other individual patients who were receiving other drugs. However, in some of the samples of plasma a small peak occurred which had a similar retention time to that of carboxy tolbutamide. The maximal contribution of this interfering peak observed in any of the various batches of plasma analyzed was of the order of 0.5 μg/ml.

Data used to establish the standard curves for tolbutamide and its metabolite are summarized in Tables I and II, respectively. Least squares linear regression analysis was carried out for each of the standard curves and the resulting coefficients of determination (r^2) were 0.9998 and 0.9996 for tolbutamide and carboxy tolbutamide, respectively. These high values of r^2 together with the constancy of the response factors (peak height divided by concentration) over the concentration ranges studied indicate good linearity for the standard curves. The percentage recovery from plasma for tolbutamide, compared to an aqueous solution, was 97 and 99% at plasma concentrations of 50 and 150 μg/ml, respectively. For carboxy tolbutamide the percentage

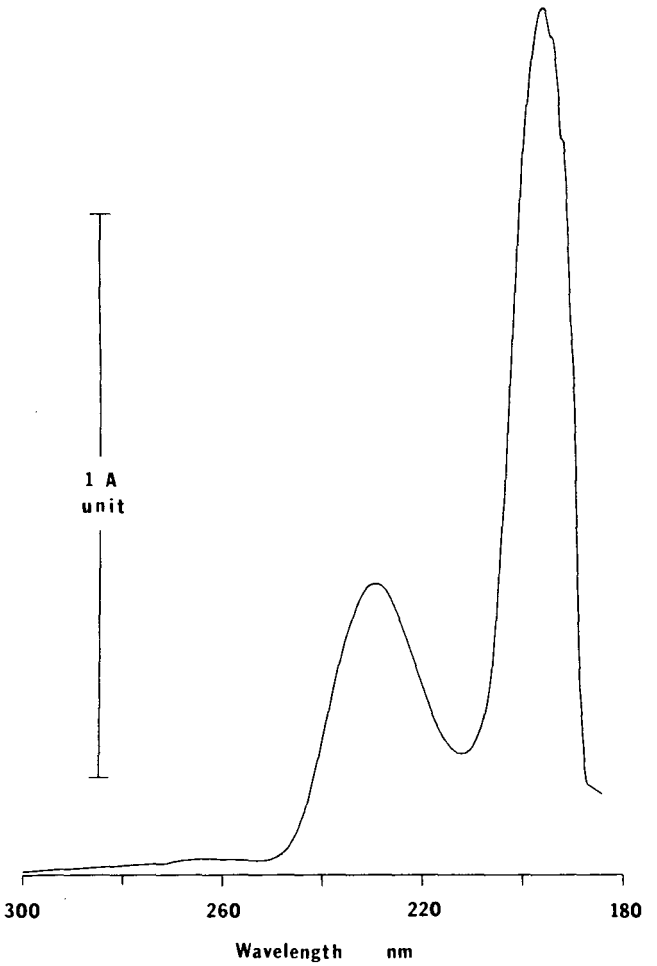

Fig. 1. UV absorption spectrum of a 1 mg% solution of tolbutamide in acetonitrile—0.05% phosphoric acid (45:55). The spectrum was recorded using a Model 200 Perkin-Elmer UV—visible recording spectrophotometer.

recovery from plasma, determined in a similar manner, was 87 and 89% at plasma concentrations of 25 and 75 μg/ml, respectively.

The within-day precision of the method was good, as assessed by conducting replicate (N = 10) analyses of the same spiked plasma sample (50 μg/ml of tolbutamide and 25 μg/ml of carboxy tolbutamide). The coefficient of variation for tolbutamide was 1.17%, while for carboxy tolbutamide the corresponding value was 2.32%. The coefficients of variation for the analysis of the same plasma sample on five days over a period of one week were 1.16% and 4.01% for tolbutamide and its metabolite, respectively. Although these data suggest that the day-to-day reproducibility of the method was good it may be prudent to include at least one standard sample on those days when patient plasma samples are being analyzed.

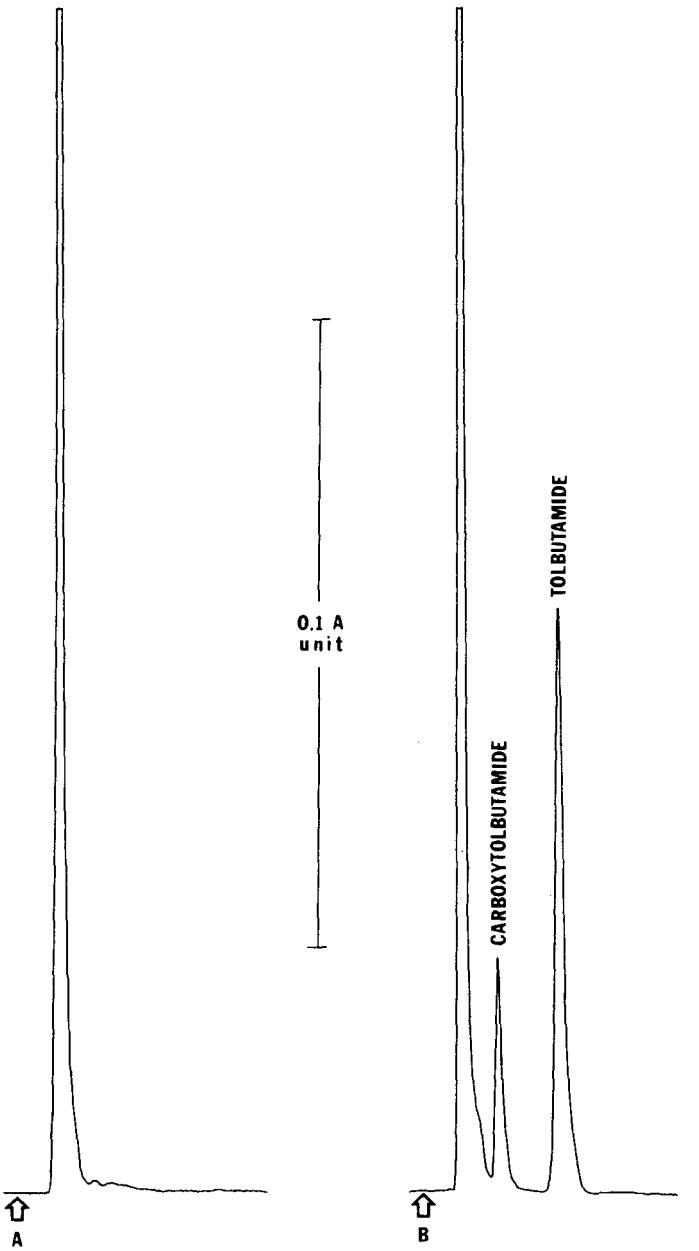

Fig. 2. Chromatograms of acetonitrile-treated blank human plasma (A) and similarly treated plasma which had been spiked with carboxy tolbutamide (25 μg/ml) and tolbutamide (50 μg/ml) (B). The arrow marks the point of injection.

TABLE I

STANDARD CURVE FOR TOLBUTAMIDE IN PLASMA

Linear regression equation: $y = 2.2909x + 1.7744$, $r = 0.9999$. One measurement was performed at each concentration.

Tolbutamide conc. in plasma (μg/ml)	Tolbutamide peak height*	Response factor**
5	11	2.21
10	23	2.28
25	57	2.29
50	119	2.38
100	237	2.37
200	457	2.28
300	689	2.30

*Peak height (mm) when detector sensitivity corresponds to 0.2 a.u.f.s.
**Peak height divided by tolbutamide concentration.

TABLE II

STANDARD CURVE FOR CARBOXY TOLBUTAMIDE IN PLASMA

Linear regression equation: $y = 1.9800x + 1.4117$, $r = 0.9998$. One measurement was performed at each concentration.

Carboxy tolbutamide con. in plasma (μg/ml)	Carboxy tolbutamide peak height*	Response factor**
2.5	5	1.93
5	10	1.99
12.5	26	2.04
25	54	2.15
50	103	2.07
100	197	1.97
150	299	1.99

*Peak height (mm) when detector sensitivity corresponds to 0.2 a.u.f.s.
**Peak height divided by carboxy tolbutamide concentration.

The addition of 2.5 volumes of acetonitrile to 1 volume of plasma followed by very brief (about 10 sec) vortex mixing results in deproteinization of the plasma. A short period of centrifugation readily separates the denatured proteins from the supernatant, which consist of plasma water and acetonitrile. Centrifugation causes the protein material to form a solid cake at the base of the culture tube and permits the supernatant to be decanted to another tube, although this latter step is not necessary. This method of preparing plasma samples for HPLC analysis is extremely simple and fast and has been used successfully in this laboratory for the analysis of a large number of other

compounds, including sulfisoxazole [15], creatinine [16], griseofulvin [17], procainamide and N-acetylprocainamide [18], theophylline [19], salicylates, and furosemide. Over a period of approx. one year many hundreds of deproteinized plasma samples have been injected into various types of HPLC columns (ion-exchange, reversed-phase, etc.) without any apparent alteration in column performance having occurred with time. No pre-columns or other special column care procedures (other than those recommended by the column suppliers) have been used during that time interval.

Different methods of deproteinizing plasma samples prior to the HPLC analysis of other drug molecules have been reported [20, 21] but the other procedures do not appear to offer simplicity and rapidity comparable with the acetonitrile deproteinization method.

It should be pointed out that a volume of plasma smaller than 100 μl can also be satisfactorily analyzed for tolbutamide and carboxy tolbutamide using the method described in this paper. The addition of 2.5 volumes of acetonitrile to 25-μl and 100-μl aliquots of the same spiked plasma sample, followed by vortexing, centrifugation, and injection of 50μl of the supernatant into the HPLC, yielded the same answer for the tolbutamide and metabolite concentration in the plasma sample. The ability to use such small plasma volumes is a great advantage in clinical situations, where capillary blood samples may be used, or for pharmacokinetic studies in small laboratory animals.

A study was made of the retention times of other sulfonylurea oral hypoglycemic drugs using the HPLC conditions described above. It was found that acetohexamide and tolazamide had almost identical retention times to that of tolbutamide, but chlorpropamide, with a retention time of 4.6 min, was resolved from the tolbutamide peak. The similarity of retention times for the four anti-diabetic drugs should in no way limit the usefulness of the tolbutamide assay since it would be very rarely that any one patient would concurrently receive more than one of the agents. It is anticipated, therefore, that tolazamide, acetohexamide, or chlorpropamide plasma concentrations could be determined using the method described here for tolbutamide, provided that the patient concerned was only receiving one of the drugs.

A large number of other drugs and drug metabolites were tested for potential interference of the assay by injecting stock solutions of the compounds into the HPLC. The compounds tested in this regard were as follows: acetaminophen, amethopterin, ampicillin, aspirin, caffeine, chloramphenicol, chlordiazepoxide, chlorpromazine, chlorthalidone, ephedrine, fluphenazine, glycinexylidide, lignocaine, methaqualone, monoethylglycinexylidide, N-acetylprocainamide, perphenazine, phenacetin, phenobarbitone, phenytoin, procainamide, prochlorperazine, salicylic acid, sulphisoxazole, tetracycline, theobromine, theophylline, triflupromazine, and trimeprazine. Methaqualone was the only compound which had a similar retention time to that of tolbutamide, although it should be noted that some of the other compounds tested would interfere with the analysis of carboxy tolbutamide. Plasma collected from eight patients who were receiving other drug therapy, which included ampicillin, chlorthalidone, digoxin, frusemide, methyl dopa, prednisone, and terbutaline, showed no interference at the retention time of tolbutamide and

only slight interference in some samples at the retention time of carboxy tolbutamide.

Fig. 3 shows chromatograms of acetonitrile-treated blank rabbit plasma and similarly treated plasma collected from a rabbit after the intravenous admini-

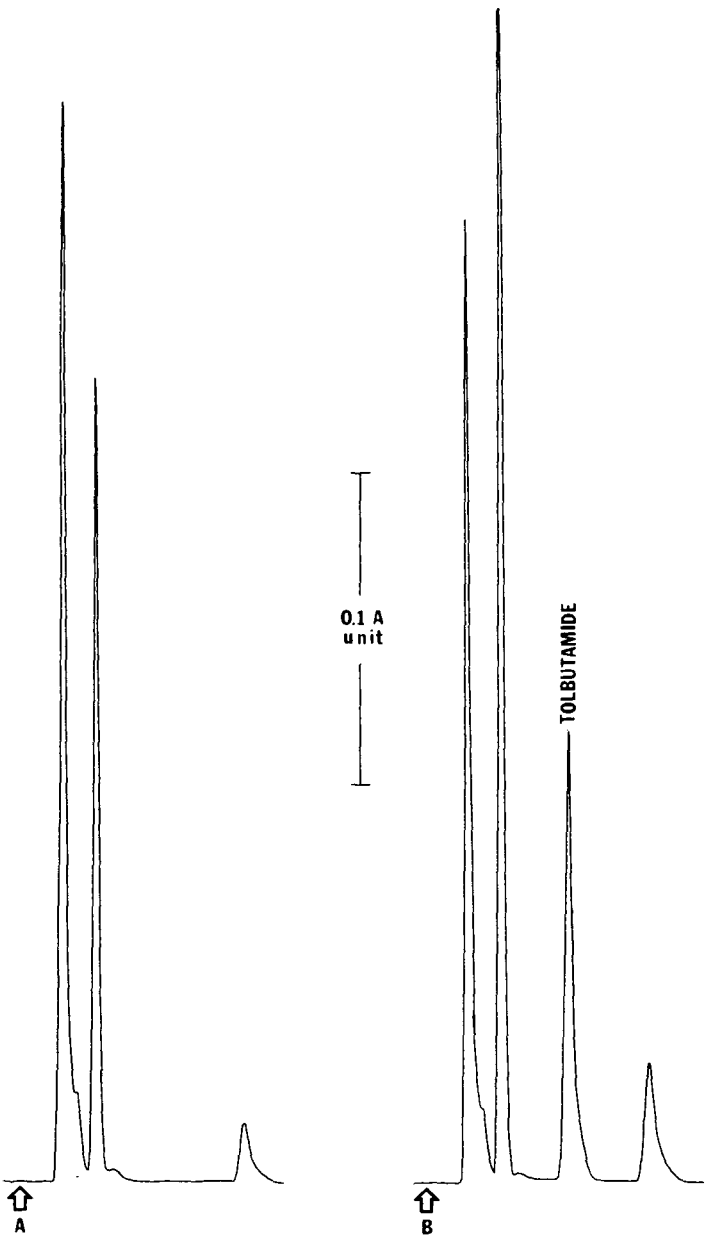

Fig. 3. Chromatograms of acetonitrile treated pre-dose (blank) rabbit plasma (A) and similarly treated plasma which was collected from the rabbit after intravenous administration of tolbutamide (B). Tolbutamide concentration in sample B was 78.6 μg/ml. The arrow marks the point of injection.

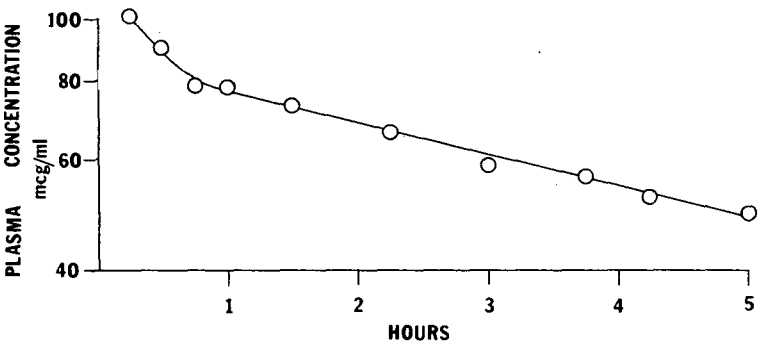

Fig. 4. Time course of tolbutamide plasma concentrations in a 4-kg rabbit following the intravenous injection of 40 mg of tolbutamide. The line represents a fit by eye to the data.

stration of tolbutamide. As can be seen from the analysis of the pre-dose (blank) plasma sample, no endogenous compounds eluted with a similar retention time to that of tolbutamide, but an interfering peak did elute near carboxy tolbutamide and made it impossible to quantitate that metabolite in rabbit plasma. The time course of tolbutamide plasma concentrations in the rabbit following intravenous administration is shown in Fig. 4.

CONCLUSIONS

The method described in this paper for the simultaneous determination of tolbutamide and carboxy tolbutamide in plasma is simple and rapid and requires only a micro volume of plasma. The total analysis time per sample is of the order of 9—10 min and no evaporation or derivatization steps are required Plasma concentrations of tolbutamide as low as 0.5 μg/ml could be quantitated, if necessary, although it should be noted that the precision of the method at that concentration has not been established. It is concluded that the analytical procedure may be valuable for the routine monitoring of plasma concentrations in patients receiving the drug and for pharmacokinetic studies in humans and animals.

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Note

Analysis of sulfonic acids by gas chromatography—mass spectrometry of trimethylsilyl derivatives

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Several sulfonic acids are known to exist in biological material. Most are formed in the metabolism of the sulfur-containing amino acids and little is known about their metabolic significance. Taurine, the sulfonic acid wich has been most extensively studied, is present in large amounts in mammalian skeletal and heart muscle, and is thought to play a role in the regulation of membrane potentials [1]. Even less is known about the biological actions of the others [1, 2].

Taurine gives a positive ninhydrin reaction, and can be determined by conventional ion-exchange amino acid analysis. The identification and determination of the other sulfonic acids was more complicated, involving laborious chromatographic, electrophoretic or ion-exchange techniques.

Methods have also been developed to determine sulfonic acids by gas chromatography (GC). Thus, Caldwell and Tappel [3] and Rosei et al. [4] described analytical methods for taurine and isethionic acid, respectively, using silylated derivatives and GC instruments equipped with hydrogen flame ionization detectors. Recently, Remtulla et al. [5] have published a GC method for isethionic acid, determined as the methyl ester.

We have repeated the above methods, and found that the methyl ester derivatives of sulfonic acids can easily be detected by flame ionization GC. However, we found that the flame ionization detectors were insensitive to the trimethylsilyl (TMS) derivatives.

In the present paper it is shown that silylated sulfonic acids can be detected by the use of combined GC—mass spectrometry (MS), where the total ion current of the ionization chamber is used as detector. Furthermore, it is

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shown that an ordinary OV-17 gas chromatographic column gives good separation among the different acids tested.

MATERIALS AND METHODS

Chemicals

Sulfuric acid, sulfosalicylic acid, sulfanilic acid, *p*-toluenesulfonic acid, and N,N-dimethylformamide were purchased from Merck, Darmstadt, G.F.R. Isethionic acid (sodium salt) and cysteic acid were obtained from Sigma, St. Louis, Mo., U.S.A. Sulfamic acid and taurine were the products of Fluka, Buchs, Switzerland. *o*-Sulfobenzoic acid was purchased from Schuchardt, Görlitz, G.F.R. BSTFA (bis (trimethylsilyl) trifluoroacetamide) and the hydrocarbon standards were obtained from Supelco, Bellafonte, Pa., U.S.A.

Instrumentation

The combined GC—MS instrument used was a Varian CH 7, manufactured by Varian-MAT, Bremen, G.F.R. It consisted of a Varian 1400 gas chromatograph with a coiled-glass column (6 ft × 1/8 in I.D.), a molecular separator of the glass frit type, and a single-focusing mass spectrometer operated with an ionization energy of 70 eV. The total ion current of the mass spectrometric ionization chamber served as detector for the gas chromatograph. Helium was the carrier gas (15 ml/min).

In addition, two Varian chromatographs model 2100 (Varian Aerograph, Walnut Creek, Calif., U.S.A.), each equipped with 2 U-columns of glass (6 ft × 1/4 in. I.D.) and one Varian 1400 chromatograph fitted with a coiled-glass column (6 ft × 1/4 in. I.D.), were used. These chromatographs had hydrogen flame ionization detectors. Nitrogen was the carrier gas (30 ml/min). The standard injection temperature was 230° and the detectors were usually kept at 250°.

The GC column material used was 10% OV-17 on Gas-Chrom Q (80—100 mesh), obtained from Applied Science Labs., State College, Pa., U.S.A.

Preparation of derivatives

In a small test tube with a PTFE screw cap were placed about 5 mg of sulfonic acid, 300 μl of dimethylformamide as solvent, and 300 μl of BSTFA. The tube was tightly capped, and placed in a sand bath at 110° for 60 min. The mixture was then ready for analysis. Heating at a temperature above 120° or at 110°for more than 2 h led to decomposition; the most unstable compounds were taurine and cysteic acid.

RESULTS

TMS derivatives were made of nine different sulfonic acids. None of these derivatives gave any response on the hydrogen flame ionization detectors. All chromatograms contained a peak with a methylene unit value of 14.5, representing a compound which was formed when dimethylformamide was heated for more than 20 min at a temperature of about 110°. The structure

TABLE I

GC—MS DATA FOR TRIMETHYLSILYL DERIVATIVES OF SULFONIC ACIDS

M.U. value = methylene unit value. The analytical details are given in the text.

Compound	TMS deriv.	Mol.wt.	M.U. value	10 most abundant *m/e* fragments									
Sulfuric acid	diTMS	242	12.8	147,	73,	227,	102,	148,	45,	59,	75,	66,	149
Isethionic acid	diTMS	270	15.1	255,	73,	147,	75,	74,	45,	66,	59,	256,	43
Taurine	diTMS	269	16.0	254,	102,	147,	73,	115,	116,	45,	59,	100,	74
	triTMS	341	17.9	326,	73,	147,	174,	327,	59,	45,	133,	100,	130
Cysteic acid	triTMS	385	18.6	73,	147,	75,	268,	45,	44,	77,	100,	59,	229
	tetraTMS	457	20.6	147,	73,	340,	241,	45,	74,	75,	268,	303,	59
Sulfamic acid	diTMS	241	14.3	147,	226,	73,	146,	148,	66,	45,	75,	59,	149
	triTMS	313	14.9	298	147,	73,	133,	148,	299,	130,	45,	59,	149
p-Toluenesulfonic acid	monoTMS	244	18.8	229,	149,	147,	91,	165,	75,	73,	65,	230,	231
o-Sulfobenzoic acid	diTMS	346	21.4	331,	147,	73,	148,	45,	75,	135,	76,	149,	119
Sulfosalicylic acid	triTMS	434	23.3	419,	73,	420,	421,	251,	331,	45,	74,	147,	149
Sulfanilic acid	triTMS	389	23.6	374,	73,	229,	375,	221,	45,	75,	149,	376,	230

of this artifact has not been determined. It has, however, no relation to any of the sulfonic acids.

When analyzed in the combined GC—MS instrument, the sulfonic acids gave rise to sharp and symmetrical peaks. Two derivatives were found for taurine, sulfamic acid and cysteic acid, representing either one or two TMS groups attached to the amino group of these compounds. The conditions used favored the formation of an amino—diTMS group over the monoTMS form. For each of the other sulfonic acids only one derivative could be detected.

In Table I the methylene unit values and the 10 most abundant *m/e* fragments of the sulfonic acids are given. A characteristic feature of the mass spectra is a dominating M—15 fragment; this is the base peak for many of the derivatives.

The mass spectra of the two taurine derivatives and of isethionic acid diTMS given in Fig. 1 illustrate the typical pattern of fragmentation for this class of compounds. The sulfonic group is present only in a small number of the fragments; most stem from the other part of the sulfonic acid molecules.

The response of the total ion current detector to the sulfonic acid derivatives is of the same magnitude as to other organic acids. The detector response to taurine (sum of both derivatives) was found to be 1.3 relative to that of nonanedioic acid.

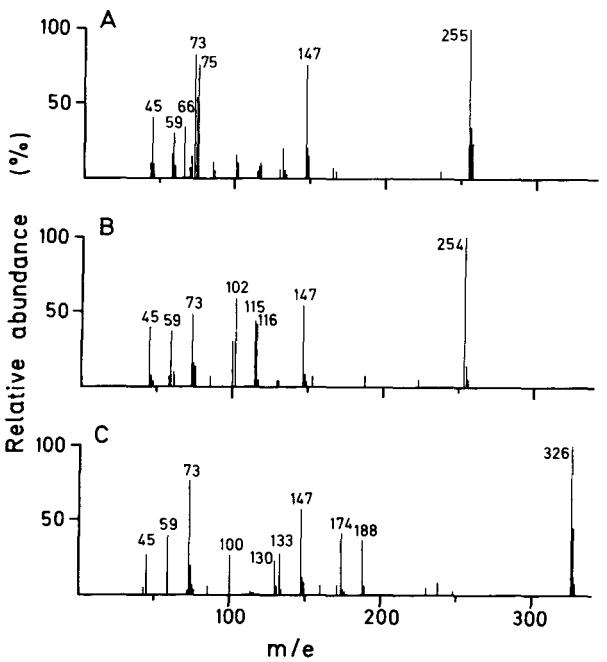

Fig. 1. Electron impact mass spectrum of (A) isethionic acid—diTMS, (B) taurine—diTMS, and (C) taurine—triTMS. The molecular ions are not visible. M—15 is base peak in all three spectra.

DISCUSSION

Sulfonic acids will easily form TMS derivatives, which are well separated by a standard gas chromatographic column. Trimethylsilylation has the advantage over methylation that a larger number of compounds becomes volatile by the former technique than by the latter. Of the nine acids analyzed in the present paper, only four could have been visualized as methyl esters (toluenesulfonic, sulfobenzoic, sulfosalicylic and isethionic acids). The sensitivity is good for both types of derivatives.

Methyl esters of the sulfonic acid are detected by the ordinary hydrogen flame ionization detector [5]. This detector is, however, insensitive to the TMS derivatives. The reason for this is unknown. Apart from containing a sulfonic group, the structures of the acids tested differed substantially from each other. It is, therefore, reasonable to conclude that the lack of response is related to some property of the sulfonic group, preventing the formation of charged radicals in the hydrogen flame.

It is difficult to understand how TMS derivatives of taurine and isethionic acid could have been detected by flame ionization in previously described methods [3, 4]. In both cases dimethylformamide was used as a solvent during derivatization. One possibility is that the artifact, which is formed when dimethylformamide is heated, has been misinterpreted and taken for the silylated derivative of the sulfonic acid in question.

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Note

Quantitative separation of bile salts from mixtures by gradient-elution high-pressure liquid chromatography

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It is being increasingly recognised that bile acids play a major role in the aetiology and course of many human diseases [1, 2]. The progress of investigation in this field, however, has been considerably hampered by the lack of a simple method for fractionating the complex bile salt mixtures found in biological materials. In earlier work liquid chromatography was used, but more recently the two main approaches have been to employ gas—liquid chromatography or thin-layer chromatography (TLC). The former necessitates elaborate processes for extraction and purification in addition to the preparation of volatile derivatives, whilst TLC requires the use of time-consuming elution procedures before further analysis can be undertaken. High-pressure liquid chromatography (HPLC) has two inherent advantages over the above techniques; it not only provides comparable resolution in a relatively short time, but it also provides the resolved fractions as eluates free from silicic acid or other chromatographic medium.

The first report of the application of HPLC to the separation of conjugated bile salts appeared as recently as 1976 [3]. Although encouraging, the results showed that human bile contains substances other than bile salts having mobilities in HPLC similar to those of the bile salts themselves. It was therefore essential that the quantitation steps following HPLC should be such as to ensure the specificity of the entire procedure. This publication was succeeded in 1977 by a paper from Parris [4] who demonstrated that reversed-phase HPLC with aqueous methanol as solvent could separate isomers of bile acids and of their conjugates. The same author showed the potential for detection of absorptiometers operating at very short wavelengths of 210 nm or less. However, the loads treated were very small, precluding recovery for confirmatory analysis, while application to clinical samples appears still to be remote. Such difficulties have been obviated by the application of gradient-

elution techniques to HPLC with silicic acid in order to resolve biological bile salt mixtures into fractions suitable for analysis by specific enzyme methods.

EXPERIMENTAL

Apparatus and materials

Stainless-steel 50 × 0.2 cm I.D. columns were packed with small-sized spherical particles coated with chemically bonded silicic acid (Vydac, 30—44 μm; Anachem, Luton, Great Britain) or with a similar pellicular material (Perisorb A, 30—40 μm; Merck, Darmstadt, G.F.R.). Samples were introduced onto the column by means of a 20-μl injection loop attached to a high-pressure change-over valve (Anachem; Fig. 1). The developing solvent was introduced by means of a Milroy pump (Milton Roy, St. Petersburg, Fla. U.S.A.) rated at 3000 p.s.i., equipped with a relief valve (V in Fig. 1; constructed in the laboratory and set to discharge at 2500 p.s.i.). A second Milroy pump was used to form the liquid gradient.

All solvents used were of Analytical Reagent Grade and obtained from BHD (Poole, Great Britain). No attempt was made to remove from the chloroform any ethanol added for stabilisation. Non-radioactive bile salts were obtained

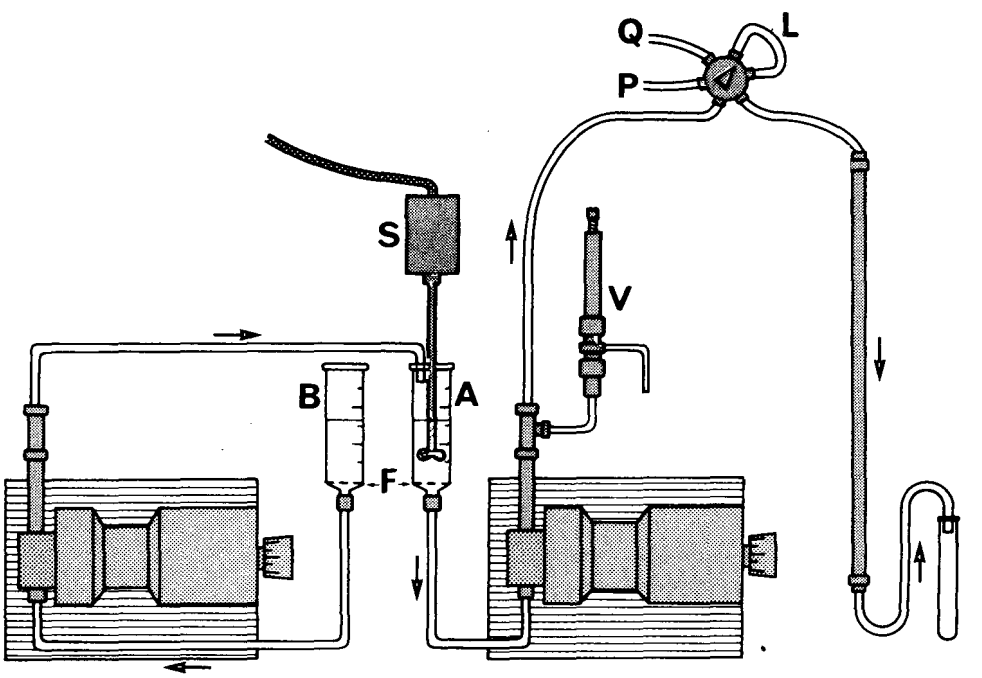

Fig. 1. System for HPLC with polarity gradient. Reservoirs A (in which the gradient forms) and B are glass 20-ml syringe barrels connected to the pumps by PTFE tubing (O.D. 3.2 mm). S is a stirrer. Reservoirs are fitted with paper filters, F. Other tubing is stainless steel of bore 0.25 mm; joints are Swagelok fittings. Loop L is loaded at P from a 100-μl syringe, excess solution being visible in the transparant tube Q while the eluent passes directly to the column. When L is placed in circuit, excess load solution can be withdrawn at P. V, relief valve.

from Maybridge, Tintagel, Great Britain, and from Steraloids (Pawling, N.Y., U.S.A.), except for glycochenodeoxycholic acid, glycocholic acid and chenodeoxycholic acid, which were kindly donated by Weddel Pharmaceuticals. The purity of each substance was assessed by TLC on silica gel G using ethylene dichloride—acetic acid—water (1:1:0.1) [5] as developing agent. Qualitative assessment was made after using sulphuric acid—ultraviolet light for detection [6]. Quantitative results were obtained by using 3α- and 7α-hydroxysteroid dehydrogenases [7, 8] following non-destructive location with iodine vapour. A stock solution (2 m*M* in methanol) of glycochenodeoxycholic acid was prepared directly from a bile acid sample which had been found to be not less than 95% pure. The other bile salts were first purified by TLC and methanolic eluates of the corresponding areas were calibrated using the enzyme method with the glycochenodeoxycholic acid solution as standard.

Radioactive bile acid preparations, [carboxyl-^{14}C]chenodeoxycholic acid, [1-^{14}C]glycocholic acid, and tauro[carbonyl-^{14}C] cholic acid were obtained from The Radiochemical Centre (Amersham, Great Britain).

Methods

All columns were equilibrated with 15 ml chloroform before each run and the flow-rate was assessed by timing the passage of 10 ml of this solvent. The output rate of the second pump was adjusted to the same value.

Bile acids were extracted from bile-rich duodenal aspirates using Amberlite XAD-2 resin on a batch procedure as described by Barnes and Chitranukroh [9]. Some 200—600 μg bile salts (20 μl methanolic solution) were applied to the column and chloroform passed for 0.5 min. The gradient elution was then commenced and half-minute fractions were collected.

The gradient was formed as follows: solution A (chloroform) of volume V, was placed in the reservoir A (Fig. 1) and solution B (ethyl acetate—ethanol, 40:15) added at the same rate (r; approx. 1 ml/min) as that at which the reservoir contents were transferred to the column. The proportion of solvent B in the reservoir A at time t (min) is given by $1\text{-}e^{-(r/V)t}$ where V is the volume (ml) at which the reservoir contents are maintained. In the present context suitable values for r were found to be in the range 0.5—1.1 ml/min, with V ranging from 5 to 15 ml; all components of the mixtures examined then eluted within 30 min. It may be shown that $(V/r)\ln 2 = \lambda$, where λ is the half-life characterising the gradient, and is the time (min) required to produce in the reservoir a mixture comprising equal volumes of A and B. Since the value for r was fixed on chromatographic criteria, V was calculated so as to obtain a half-life of 4—15 min, this range having been determined as optimal in prior experiments with standard mixtures.

In qualitative studies the fractions obtained were evaporated to dryness under a steam of nitrogen with the tubes in water at 60°, the residues were dissolved in methanol—chloroform (3:1) and subjected to TLC using ethyl acetate—ethanol—acetic acid (40:20:2) as mobile phase and with phosphomolybdic acid for detection. In quantitative studies the fractions obtained were evaporated to dryness and assessed using, for bile extracts, the enzymatic methods mentioned above.

RESULTS

Qualitative studies with various mixtures of bile salt standards indicated that with a gradient half-life of 4—8 min, satisfactory resolution of the common bile acids could be obtained within 30 min. Bile salts were clearly separated according to whether they were taurine- or glycine-conjugated, and di- or trihydroxy acids (Table I). The maximum amount of bile acid mixture that could be applied to the present column if "tailing" and other overload effects were to be avoided was about 500 μg for roughly equal amounts of six components.

With human duodenal aspirates it was found essential that some initial extraction be made, and in all subsequent work the method of Barnes and Chitranukroh [9] was used. Bile acids were assayed before extraction and in each half-minute fraction obtained from HPLC. In this way it was possible to monitor the recovery. In six duodenal aspirates values for total 3α-hydroxy bile salts obtained by summation of the HPLC fractions were some 92.3±1.7 (S.D.) % of those obtained by analysis of the unchromatographed extracts.

The absence of cross-contamination between eluted peaks was demonstrated by the addition of radioactive bile acids (approx. 0.1 μCi) to each of

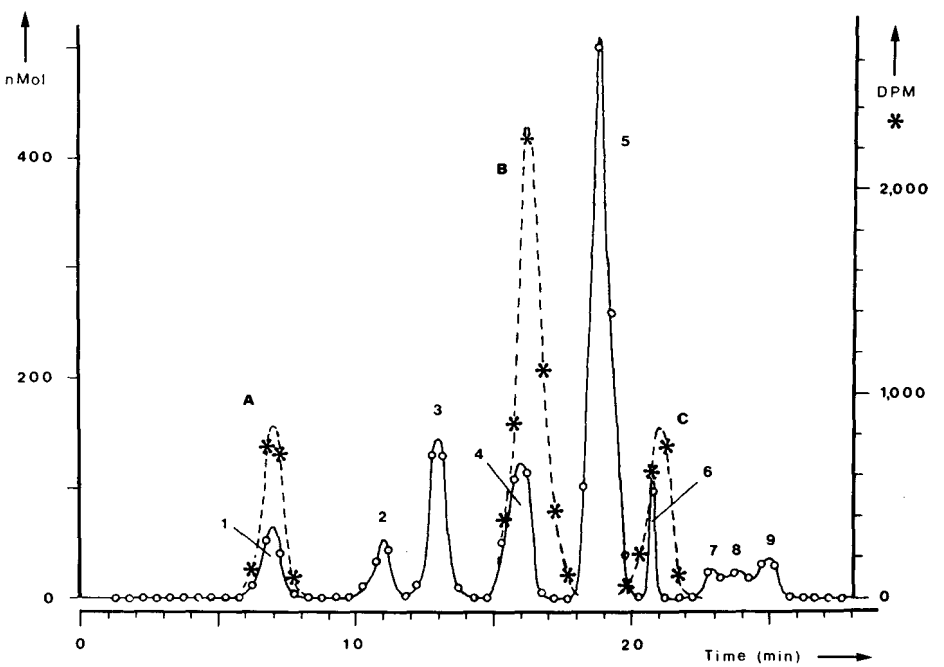

Fig. 2. Separation by gradient HPLC with Perisorb A (30—40 μm) of extract from bile-rich duodenal fluid. Peaks: 1 = dihydroxy bile salts; 2 = cholate; 3 = taurine conjugated dihydroxy bile salts; 4 = taurocholate; 5 = glycochenodeoxycholate; 6 = glycocholate; 7—9, unknown; A = [^{14}C]chenodeoxycholic acid; B = tauro[carbonyl-^{14}C]cholic acid; C = [1-^{14}C]glycocholic acid. Curves drawn through the points conform with those seen on the absorption patterns (280 nm) obtained by chromatography, on the same column and in similar conditions, of aromatic solutes.

TABLE I

SEPARATION OF COMPONENTS OF A 6-PART MIXTURE OF BILE SALTS

Load, 800 μg total. A: Perisorb A; gradient half-life, 3.4 min; flow-rate, 0.79 ml/min. B: Vydac; gradient half-life, 8 min; flow-rate, 0.78 ml/min. V_e = elution volume.

Substance	A		B	
	V_e(ml)	t(min)	V_e(ml)	t(min)
Na deoxycholate	6.2	7.9	3.2	4.1
Cholic acid	8.1	10.3	5.5	7.1
Na taurodeoxycholate	10.7	13.5	8.8	11.3
Na taurocholate	13.1	16.6	11.4	14.6
Na glycodeoxycholate	15.5	19.6	12.9	16.5
Na glycocholate	16.9	21.4	18.1	23.2

three samples. Recovery was 96.8±1.4% and occurred in a single peak. To one sample all three radioactive labels were added, the conjugated markers being eluted with the corresponding bile acids in the sample (Fig. 2).

As part of an otherwise unrelated investigation [10] the columns presently described were used in similar conditions for chromatography of esters of phthalic acid and of cholesterol and its esters. In that work, effluent was passed directly through the cell of a Uvicord II monitor (LKB) before collection, thus providing a continuous strip-chart of absorption at 281 nm. Sharp symmetrical peaks of Gaussian form were seen; this evidence was considered to justify the drawing of the continuous curves in Fig. 2 through the scattered points actually obtained.

DISCUSSION

The success of any method for the quantitative resolution of the bile acid mixture present in biological samples depends on the homogeneity of each fraction and the specificity of the final reaction. With the possible exception of gas chromatography—mass spectrometry (GC—MS) [11] few of the methods described to date fulfil these criteria. GC—MS, however, necessitates the preparation of volatile derivatives and the use of technically exacting and time-consuming techniques [12]. Even then, the possibility of the formation of artefacts during these chemical preparative steps is not easily precluded. All previously established methods for fractionating bile acid mixtures are compromises and clearly that which we now describe is no exception. The level of specificity now attained, however, is comparable with that achieved with TLC in conjunction with 3α-hydroxysteroid dehydrogenase [13]. Its advantages over TLC are those of higher resolution and rapidity, and reside also in the fact that the fractions are obtained directly as eluates in solvents that are free of silicic acid, volatile and which do not react with bile salts.

The resolution obtained is similar to that described by Shaw and Elliot [3] and, very recently, by Parris [4]. Obviously, our use of discrete fractions must confer the effect of broadening the peaks, and thus of impairing resolution. This effect could be reduced by taking fractions at intervals of less

than 0.5 min, or would be avoided entirely if a flow-through detector could be employed. Application of short-wave ultraviolet absorption, the feasibility of which was established by Parris [4], does however impose crippling limitations on the choice of solvents, as that author points out. On balance, however, it might appear that the useful future for HPLC in the analysis of bile salts could be exploited most profitably by reserving maximum flexibility of conditions, meanwhile taking advantage of the highly specific enzymatic or radioimmunological methods already available.

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Note

The gas—liquid chromatographic analysis of trimethoprim in plasma and urine

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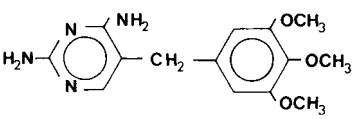
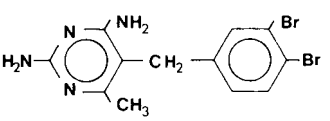
Trimethoprim (I) has been used in combination [1, 2] with various sulphonamides as a broad-spectrum antibacterial agent. Assays based on microbiological [3], differential pulse polarography [4], spectrofluorimetric [5—7] and gas chromatographic [8] methods have been reported. The assay from plasma and urine described here employs GC with nitrogen-selective detection, and obviates derivatisation.



I-TRIMETHOPRIM



II-BW 214U

EXPERIMENTAL

Materials

Trimethoprim (TMP; 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)-pyrimidine) and 2,4-diamino-5-(3′4′-dibromophenyl)-6-methyl pyrimidine (BW51-214) (II) were obtained from the Wellcome Research Labs. (Beckenham, Great Britain). Chloroform of AnalaR grade (BHD, Poole, Great Britain), isopropanol (Rathburn Chemicals, Walkerburn, Great Britain) and freshly deionised water were also used.

Glassware

Soveril 20-ml stoppered test tubes (type 611-03; V.A. Howe, London, Great Britain) were used for the extraction and Quickfit BC24/C14T (Q and Q) tapered centrifuge tubes were used in the drying down stage. Micro-vials (Hewlett-Packard, Winnersh, Great Britain) were used for the gas chromatograph auto-sampler. All glassware was hydrochloric acid-washed before use.

Gas chromatograph

A Perkin-Elmer (Beaconsfield, Great Britain) F-30 gas chromatograph equipped with a nitrogen—phosphorous detector (Part No. 3006—7178) was used. The injection port was modified to take a Hewlett-Packard 7670A/002 auto-sampler.

Method

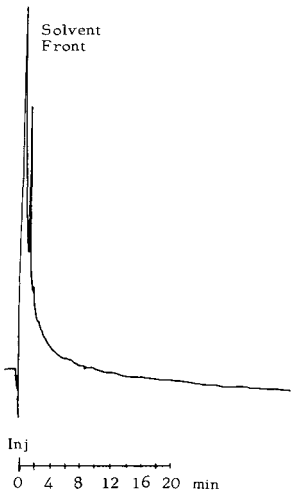
Standard solutions of TMP in plasma or urine were prepared by adding the appropriate volume from a 1 mg/ml stock solution in water containing HCl (pH 4.5).

Plasma or urine samples (0.5 ml) containing TMP were placed in 20-ml extraction tubes to which were added 20 μl of a 100 μg/ml solution of BW51-214 in isopropanol, 4 ml of 0.1 mol/l sodium carbonate and 10 ml of chloroform. The test tubes were then stoppered and mixed for 30 min along their long axes at 25 oscillations per min. The liquid phases were then separated by centrifugation at 100 *g* for 20 min, the aqueous layer was removed by suction and discarded. An 8-ml aliquot from the remaining organic layer (chloroform) was then transferred to the tapered centrifuge tubes. The chloroform was evaporated by heating the tubes at 60° under a stream of nitrogen. The residue was then taken up in 100 μl of isopropanol and transferred to a microvial followed by two further 50-μl washes of isopropanol. The vial was capped for subsequent GC analysis the same day.

Gas—liquid chromatographic conditions

A 1.8 m × 4 mm I.D. glass column was hand packed with 10% Poly S-179 on Chromosorb W HP, 80—100 mesh (Field Instruments, Richmond, Great Britain) and conditioned at 350° with a 30 ml/min helium carrier-gas flow for 24 h before use.

The detector was used in the nitrogen mode under the following conditions: hydrogen (3.8 ml/min); air (105 ml/min); heating position (680 in the NP-mode); carrier gas (helium, 45 ml/min); manifold, oven and injection port (350°, 330° and 350° respectively). Chromatography was good (see Figs. 1 and 2) with BW51-214 having a retention time of 6.3 min and TMP having a retention time of 8.5 min. No interfering peaks were seen in human plasma or urine, even from the sulphonamides commonly administered in conjunction with TMP, namely sulphamethoxazole and sulphadiazine. A computer-based data system (Hewlett-Packard Model 3352) was used to calculate peak areas and their ratios, and also it controlled the operation of the automatic liquid sampler.



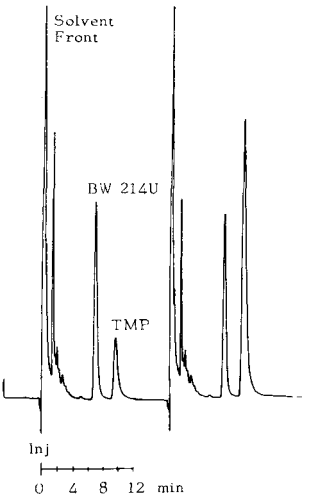


Fig. 1. Chromatogram produced from a urine "blank" sample analysed by this procedure.

Fig. 2. Chromatograms produced by plasma samples containing 5 μg/ml and 20 μg/ml TMP, respectively, with internal standard.

RESULTS

Calculation of results

A known mass of internal standard (2 μg of BW51-214) was added to a range of TMP standard solutions. A calibration curve was constructed with the concentration of TMP on the abcissa and the ratio of peak area TMP to peak area BW51-214 on the ordinate. Since the same mass of internal standard was added to the unknown samples the unknown concentrations can be determined from the calibration curve.

Validation

The method was tested by analysing samples of plasma to which quantities of TMP unknown to the analyst had been added. Six determinations were carried out on each sample and the precision is shown in Table I.

Applications

An example of the plasma concentration curve in a healthy female volunteer after taking 200 mg TMP by mouth is shown in Fig. 3.

Linearity, sensitivity and recovery

The detector gave a linear response from 2 to 400 ng of TMP injected on column. The lower limit of sensitivity being 1 ng under the standard conditions. Under the operating conditions described the method is capable of routinely detecting down to 0.1 μg/ml of TMP in plasma or urine. Total recovery was 78% because of extraction and transfer losses.

TABLE I

ANALYSIS OF TRIMETHOPRIM ADDED TO PLASMA

Each result is the mean of six determinations

Trimethoprin conc. (μg/ml)		Standard deviation (μg/ml)	Sample coefficient of variation (%)	Deviation from theory (%)
Added	Assayed			
0.85	0.85	0.11	12.9	0
2.13	2.14	0.08	3.74	+0.47
3.25	3.21	0.31	9.66	-1.23
4.33	4.47	0.27	6.04	+3.23
5.63	5.89	0.28	4.75	+4.62
7.50	7.73	0.40	5.17	+3.07

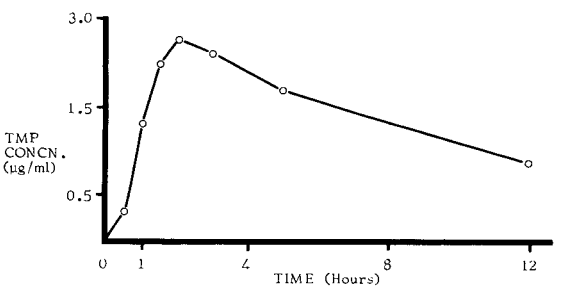


Fig. 3. A typical plasma profile from a healthy female after 200 mg of TMP was given orally.

DISCUSSION

The described method was found to be simple and specific for unchanged TMP and also extremely sensitive, 1 ng of TMP injected on column being readily detected. Several precautions were necessary to ensure reproducible results. Firstly, some TMP adsorption occurs to glass, which causes carry-over from the glass injection syringe of the auto-sampler. The use of a syringe wash in chloroform between injections overcame the problem. Secondly, sensitivity could be increased by increasing the bead temperature of the detector, however this was detrimental to reproducibility. Finally, the use of an internal standard reduces error from transfer losses.

The method is in routine use for human pharmacokinetic and bioavailability studies.

ACKNOWLEDGEMENTS

We would like to express thanks to Dr. Graham Taylor, Wellcome Foundation Ltd., Dartford, England, for suggesting the use of 10% Poly S-179 on Chromosorb W HP for the gas chromatography of Trimethoprim.

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Note

Determination of metformin in plasma at therapeutic levels by gas—liquid chromatography using a nitrogen detector

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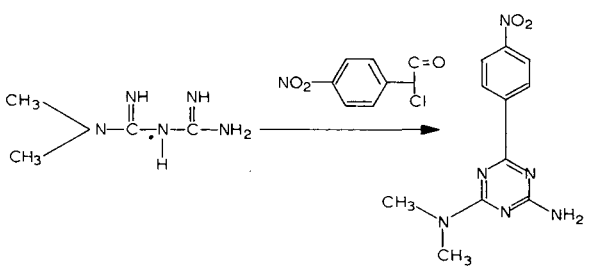
In the literature dealing with the identification and quantification of the hypoglycaemic biguanides in biological fluids there is little information concerning specific methods for metformin (N,N-dimethylbiguanide) [1—7]. Unfortunately, even the most sensitive of these do not demonstrate sufficient reproducibility; this is due principally to difficulties in extraction, derivatization and linearity of the internal standardisation. In order to overcome these extraction difficulties Ross [8] described a technique for derivatization in aqueous medium using high-performance liquid chromatography for the determination of urinary levels of metformin. However, this has limited application to other biological fluids, due to the sensitivity of the detector. We have adapted this useful technique of derivatization to gas—liquid chromatography (GLC). By using a nitrogen detector and a linear internal standard, a reproducible, specific and quantitative analysis of metformin has been possible with a sensitivity suitable for plasma and tissue levels of the drug after the administration of therapeutic doses.

PRINCIPLE

Metformin is derivatized directly in the biological medium with *p*-nitrobenzoyl chloride to form the corresponding substituted triazine derivative: 2-amino-6-dimethylamino-4-(4′-nitrophenyl)-1-3-5-triazine, thus:



The reaction is carried out in the presence of an excess of acetonitrile and as soon as the triazine is formed it passes into the acetonitrile phase. The extract is analysed by GLC. A thermionic detector sensitive to nitrogen assures specific detection of the molecule and its quantitative analysis with good sensitivity. The calculation of the metformin level is performed using propylbiguanide hydrochloride as an internal standard.

EXPERIMENTAL

Reagents

Metformin and propylbiguanide hydrochloride were kindly supplied by S.N.E.L. Aron (Suresnes, France).

The preparation of derivatives of metformin: the 2-amino-6-dimethylamino-4-(4'-nitrophenyl)-1-3-5-triazine and of propylbiguanide: the 2-amino-6-propylamino-4-(4'-nitrophenyl)-1-3-5-triazine were carried out using the technique described by Ross [8].

Gas—liquid chromatography

The analyses were performed on a Carlo Erba Chromatograph, Fractovap series 2350, equipped with a nitrogen detector (KCl salt). The glass columns (200 cm × 3 mm I.D.) were packed with 3% OV-17 on silanised Chromosorb W (80—100 mesh). Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min. The oven temperature was maintained at 250°.

Extraction and derivatization of metformin

Samples of 2 ml of plasma were placed into 10-ml stoppered test tubes. After addition of 2.5 μg propylbiguanide hydrochloride (in 100 μl of water) as the internal standard, sodium chloride was added to saturation and 1 ml of acetonitrile. The tubes were agitated for 15 min on a roto-reciprocating stirrer. After centrifugation the acetonitrile layer was removed using a Pasteur pipette and rejected. 1 ml of 5 *N* sodium hydroxide, sodium chloride to saturation point, 1 ml of acetonitrile and about 10 mg of *p*-nitrobenzoyl chloride were added. The stoppered tubes were shaken for 15 min. A further 10 mg of *p*-nitrobenzoyl chloride was added and the tubes were shaken again for 15 min. After centrifugation the acetonitrile layer was transferred to a "minivial" and evaporated to dryness in a water bath at 60°. The residue was taken up in tetrahydrofuran (usually 100 μl) and the solution was injected into the chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows the trace obtained from 2 ml of control dog plasma. Fig. 2 shows the trace obtained from 2 ml of treated dog plasma taken 1 h after the intravenous administration of metformin at a dose of 10 mg/kg body weight with the addition of 2.5 μg of propylbiguanide hydrochloride. The derivatives of metformin and propylbiguanide hydrochloride were characterized by their Kováts retention indices obtained from an injection containing the two syn-

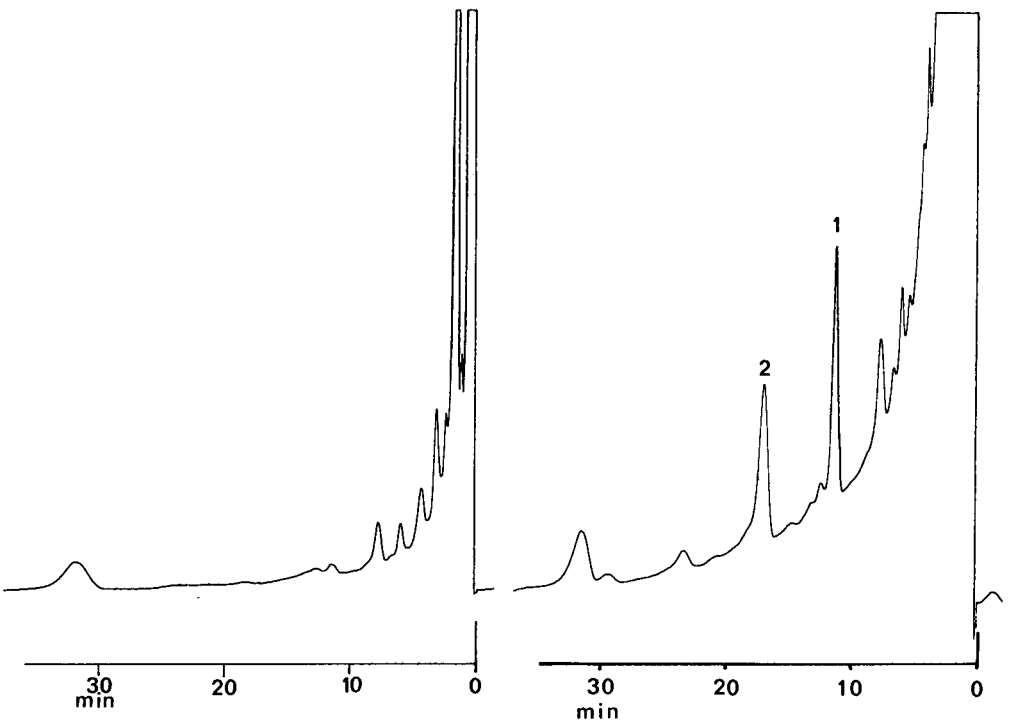


Fig. 1. Gas chromatogram of an extract of 2 ml of dog plasma containing no drug.

Fig. 2. Gas chromatogram of 2 ml of dog plasma previously treated with 10 mg/kg metformin i.v. sampled after 1 h. 2.5 μg propylbiguanide hydrochloride added as internal standard. 1 = Metformin derivative; 2 = propylbiguanide derivative.

thesised *p*-nitrobenzoyl derivatives and selected normal saturated hydrocarbons (C28 and C32):

p-nitrobenzoyl derivative of metformin: IRK = 2975
p-nitrobenzoyl derivative of propylbiguanide: IRK = 3120

Considering the large variations in recovery observed between the two preparations it was necessary to use an internal standard. The area under the peaks corresponding to each of these derivatives is measured on the chromatogram. The concentration of metformin is directly proportional to the areas under the peaks. Experimental points obtained by adding known quantities of metformin and propylbiguanide hydrochloride to rat plasma fall on a straight line, the equation of which has been mathematically calculated, (y = $1.35\,x - 0.04$) with an excellent correlation ($r = 0.994$).

The derivatization techniques for metformin previously described [1, 2] necessitated a preliminary extraction procedure. This extraction from blood plasma has a poor recovery, of the order of 40% under the best conditions

giving a very poor overall recovery of about 10%. In this new derivatization technique, performed directly in aqueous solution without previous extraction, it is possible to obtain a total recovery of 88% (10 determinations) with a standard variation of 15%.

In order to use GLC for this determination it is important that the extract from plasma should be as pure as possible while consistent with minimal loss of metformin. For this reason, an initial washing of the plasma sample with acetonitrile was carried out. This solvent is, however, incompatible with a nitrogen detector and the dried extract was dissolved in tetrahydrofuran for injection into the chromatograph. Using this procedure levels of metformin below 25 ng/ml could be easily determined, providing adequate sensitivity for the determination of therapeutic blood levels. It should be noted that the procedure is also applicable to other biological fluids such as urine and tissue homogenates.

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Note

Determination of the thiazide diuretic bemetizide in the plasma and urine of humans by high-performance liquid chromatography

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Thiazides are among the most commonly used diuretic and antihypertensive agents, but until recently few methods have been reported for their determination in biological fluids. Earlier methods of measurement, involving hydrolysis and colorimetric determination of their diazotized amino degradation products [1], lacked sensitivity and specificity, Fluorimetric thin-layer chromatography [2] and spectrofluorimetry [3] have also been employed. Gas chromatographic determinations [4—6] utilising electron-capture detection are somewhat lengthy, involving several extraction steps as well as derivatisation. More recently, high-performance liquid chromatography (HPLC) has been applied to the measurement of hydrochlorothiazide in serum after gel filtration [7] and in serum and urine after solvent extraction and back extraction into alkali [8].

This paper describes a method for the measurement of the thiazide diuretic bemetizide [3-(α-methylbenzyl)-6-chloro-7-sulphamoyl-3,4-dihydro-1,2,4-benzothiadiazin-1,1-dioxide; Fig. 1]* in plasma and urine by HPLC in a reversed-phase mode. An internal standardisation technique was employed using the structurally related thiazide, cyclopenthiazide as the internal standard. The method is simple, rapid and sensitive and has been applied to the measurement of drug in human plasma and urine.

*Present in Diucomb® produced by Sanol Schwarz-Monheim GmbH, Monheim, G.F.R.

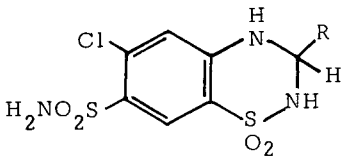

Fig. 1. Chemical structure of bemetizide (R = $-CH(CH_3)C_6H_5$) and cyclopenthiazide (R = $-CH_2C_5H_9$).

EXPERIMENTAL

Materials

Standard solutions of bemetizide in methanol were prepared at concentrations of 5 μg/ml and 20 μg/ml, and cyclopenthiazide (3-cyclopentylmethyl-6-chloro-7-sulphamoyl-3,4-dihydro-1,2,4-benzothiadiazin-1,1-dioxide) (the internal standard) was prepared at 20 μg/ml. Both thiazides were supplied by Sanol Schwarz-Monheim (Monheim, G.F.R.). Reagents were of analytical grade and inorganic reagents were prepared in freshly glass-distilled water.

Extraction

Plasma samples (2 ml) were pipetted into centrifuge tubes and spiked with internal standard (5 μl, 100 ng). Urine samples (1 ml) were spiked with internal standard (20 μl, 400 ng). Sodium hydrogen carbonate (200 mg) was then added, and the mixture was extracted by shaking it with diethyl ether (5 ml) for 1 min. After centrifugation, the ether layer was carefully transferred to a pointed centrifuge tube and evaporated to dryness under a stream of nitrogen at ambient temperature. The residue was washed to the bottom of the tube by the addition of further small volumes of diethyl ether, which were evaporated to dryness. The residue was dissolved in methanol (20 μl), and as much as possible injected into the chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M 6000 A pump (Waters Assoc., Stockport, Great Britain) fitted to a Cecil 212 variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) operated at 271 nm. Injection was by syringe (50 μl) via a Waters Assoc. U6K universal injector. The column was 30 cm × 4 mm I.D., prepacked with μBondapak C_{18} (particle size 10 μm) (Waters Assoc.). Chromatography was performed in reversed-phase mode using a solvent system of methanol—0.01 *M* potassium dihydrogen orthophosphate (1 : 1, v/v), at a flow-rate of 2 ml/min. Under these conditions, bemetizide and cyclopenthiazide were eluted with retention times of 4 and 5 min, respectively (Fig. 2). As used in therapy, bemetizide is a mixture of stereoisomers in a 5 : 1 ratio, which are capable of being resolved by HPLC, but the conditions were adjusted so that the isomers were eluted from the column as a single peak, and total unchanged drug concentrations were measured.

Collection of samples

The method of analysis was applied to samples taken from six male

volunteer subjects who were participating in pharmacokinetic studies of bemetizide. The subjects were screened clinically before and after the study and remained under medical supervision in a clinical pharmacology unit throughout its duration. The studies were carried out following consent from the volunteers and from the appropriate ethics committees. After an overnight fast, each subject received a dose of 25 mg of bemetizide which was finely suspended in 150 ml of water. Fasting was continued for 3 h after dosing. Blood samples were withdrawn into heparinised tubes before dosing and at several times thereafter. Plasma was separated by centrifugation. Urine samples were collected from each subject before dosing and during the subsequent 12 h after dosing.

RESULTS AND DISCUSSION

Concentrations of bemetizide were calculated from calibration curves constructed by plotting the peak height ratios of drug to internal standard over the concentration range 10 to 100 ng/ml in plasma and 200 to 1000 ng/ml in urine. The recovery of internal standard from plasma was 95% ± 0.9 S.D. (n = 5) and from urine was 99% ± 0.0 S.D. (n = 5). The recoveries of bemetizide from plasma (means of 94% at 10 ng/ml and 88% at 100 ng/ml) and urine (means of 98% at 200 ng/ml and 99% at 1000 ng/ml) were calculated by comparing peak height ratios of standards to those of standards extracted from plasma and corrected for any losses of internal standard (Table I). The overall recovery of bemetizide from plasma in the concentration range 10 to 100 ng/ml was 90% ± 3.4 S.D. and from urine was 98% ± 0.7 S.D. in the concentration range 200 to 1000 ng/ml.

The calibration curves were constructed from five replicate measurements at five concentrations over the ranges, and plots of peak height ratios against concentration were linear ($y = a + bx$; where a = 0.0172 ± 0.0110 S.D. and b = 0.0117 ± 0.0002 S.D. for plasma, 10 to 100 ng/ml, and a = 0.0304 ± 0.0073 S.D. and b = 0.0024 ± 0.00001 S.D. for urine, 200 to 1000 ng/ml). The intercept of the plasma calibration curve on the y axis was not significantly different from zero. The 95% confidence limits of the least-squares regression line for plasma forced through the origin were ± 36% at 10 ng/ml, ± 7% at 50 ng/ml and ± 4% at 100 ng/ml. The precision of the method for measurement of bemetizide in plasma ranged from ± 9.3% at 10 ng/ml and ± 1.5% at 100 ng/ml (Table I). The 95% confidence limits of the regression line for urine were ± 8% at 200 ng/ml, ± 2% at 600 ng/ml and ± 1% at 1000

TABLE I

RECOVERIES OF BEMETIZIDE FROM PLASMA AND URINE

Concentration added to plasma (ng/ml)	Recovery (%)	Coefficient of variation (%)	Concentration added to urine (ng/ml)	Recovery (%)	Coefficient of variation (%)
10	94	9.3	200	98	2.5
25	93	8.9	400	98	1.8
50	89	2.2	600	98	1.6
75	86	2.7	800	97	1.0
100	88	1.5	1000	99	0.5

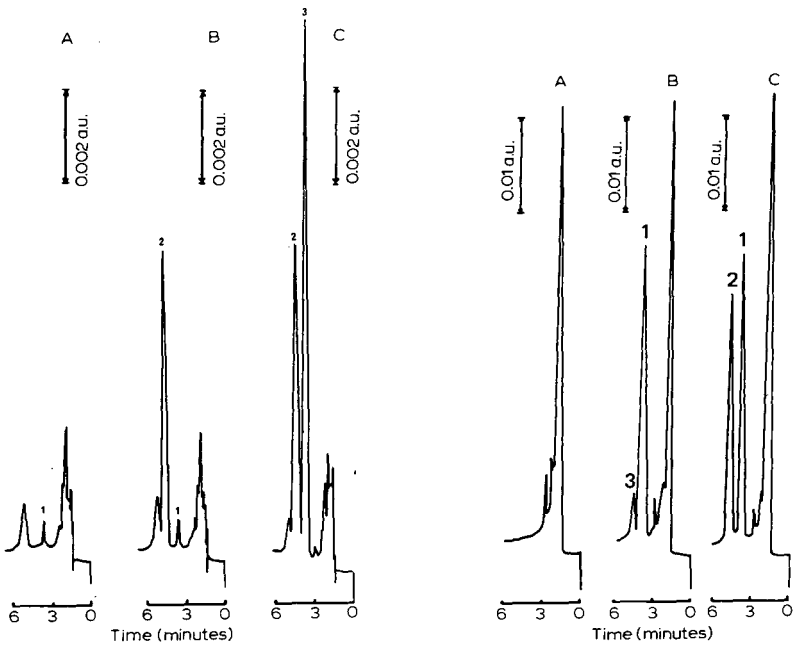

Fig. 2. Chromatograms of (A) predose control plasma, (B) predose control plasma containing internal standard (50 ng/ml) and (C) plasma sample at 4 h after dosing containing 94 ng/ml of bemetizide. Column (30 cm × 4 mm I.D.) prepared with μBondapak C_{18}; flow-rate, 2 ml/min; attenuation, 0.02 a.u.f.s.; solvent system, methanol—0.01 *M* potassium dihydrogen orthophosphate (1 : 1, v/v); wavelength, 271 nm. Peaks: 1 = plasma component (equivalent to 6 ng/ml of bemetizide); 2 = internal standard (cyclopenthiazide); 3 = bemetizide.

Fig. 3. Chromatograms of (A) predose control urine, (B) 0—12 h urine sample without internal standard and (C) 0—12 h urine sample with internal standard (400 ng/ml) containing 590 ng/ml of bemetizide. Conditions as for Fig. 1, except attenuation, 0.1 a.u.f.s. Peaks: 1 = bemetizide; 2 = internal standard (cyclopenthiazide); 3 = possible urinary metabolite with same retention time as internal standard.

ng/ml. The precision of the method for measurement of bemetizide in urine ranged from ± 2.5% at 200 ng/ml to ± 0.5% at 1000 ng/ml (Table I).

An interfering peak with the retention time of bemetizide was present in extracts of predose plasma (Fig. 2) equivalent to 6 ng/ml ± 1.5 S.D. (n = 6). This "blank" value was subtracted from measured concentrations in postdose plasma and the upper 95% confidence limit of the "blank" (10 ng/ml) was considered to be the limit of detection. No interfering peak was present with the retention time of bemetizide in extracts of urine (Fig. 3), but a peak was present in urine extracts of postdose urine (possibly a metabolite of bemetizide as it did not occur in extracts of predose urine) with the retention time of the internal standard. To determine the contribution of this material to the final peak height of the internal standard, urine was assayed with and without addition of the internal standard. The limit of detection of bemetizide in urine was not determined since measured concentrations exceeded 200 ng/ml.

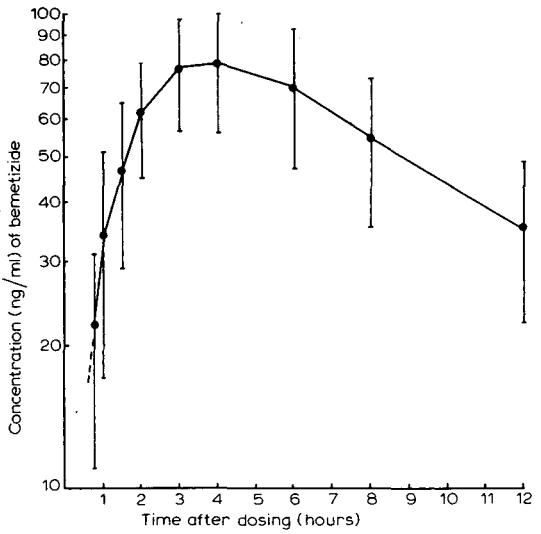

Fig. 4. Semi-logarithmic plot of mean plasma concentrations of bemetizide with time after an oral dose of 25 mg to six human subjects. Standard deviations at each time interval are shown.

When applied to the collected samples, the method showed that the peak of mean concentrations of bemetizide occurred at 4 h after dosing (78 ng/ml ± 22 S.D.) and declined to 35 ng/ml ± 13 S.D. at 12 h after dosing (Fig. 4). In urine, concentrations of bemetizide of 325—590 ng/ml were measured in samples collected during 0—12 h after dosing. During this period, a mean of 3% of the dose was excreted in the urine as unchanged drug [9].

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CHROMBIO. 169

Note

Determination of sulfapyridine and its major metabolites in plasma by high-pressure liquid chromatography

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Sulfapyridine (SP) is one biologically active moiety of salicylazosulfapyridine (trade name Azulfidine®; Pharmacia, Uppsala, Sweden) [1, 2]. This drug consists of 5-aminosalicylic acid and sulfapyridine which are linked by an azo bond. It is split in the colon by gut bacteria [1]. The sulfonamide is widely used in the treatment of Crohn's disease and ulcerative colitis [3, 4]. Similar to other sulfonamides, the metabolism of SP includes acetylation of the free amino group and hydroxylation of the benzene ring followed by conjugation with glucuronic acid to their corresponding glucuronides (Fig. 1) [4, 5]. Since the rate of acetylation of SP is genetically controlled [6, 7] and the observed side effects are found primarily in patients of the slow acetylator phenotype, it

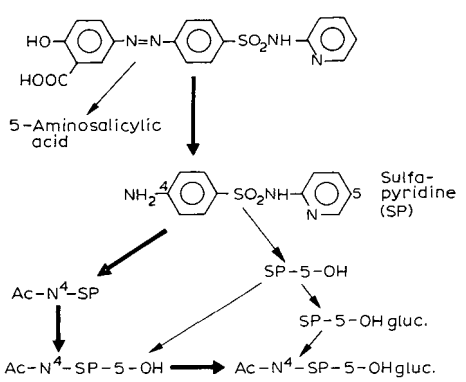

Fig. 1. Structure and metabolism of salicylazosulfapyridine (Azulfidine) in man as described by Das and Dubin [4].

seems advisable to determine the phenotype of the treated patients. In addition, a therapeutic range of 20—50 μg/ml has been postulated for the total SP [4]. This necessitates a specific method for the determination of SP and its acetylated metabolite. In the past plasma level monitoring was performed by a time-consuming and rather unspecific photometric method [10]. Therefore we have developed a fast and specific high-pressure liquid chromatography (HPLC) method for the simultaneous measurement of SP and its major metabolites in human plasma.

EXPERIMENTAL

Apparatus

The chromatographic separations were performed with an HPLC apparatus (Spectra-Physics, Darmstadt, G.F.R.) equipped with a 250×4.6 mm I.D. column packed with Nucleosil RP-18, 10 μm (Macherey & Nagel, Düren, G.F.R.) and a UV detector (254 nm; Spectra-Physics; cell volume 8 μl). Flow-rate was 1.4 ml/min at ambient temperature. The same results have been obtained with a self-packed 250×3 mm I.D. column filled with Nucleosil RP-18, 5 μm.

Reagents

All solvents were of analytical reagent grade (Merck, Darmstadt, G.F.R.). The mobile phase consisted of methanol—1% acetic acid (20:80, v/v). SP and the internal standard sulfadimidine (SD) were purchased from Serva (Heidelberg, G.F.R.). N^4-acetylsulfapyridine (AcSP) and 5-hydroxysulfapyridine (SPOH) were gifts from Pharmacia. A stock solution of 0.1 mg per ml of twice-distilled water was prepared for each compound. To achieve complete dissolution 50 μl of 0.1 *N* NaOH in 10 ml of twice-distilled water were added. The solutions were stable when stored at 4°. Plasma samples were kept frozen at −20° until analysis.

Calculations

Quantification of the plasma concentrations of SP, AcSP and SPOH was performed by calculating the peak height ratios of the drug/metabolites to the added internal standard SD and relating these to previously constructed calibration curves. These curves were computed by a linear regression program. All samples were run in duplicate.

Extraction procedure

Plasma samples and the added internal standard SD were extracted from acetate buffer by chloroform. The separated organic phase was evaporated to complete dryness. The residue was redissolved in methanol—water and injected into the HPLC system. The details of the complete extraction procedure are given in Fig. 2.

RESULTS

All the various compounds (SP, AcSP, SPOH and SD; see Fig. 1) were extracted with chloroform in a single step from 100 μl human plasma. The evapo-

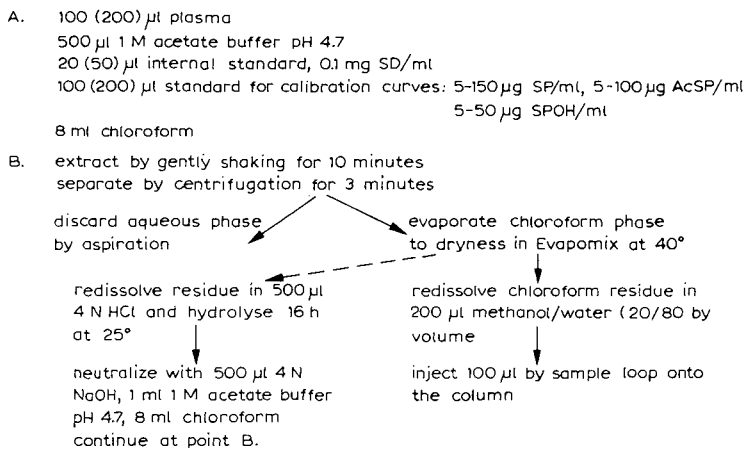

Fig. 2. Scheme of the extraction procedure for the determination of SP, AcSP and SPOH. The specifications given in parentheses refer to the determination of total SP including hydrolysis of AcSP to SP.

rated and redissolved chloroform extracts gave well-resolved, sharp and symmetrical peaks for SP and its metabolites after HPLC separation on an RP-18 column. Typical chromatograms are given in Fig. 3. To exclude any interference from other endogenous substances, a plasma sample drawn prior to the drug administration and without the addition of the internal standard served as blank.

No disturbing peaks were found during measurement of samples of more than 30 patients. Generally, elution was completed within 10—15 min depending on late peaks in some specimens. The calibration curves are linear from 5—150 µg/ml for SP, from 5—100 µg/ml for AcSP and from 5—50 µg/ml for SPOH. The lower limit of sensitivity was 5 µg/ml. The substances were stable if the plasma was stored for 7 days at room temperature. The accuracy and recoveries of the assay are summarised in Tables I and II, respectively.

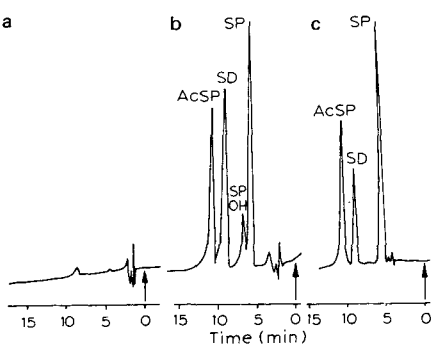

Fig. 3. Typical chromatograms of: (a) patient's blank plasma; (b) blank plasma containing 20 µg SP per ml, 20 µg AcSP per ml, 20 µg SPOH per ml and 20 µg SD per ml; (c) sample of a patient treated chronically with 3 g salicylazosulfapyridine per day.

TABLE I

DETERMINATION OF THE ACCURACY OF TWO DIFFERENT CONCENTRATIONS OF SP, AcSP AND SPOH

Drug	Concentration (μg/ml)	n	Mean ± SD
SP	10	10	9.88 ± 0.43
SP	50	4	51.29 ± 1.18
AcSP	10	4	9.58 ± 0.26
AcSP	50	7	49.34 ± 0.95
SPOH	10	10	9.41 ± 1.48
SPOH	20	10	18.65 ± 1.90

TABLE II

RECOVERY OF SP AND AcSP INCLUDING THE RESULTS AFTER HYDROLYSIS

Drug	Concentration (μg/ml)	Recovery (%)	Recovery after hydrolysis (%)
SP	20	96 (n=6)	76 (n=2)
AcSP	20	91 (n=4)	83 (n=4)

In contrast with our HPLC method (direct and simultaneous SP and AcSP measurement without hydrolysis) the photometric assay of Hannson and Sandberg [10] utilizes two measurements, one with boiling in 4 *N* hydrochloric acid to hydrolyse AcSP into SP. To compare our results with this assay, several samples were measured directly and split according to the above procedure. The conditions for this extraction and for the hydrolysis are included in Fig. 2.

Following the aggressive hydrolysis the resulting chromatograms exhibited (after extraction with ether—acetone (6 ml + 0.5 ml)) some additional peaks indicating decomposition of SP by the hydrochloric acid (Fig. 4a). If chloroform was used as extraction solvent these degradative products were not visible (Fig. 4b). If the hydrolysis was performed at 25° for ca. 16 h, easily measurable peaks were obtained when either ether or chloroform was used (see Fig. 4c). Under these conditions the total amount of SP (free SP and SP derived from AcSP) equalled the sum of the separately determined concentrations of SP and AcSP (Table III). Therefore this milder hydrolysis seems to be an alternative pathway for evaluation of the amounts of total SP and AcSP (=total SP — free SP) if no direct and specific method for AcSP is available.

The normal daily maintenance dose for the treatment of Crohn's disease or ulcerative colitis averages 3 g salicylazosulfapyridine (containing 1.83 g SP). With this dosage regimen we could not detect any free SPOH in plasma, but in the form of its glucuronide minor amounts were visible after incubating 100—200 μl plasma with β-glucuronidase—arylsulfatase for 3 h at 37°. However, only at the highest sensitivity setting of the UV detector could concentrations of less than 1 μg/ml be detected.

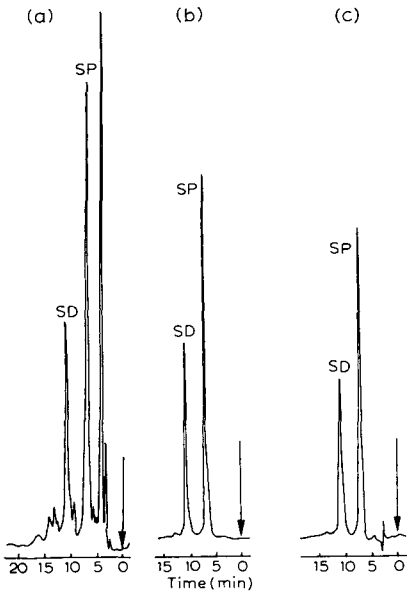

Fig. 4. Typical chromatograms of a patient's plasma sample for the determination of total SP. The extracts were hydrolyzed according to ref. 10 with 4 *N* HCl for 10 min at 100° and extracted with (a) ether—acetone or (b) chloroform: In (c) our milder conditions (see Fig. 2) for the hydrolysis of AcSP were used.

TABLE III

HPLC DETERMINATION OF TOTAL SP BY TWO DIFFERENT PROCEDURES

Patients	Total SP measured after hydrolysis* (μg/ml)	Free SP** (μg/ml)	AcSP** (μg/ml)	Free SP + AcSP (μg/ml)
A.D.	25.6	6.9	19.0	25.9
N.D.	32.3	7.1	29.2	36.3
S.R.	64.5	28.3	31.7	60.0
H.Sch.	46.0	29.9	12.0	41.9
N.St.	31.6	16.0	12.2	28.8

*Hydrolysed by 4 *N* HCl at 25° for 16 h.
**Measured directly; without hydrolysis.

DISCUSSION

The photometric determination of SP as described by Hannson and Sandberg [10] represents a well-established method. However, they measured the major metabolite, AcSP, in the form of SP after hydrolysis in strong hydrochloric acid at 100°, which might also partly destroy SP or other metabolites. Our experiments under different conditions of hydrolysis support this assumption.

We have therefore developed a method which allows the simultaneous and direct detection of SP and its intact metabolites by a specific chromatographic

measurement without any derivatization. The detection limit of our method is adequate for measurement of SP and AcSP in 100-μl plasma samples within 10—12 min. The rate of acetylation of the sulfonamide SP is dependent on the phenotype [6]. This parameter can be determined by the measurement of free SP and AcSP in the same single plasma sample.

According to the ratio AcSP:total SP, the population can be divided into rapid and slow acetylators [4—6, 11]. Patients with Crohn's disease or ulcerative colitis are treated with this sulfonamide for long periods, sometimes even for their whole life. The incidence of side effects is much more pronounced and occurs almost exclusively among patients characterized as slow acetylators. About 80% of the side effects were observed in slow acetylators with plasma levels higher than 50 μg/ml [4, 7—9].

In addition to the toxic manifestations, therapeutic efficacy seems to be related to a therapeutic range of 20—50 μg/ml of total SP. Therefore determination of the acetylation phenotype and of the plasma levels seems to be of benefit to the patients.

The method described is a relatively simple and specific way of obtaining rapid information about the phenotype and effective plasma concentrations from one small plasma sample. From the 33 patients tested so far, 17 patients could be regarded as slow acetylators (acetylation range 11—35%) and the remaining 16 as fast acetylators (acetylation range 41—85%). In 11 patients plasma levels of total SP were in the toxic range (>50 μg/ml), while in 8 patients these concentrations were below the therapeutic range (<20 μg/ml). These routine measurements may help in improving therapy with salicylazosulfapyridine.

In summary, a specific and sensitive HPLC procedure for the simultaneous determination of SP, AcSP and SPOH in human plasma was developed. Following a single extraction step with chloroform the compounds and the added internal standard SD are eluated from a reversed-phase column by methanol—1% acetic acid (20:80).

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Note

Determination of metronidazole and misonidazole and their metabolites in plasma and urine by high-performance liquid chromatography

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The nitroimidazole derivatives, metronidazole and misonidazole are currently being investigated for their ability to sensitize hypoxic cells to radiation damage as well as for their selective toxicity against hypoxic cells [1—3].

Quantitative correlations between nitroimidazole cell concentrations and radiation enhancement have been demonstrated [4]. This finding, along with the known central nervous system toxicity [5] and potential carcinogenicity [6] make it imperative to monitor drug levels in plasma and urine.

Analytical methods currently available utilizing UV spectrophotometry [7] and polarography [8] do not differentiate between these drugs and their potentially active nitroimidazole metabolites. A previously reported gas—liquid chromatography—electron capture assay [9] is sensitive and specific for the parent drug but too lengthy for routine use. This report presents a reversed-phase high-performance liquid chromatography (HPLC) method with direct analysis of deproteinized serum and urine and UV detection at 324 nm. This procedure is specific for metronidazole or misonidazole and their metabolites with the unchanged nitroimidazole moiety.

MATERIALS AND METHODS

Apparatus and reagents

A Waters Assoc. (Milford, Mass., U.S.A.) OT757 high-performance liquid chromatograph was used, equipped with a Model 6000A pump, UK6 injector and reversed-phase μBondapak C_{18} column (30 cm × 4 mm I.D., average par-

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ticle size 10μm). Eluents were monitored by UV absorbance at 324 nm utilizing a Spectroflow monitor model SF770 from Schoeffel Instrument Co. (Westwood, N.J., U.S.A.). The HPLC eluent was 8% acetonitrile in 10^{-3} *M* phosphate buffer, pH 4.0. The flow-rate was 2.0 ml/min.

Mass spectra were obtained on an AEI-901 chemical ionization (isobutane) mass spectrometer (Kratos Ltd., AEI, Manchester, Great Britain) using direct insertion.

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol] and desmethylmisonidazole [1-(2-nitro-1-imidazolyl0-2,3-propanediol] were obtained from the National Cancer Institute (Bethesda, Md., U.S.A.). All chemicals were of analytical reagent grade quality.

PROCEDURE

Serum and diluted (1:2 in water) urine samples were mixed with equal volumes of ethanol, vigorously shaken for 10 sec and proteins allowed to precipitate for 15 min at room temperature. The mixture was then centrifuged at 1700 *g* for 10 min and 20 μl of the supernatant were injected onto the HPLC column. Peaks eluting from the column were quantitated using peak heights and compared with an external standard curve at three different drug concentrations.

RESULTS AND DISCUSSION

Metronidazole and misonidazole were not separable on the C_{18} reversed-phase column under the experimental conditions. As little as 5 ng of these drugs were detectable when using UV absorption at 324 nm. The direct serum and urine assay after deproteinization without further purification steps was sufficiently sensitive for pharmacological studies. The sensitivity of the assay is about 0.5 μg/ml for both drugs when 10 μl of serum or urine are analyzed. The sensitivity limit can be further lowered by injecting larger volumes since biological background is negligible. None of the control or patient samples showed any interfering peaks.

Typical HPLC records obtained from human urine and serum samples are shown in Fig. 1 which includes analysis of a blank serum and a serum sample to which misonidazole and its major metabolite desmethylmisonidazole (10 μg/ml each) have been added. Standard curves of misonidazole, desmethylmisonidazole and metronidazole are linear from 0.5 μg/ml to at least 100 μg/ml serum. Precision was determined for misonidazole and desmethylmisonidazole at 10 μg/ml and 20 μg/ml respectively and gave $\bar{X}$ = 9.94 μg/ml (S.D. 0.15; C.V. 1.5%; n = 5) for misonidazole and $\bar{X}$ = 20.19 μg/ml (S.D. 0.45; C.V. 4.5%; n = 5) for desmethylmisonidazole.

Chromatograms obtained from serum and urine samples of patients receiving oral doses of 1 g/m^2 misonidazole and 6 g/m^2 metronidazole are also included with Fig. 1. It can be noted that misonidazole is more concentrated than its metabolite desmethylmisonidazole in serum (Fig. 1C) of which the opposite is true in urine (Fig. 1D). This indicates a higher renal clearance for the more po-

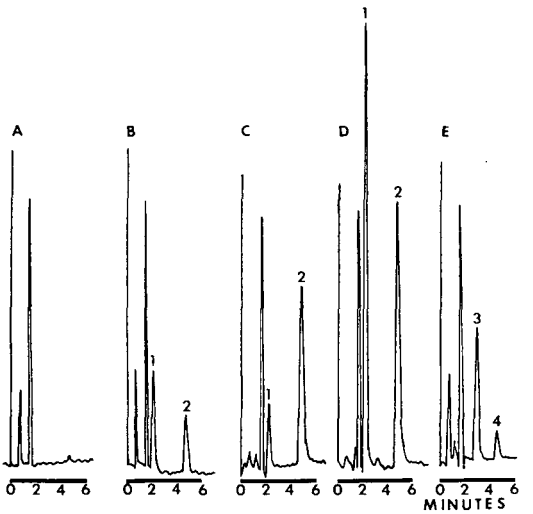

Fig. 1. Misonidazole and metronidazole HPLC records. (A) Drug-free patient serum; (B) drug-free serum spiked with 10 μg/ml of misonidazole and desmethylmisonidazole; (C) serum sample collected 12 h after a dose of 1.0 g/m² misonidazole (misonidazole 28 μg/ml; desmethylmisonidazole 6 μg/ml); (D) diluted (1:2) urine sample collected 15 h after a dose of 1.0 g/m² misonidazole (misonidazole 68 μg/ml; desmethylmisonidazole 60 μg/ml; (E) serum sample collected 27 h after a dose of 6 g/m² metronidazole (metronidazole 2.8 μg/ml and approximately 10 μg/ml of metronidazole metabolite. Peaks: 1=desmethylmisonidazole; 2=misonidazole; 3=metronidazole; 4=metronidazole metabolite.

lar desmethyl metabolite. The urine sample was rechromatographed by HPLC on the same column using the aqueous eluent buffer without acetonitrile in order to separate desmethylmisonidazole from potentially present glucuronide metabolites. Only a single peak was observed with a retention time identical to that of authentic desmethylmisonidazole (7.2 min) indicating absence of further metabolites.

A patient serum sample taken 27 h following a metronidazole dose of 6 g/m² (Fig. 1E) gave a metronidazole concentration of 14.0 μg/ml using the previously described UV assay [7] and 2.8 μg/ml by HPLC. The discrepancy between these assays can be accounted for by the level of a major metabolite (Fig. 1E), which was analyzed by mass spectrometry. The collected HPLC fraction corresponding to this metabolite of metronidazole was purified by silica gel column chromatography (eluent chloroform—methanol, 10:1) and analyzed by chemical ionization mass spectrometry. A quasi-molecular ion, *m/e* 188, indicated the insertion of one oxygen atom into metronidazole. A major hydroxylated metabolite of metronidazole (M.W. 187) has been previously isolated from human urine as the only metabolic species corresponding to the addition of one oxygen atom [10]. We therefore conclude the identity of the material isolated by HPLC to be 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole.

No standard curve was constructed for the quantitation of this metabolite due to the lack of an appropriate reference compound. The ratio between metronidazole and its metabolite varied considerably between patients.

The presented HPLC method will be utilized to determine the pharmacoki-

netics of misonidazole and metronidazole in patients undergoing radiation therapy in conjunction with these nitroimidazole radiosensitizers.

ACKNOWLEDGEMENTS

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Note

Rapid quantitative method for the simultaneous determination of carbamazepine, carbamazepine-10,11-epoxide, diphenylhydantoin, mephenytoin, phenobarbital and primidone in serum by thin-layer chromatography

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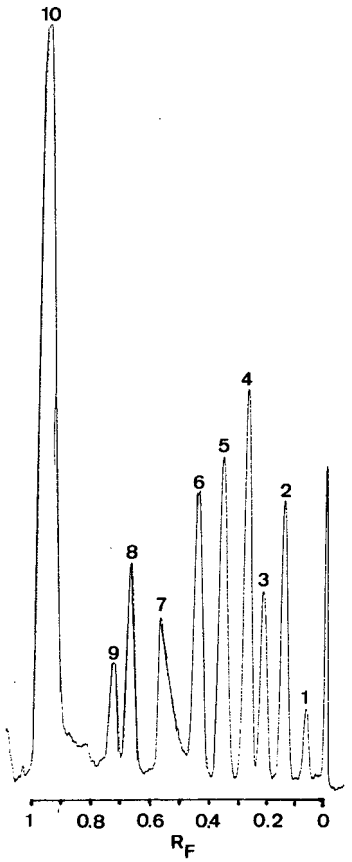
This note describes the adaptation of our recently published method [1] to the analysis of carbamazepine-10,11-epoxide. The epoxide was kindly donated by Ciba-Geigy, Basel, Switzerland. A working standard solution was prepared containing 12.5 mg each of caffeine, carbamazepine and carbamazepine-10,11-epoxide together with 25 mg each of diphenylhydantoin, mephenytoin, phenobarbital and primidone per 100 ml absolute ethanol. This standard solution was applied to drug-free serum, extracted and chromatographed as described by us [1]. A scan of this thin-layer separation is shown in Fig. 1. The epoxide metabolite exhibits the absorption spectrum shown in Fig. 2. The recovery of the epoxide metabolite added to serum was found to be 80%.

With this method we measured the concentration of carbamazepine and its epoxide in 31 serums from patients treated with carbamazepine and other anticonvulsants. The mean value of carbamazepine in these serums was found to be 4.2 mg/l and the epoxide metabolite 1.1 mg/l which is 26.2% of the carbamazepine concentration.



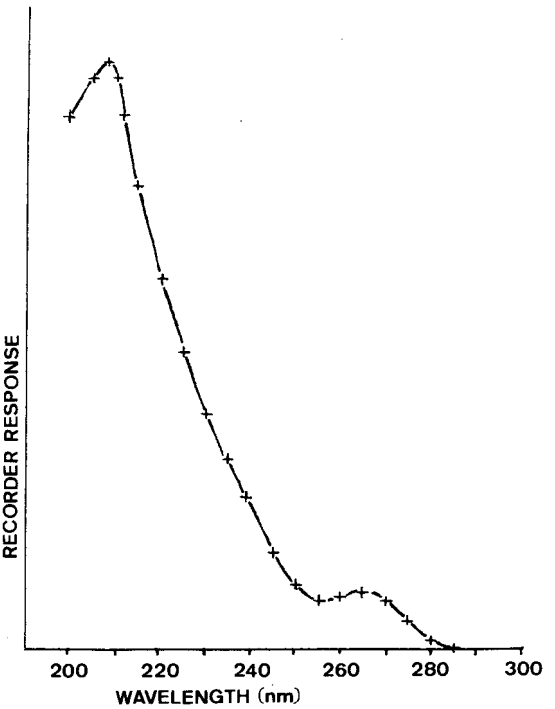


Fig. 1. Results obtained from a scan at 215 nm of a TLC plate after the separation of a serum containing 8.2 mg/l each of carbamazepine-10,11-epoxide (2), caffeine (3), carbamazepine (4), and 16.5 mg/l each of primidone (1), diphenylhydantoin (5), phenobarbital (6), mephenytoin (8). Peaks (7) and (9) are unidentified serum peaks and peak (10) is the solution front.

Fig. 2. Ultraviolet absorption spectrum of a thin-layer chromatogram containing carbamazepine-10,11-epoxide, applied directly to the plate, separated chromatographically and measured in situ with the Zeiss chromatogram-spectrophotometer.

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Note

Fluorimetrische Bestimmung von Flufenaminsäure aus Plasma durch direkte Auswertung von Dünnschichtchromatogrammen*

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Flufenaminsäure (N-(*m*-Trifluormethylphenyl)-anthranilsäure) wird als Antiphlogistikum und Antirheumatikum therapeutisch häufig verwendet. Für die quantitative Analyse dieses Wirkstoffes wurden bereits mehrere Methoden entwickelt [1—11] .Von diesen eignen sich jedoch nur wenige für Messungen des Flufenaminsäuregehaltes in biologischem Material. Die wesentlichen sind nachstehend angeführt.

Panse et al. [4] bestimmten Flufenaminsäure im Harn durch direkte quantitative Dünnschichtchromatographie bei 288 nm in Remission. Eigene Untersuchungen ergaben jedoch, dass die Messung in Remission bei einem molaren Extinktionskoeffizienten von 19108 (l/mol × cm) eine höhere Nachweisgrenze besitzt als die fluorimetrische Bestimmung und eine direkte Messung der Plasmaspiegel ohne vorherige Anreicherung der Substanz durch Extraktion nicht möglich wäre.

Metha und Schulman [5] bezeichnen die native Fluoreszenz der Flufenaminsäure in organischen Lösungsmitteln für Fluoreszenzmessungen als durchaus ausreichend, was jedoch in mehreren Arbeiten anderer Autoren, die sich deshalb um eine Verbesserung der Fluoreszenzeigenschaften z.B. durch Säurezusatz bemühten, angezweifelt wurde.

Zur fluorimetrischen Bestimmung in Lösung verwendeten Glatzko [1], Buchanan et al. [6] sowie Panse et al.[7] Tetrachlorkohlenstoff als Lösungsmittel. Die Fluoreszenz der Flufenaminsäure wurde dabei durch Zugabe von Halogenessigsäuren — teilweise nach vorausgehender Oxidation mit Kaliumdichromat — verstärkt.

*Teilergebnisse der Dissertation A. Schumacher, in Vorbereitung.
**An den Anforderungen von Sonderdrucken zu richten sind.

Nach Angaben von Dell und Kutschbach [8] sind als Lösungsmittel für Flufenaminsäurebestimmungen solche mit einer Dielektrizitätskonstanten von Null geeignet, wenn ihnen Halogenessigsäuren zugesetzt werden.

Gute Ergebnisse erzielten Hattori et al. [9] bei der fluorimetrischen Bestimmung der Flufenaminsäure als Aluminiumkomplex. Die Nachweisgrenze lag bei 4 ng/ml, wobei die Bestimmung allerdings nur in Pufferlösung und nicht aus Plasma erfolgte. Nachteilig ist hierbei der grosse Arbeitsaufwand, die notwendige Elution von der Platte und die Herstellung einer Blindlösung.

Eine Derivatisierung der Flufenaminsäure zu besser fluoreszierenden Produkten wurde erstmals von Dell und Kamp [10] durchgeführt. Sie setzen entweder die Substanz mit konzentrierter Schwefelsäure zu gut fluoreszierendem Trifluormethylacridon um oder führten eine Cyclisierungsreaktion mit Formaldehyd durch.

Mit Formaldehyd cyclisiert Flufenaminsäure zu 1-(*m*-Trifluormethylphenyl)-4-oxo-1,2-dihydro-3,1,4-benzoxazin. Diese Reaktion wird sauer katalysiert (Fig. 1). Diese Umsetzung benutzten Schmollack und Wenzel [11] zur quantitativen Flufenaminsäurebestimmung. Da hierbei jedoch keine chromatographische Auftrennung erfolgt, ist die Methode zur Blutspiegelbestimmung der Flufenaminsäure nicht geeignet.

Die in der Literatur beschriebenen Methoden zind zum Teil nicht selektiv, da keine Abtrennung von störenden Begleitsubstanzen erfolgt. In den Fällen, in denen eine Dünnschichtchromatographie (DC) durchgeführt wird, schliesst sich eine Elution der Substanz von der Platte vor der Messung an. Ein solches Verfahren ist arbeitsaufwendig und eventuell mit Fehlern behaftet. Infolge einer zu hohen Nachweisgrenze sind viele Verfahren wegen der notwendigen Konzentrierung der Substanz nach Elution aus dem Plasma ebenfalls sehr arbeitsintensiv. Unser Ziel war es daher, die Flufenaminsäure direkt aus dem Plasma nach DC-Abtrennung von Metaboliten und Plasmabestandteilen auf der Platte zu bestimmen.

Nachdem auf Grund von Vorversuchen die von Dell und Kamp [10] beschriebene Umsetzung mit Formaldehyd sich als erfolgversprechend erwiesen hatte, versuchten wir die Reaktionsbedingungen so zu verbessern, dass für quantitative Analysen brauchbare Ergebnisse erhalten werden. Dell und Kamp setzten die Flufenaminsäure nach Chromatographie mit Paraformaldehyd und konzentrierter Schwefelsäure während 2 h bei 80° um. Wir konnten zeigen, dass eine Zugabe von Ameisensäure zu den genannten Substanzen die notwendige Reaktionszeit auf 45 min verkürzt, die Fluoreszenzintensität erhöht

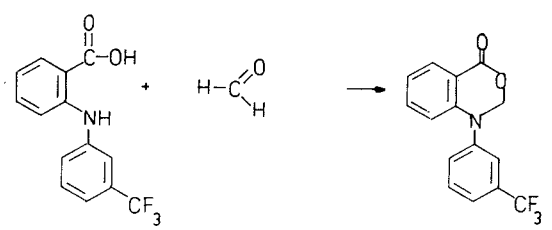

Fig. 1. Umsetzung der Flufenaminsäure mit Formaldehyd zu 1-(*m*-Trifluormethylphenyl)-4-oxo-1,2-dihydro-3,1,4-benzoxazin.

und die relative Standardabweichung bei einer Bestimmung von 10 μg/ml Plasma von 6.9% auf 1.5% verbessert. Die Nachweisgrenze ist dabei so niedrig, dass Mengen ⩽ 2 ng/Fleck messbar sind. Eine Extraktion aus dem Plasma ist daher nicht notwendig, die Elution von der Platte entfällt ebenfalls, wodurch das Verfahren wesentlich vereinfacht wird.

METHODIK

Die zu chromatographierende Lösung wird wie folgt hergestellt: Ein Volumenteil Plasma wird mit zwei Volumenteilen Methanol versetzt und scharf zentrifugiert, wobei das gefällte Eiweiss sedimentiert. Entsprechend der zu erwartenden Konzentration werden von der überstehenden Lösung 10—50 μl strichförmig mit dem Linomaten III (Camag) auf Kieselgel-60-Fertigplatten, 20 × 20 cm, ohne Fluoreszenzindikator (Merck, Darmstadt, B.R.D.), aufgetragen. Bei einer Strichbreite von 10 mm pro Fleck und einem 25-mm-Abstand der äusseren Startlinien vom Plattenrand können acht Proben und drei Standards auf eine Platte aufgetragen werden. Die drei Standards werden durch Zusatz von Flufenaminsäure zu gepooltem Plasma hergestellt.

Dazu werden 60.0 mg Flufenaminsäure in 100.0 ml Aceton gelöst und 1.0 ml dieser Lösung wird nochmals auf 10.0 ml verdünnt, entsprechend einer Konzentration von 600.0 μg pro 10.0 ml Aceton. Eine 5.0-ml-Probe dieser Lösung wird nun durch Einblasen von Stickstoff bis zur Trockne eingeengt, dann werden 50.0 ml gepooltes Plasma zugegeben. Dadurch ergibt sich eine Konzentration von 6.0 μg Flufenaminsäure/ml Plasma. Nach Zugabe von zwei Volumenteilen Methanol und Zentrifugation wird der Überstand als Standardlösung verwendet.

Die DC erfolgt unter Standardbedingungen mit dem Fliessmittel Chloroform—Methanol (70:30, v/v) in Ammoniakatmosphäre Fliesstrecke 12 cm. In diesem System wird die Substanz von ihren Metaboliten und Plasmabestandteilen getrennt.

In Fig. 2 ist die Fluoreszenzintensitäts-Ortskurve des Chromatogramms eines 3.9 μg/ml Flufenaminsäure enthaltenden Plasmas dargestellt.

Nach dem Trocknen der Platte (ca. 5 min nach der Chromatographie) wird die Flufenaminsäure mit Formaldehyd umgesetzt. Dabei wird die Platte in eine Trennkammer gestellt, auf deren Boden ein Glasschiffchen, gefüllt mit 2 g Paraformaldehyd und 0.25 ml konzentrierter Schwefelsäure, sowie ein Glasschiffchen mit 0.1 ml Ameisensäure stehen. Die geschlossene Kammer wird für 45 min bei 100° in einen Wärmeschrank gestellt. Nach Abkühlen der Platte (ca. 10 min nach Herausnahme aus dem Wärmeschrank) wird eine direkte quantitative Messung der Fluoreszenz mit dem Chromatogramm-Spektralphotometer KM3 der Firma Zeiss vorgenommen. Die Anregung der Fluoreszenz erfolgt durch die Hg-Linie 365 nm (27.4×10^3 cm^{-1}) der Hg-Mitteldrucklampe St 41 bei einer Spaltgrösse von 1 × 8 mm.

Wie aus dem abgebildeten Emissionsspektrum des an das Sorbens gebundenen Benzoxazin Derivates (Fig. 3) hervorgeht, liegt das Fluoreszenzmaximum bei 455 nm. Als Sperrfilter für die Exzitationsstrahlung verwendeten wir den Kantenfilter Fl 43.

Die Intensität der Emissionsstrahlung wird durch einen Photoelektronenvervielfacher gemessen, die Aufzeichnung der Fluoreszenzintensitäts-Ortskurve

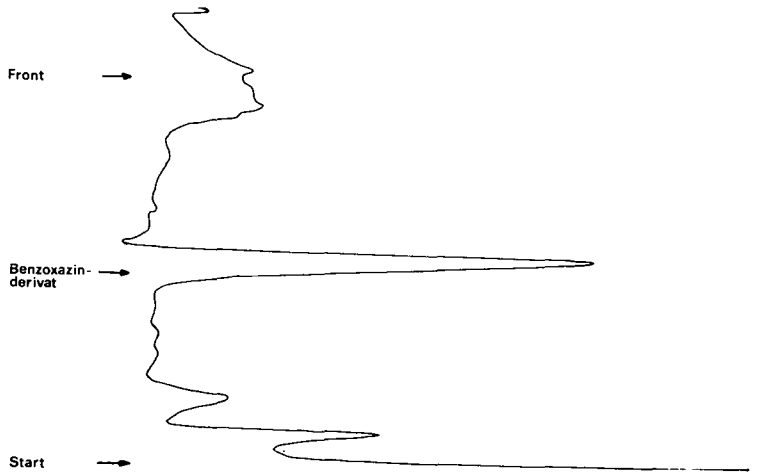

Fig. 2. Fluoreszenzintensitäts-Ortskurve des Chromatogramms eines 3.9 µg/ml Flufenaminsäure enthaltenden Plasmas. Die Plasmaprobe wurde 5 h nach der letzten Einnahme von 200 mg Flufenaminsäure bei einer Erhaltungsdosis von 600 mg pro die gewonnen. Aufgetragen wurden 50.0 µl eines Überstandes von 1.0 ml Plasma und 2.0 ml Methanol. Die Fluoreszenzreaktion erfolgte nach der Chromatographie direkt auf der Platte mit Formaldehyd.

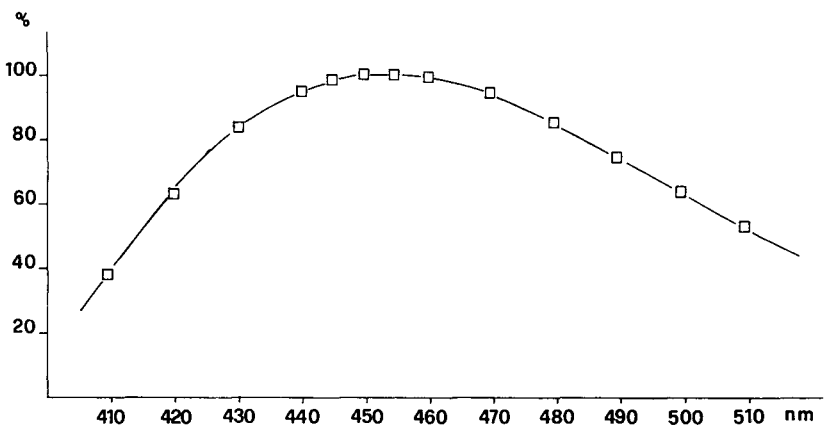

Fig. 3. Emissionsspektrum des Benzoxazin-Derivates der Flufenaminsäure. Exzitation: Hg-Linie 365 nm.

erfolgt durch einen Perkin-Elmer-Recorder 56, Tischgeschwindigkeit 100 mm/min, Schreibervorschub 120 mm/min. Unter den genannten Bedingungen besteht in Konzentrationen von 2 bis 200 ng pro Fleck sowohl zwischen den Höhen der aufgezeichneten Fluoreszenzintensitäts-Ortskurven und den aufgetragenen Substanzmengen als auch zwischen den Flächen unter den Ortskurven und den aufgetragenen Substanzmengen Linearität, die Eichkurven gehen durch den Nullpunkt. Zur Erstellung der Eichgeraden genügt daher ein einziger Kurvenpunkt, der zur Erhöhung der Genauigkeit aus drei Messwerten ermittelt wird. Bei unseren Untersuchungen benutzten wir zur Auswertung die

Höhe der Fluoreszenzintensitäts-Ortskurven.

Zur Überprüfung der Methode wurde einer Versuchsperson eine einmalige Dosis von 200 mg Flufenaminsäure verabreicht und 6, 12 und 24 h nach der Einnahme Blut entnommen. Die Proben wurden, zusammen mit einem 3.0 µg Flufenaminsäure/ml Plasma enthaltenden Standard, nach der beschriebenen Methode aufgearbeitet. Die Auftragemenge betrug jeweils 50.0 µl des Überstandes der Methanolfällung. Es ergaben sich Blutspiegel von 1.92 µg/ml nach 6 h und 0.225 µg/ml nach 12 h. Nach 24 h waren nur noch Spuren von Flufenaminsäure nachweisbar.

Die Bestimmung störende Metaboliten wurden nicht beobachtet. Ein DC-Fleck im hR_F-Bereich von 15 (hR_F von Flufenaminsäure: 37) könnte mit einem der von Glatzko [1] beschriebenen hydroxylierten oder konjugierten Metaboliten identisch sein.

PRÄZISION UND RICHTIGKEIT DES VERFAHRENS

Bei der mittleren therapeutischen Tagesdosis von dreimal 200 mg Flufenaminsäure werden Blutspiegelmaxima bis 6 µg/ml erreicht. Die Präzision des Verfahrens wurde daher für die Konzentrationen (a) 10.0 µg, (b) 1.0 µg, (c) 0.5 µg Flufenaminsäure/ml Plasma bestimmt.

Nach der oben beschriebenen Methode wurden (jeweils n=8) (a) 10.0 µg, (b) 1.0 µg, (c) 0.5 µg Flufenaminsäure/ml Aceton unter Stickstoff zur Trockne eingeengt und in 1.0 ml Plasma aufgenommen. Die Eiweissfällung erfolgte jeweils mit 2.0 ml Methanol. Aufgetragen wurden dann von der Lösung (a) 10 µl, (b) 50 µl, (c) 50 µl.

Die weitere Behandlung der Proben erfolgte wie oben beschrieben, wobei die Registrierung der Kurven bei Lösung a und b mit 0.5 V und bei Lösung c mit 0.2 V Eingangsspannung am Schreiber durchgeführt wurden (Ausgang des KM3 0–1 V).

Die Ergebnisse sind in Tabelle I aufgeführt.

TABELLE I

PRÄZISION DES VERFAHRENS ZUR BESTIMMUNG VON FLUFENAMINSÄURE

Konzentration im Testplasma (µg/ml)	Relative Standardabweichung (%)
10.0	1.5
1.0	3.1
0.5	8.5

Bei den Untersuchungen auf Richtigkeit des Verfahrens, bei denen als Standard in Methanol–Wasser (2:1) gelöste Flufenaminsäure benutzt wurde, lagen die Wiederfindungsraten über 100%, was einerseits durch eine Volumenkontraktion des Plasma–Methanol-Gemisches und andererseits durch eine Volumenverminderung infolge des ausgefallenen Eiweisses erklärt wird. Wird die ursprüngliche im Plasma vorhandene (durch Einwaage bekannte) Flufen-

aminsäure-Menge auf das Volumen des Plasma—Methanol-Gemisches nach der Zentrifugation berechnet, so liegt die Wiederfindungsrate etwa bei 100%.

Geringe Schwankungen sind bei verändertem Eiweissgehalt des Plasmas zu erwarten. Deshalb erschien es am günstigsten, als Standard gepooltes Plasma zu benutzen, und dies genauso wie die zu bestimmende Probe zu behandeln. Dadurch wird der Fehler infolge der Volumenkontraktion ausgeschlossen und der Fehler durch schwankenden Eiweissgehalt so klein wie möglich gehalten.

Die vorstehend beschriebene Bestimmungsmethode für Flufenaminsäure aus Humanplasma ist wegen ihrer einfachen Durchführung und ihres geringen Zeit- und Arbeitsaufwandes bei hoher Selektivität und guter Genauigkeit für Reihenuntersuchungen geeignet.

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Biomedical Applications

CHROMBIO. 170

Note

Micro-method for the determination of caffeine and theophylline allowing direct application of biological fluids to thin-layer chromatography plates

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Theophylline (1,3-dimethylxanthine) is often used in children in the treatment of bronchial asthma. Levels of 10—20 μg/ml are considered to be "therapeutic serum concentrations" [1, 2]. The clearance and half-life show considerable variations from one patient to another. Thus, determination of theophylline serum concentrations is important in the avoidance of either ineffective or toxic serum levels. Contrary to theophylline, caffeine (1,3,7-trimethylxanthine) is rarely given therapeutically.

Recently the efficacy of theophylline and caffeine for treatment of apneic spells of prematures has been reported [3, 4]. Sufficient knowledge of pharmacokinetics, efficacy and toxicity of new drugs is mandatory, before their general clinical use, particularly in prematures, can be recommended. Multiple determinations of theophylline and caffeine serum concentrations for pharmacokinetic studies in prematures are only possible using a micro-method.

Existing spectrophotometric methods [5—7] for the quantitation of xanthines require relatively large amounts of serum (1—3 ml) and are of reduced specificity due to the interference of endogenous and exogenous substances. Some gas chromatographic [8—10] and high-pressure liquid chromatographic [11—13] methods, which require amounts of 0.05—1.0 ml serum, are known, especially for the determination of theophylline, but require extraction from biological material. Spectrodensitometric methods for theophylline after serum extraction are described [14, 15], but because large amounts of serum are required (1—5 ml), these methods are not suitable for use in prematures and children.

In the present paper a micro-method for the quantitative measurement of theophylline and caffeine in 5—10 μl biological fluid is described. Xanthine derivatives may be determined in nanogram amounts by their UV absorption, without extraction, after direct application of serum, saliva and urine to thin-

layer chromatography (TLC) plates. Using this method in pharmacokinetic studies some interesting results have been found, e.g. a markedly prolonged half-life of theophylline and caffeine in prematures as compared with older children.

EXPERIMENTAL

Materials

Reagent-grade chloroform, methanol, absolute ethanol, ethyl acetate and ammonia (25%) were obtained from Merck (Darmstadt, G.F.R.). The TLC plates were Kieselgel $60_{F_{254}}$ DC-Fertigplatten (20×20 cm, with a layer thickness of 0.25 mm; Merck). Theophylline and caffeine were used as DAB 7 substances and theobromine (3,7-dimethylxanthine) as Ph. Eur. II substance*. Stock solutions were prepared by dissolving 40 mg of theophylline or caffeine in 100 ml of methanol and 40 mg of theobromine in 100 ml of 0.2 *N* NaOH. Theophylline and caffeine standards of 25, 50, 100, 200 and 250 ng in 10 µl serum (plasma), saliva and urine, for the preparation of calibration curves, were obtained by appropriate dilutions of the stock solution. These reference standards were stored at −20° and used for 2 weeks. The stock solutions are stable at 4° for at least 2 months.

TLC

Six samples (two reference standards of 50 and 200 ng xanthine and four samples from patients) were applied to the TLC plate. The distance between the spots and to the side of the plate was about 2.5 cm and to the lower edge 1.5 cm. First, 10 µl of ethanol were spotted onto the plate with a micro-pipette (Brand, Wertheim, G.F.R.). Immediately afterwards, either 10 µl of plasma from a second micro-pipette, or 10 µl of saliva from a constriction pipette, were applied to the centre of the wet ethanol spot. After application of the last sample the plates were air dried for 20 min and subsequently developed. Urine (10 µl) was applied to the plate directly, without ethanol. The solvent system was chloroform—methanol (90:10), the development being carried out in a saturated tank (Desaga, Heidelberg, G.F.R.) with an elution time of 45—50 min and an elution distance of 15 cm. After TLC the plates were dried in an oven for 10 min at 100°. Subsequently caffeine (hR_F value: 44) and theophylline (hR_F value: 30) were marked under a UV lamp (254 nm) by their fluorescence quenching. For separation of theobromine and theophylline the solvent ethyl acetate—methanol—25% ammonia (80:20:10) must be used with an elution distance of 15 cm and hR_F values of 24 (theophylline), 43 (theobromine) and 53 (caffeine). Down to 50 ng of xanthines could be detected as fluorescence-quenching spots. Blanks of plasma, saliva and urine did not show UV absorption in the region of xanthines.

Spectrodensitometry

The UV absorption of the xanthine derivatives was measured using a dual-wavelength TLC scanner (CS-910; Shimadzu, Kyoto, Japan) in reflection mode

*DAB 7 = Deutsches Arzneibuch, Ausgabe 7; Ph. Eur. II = Pharmacopoea Europea, Band II.

with a sample wavelength of 273 nm and a reference wavelength of 315 nm. The zigzag scanning method with a light beam of 1.25×1.25 mm^2 was used. The integration range was 2 cm and the scan speed 5 mm/min. The integration zero point was adjusted by background correction and suppression in front of every spot. Absorption peaks and their integrals were simultaneously recorded by a two-pen recorder (Colora 1200-01, Lorch, G.F.R.) with aninput voltage of 50/50 mV and a paper speed of 1 cm/min. Each spot was measured twice. Storage of developed plates for up to two weeks did not change the results.

RESULTS AND DISCUSSION

When from 25 to 250 ng caffeine and theophylline in 10 μl plasma, saliva or urine were applied to TLC plates, linear calibration curves were obtained (Figs. 1 and 2). Thus, "therapeutic theophylline serum concentrations" of 6—14 μg/ml [3, 17] in apneic prematures and of 10—20 μg/ml in patients with asthma bronchiale [1, 2] could be determined in 10-μl samples. For concentrations of above 10 μg/ml xanthine, 5 μl biological fluid were sufficient for quantitation. Down to 1 μg/ml xanthine derivate could be determined by changing the scan speed.

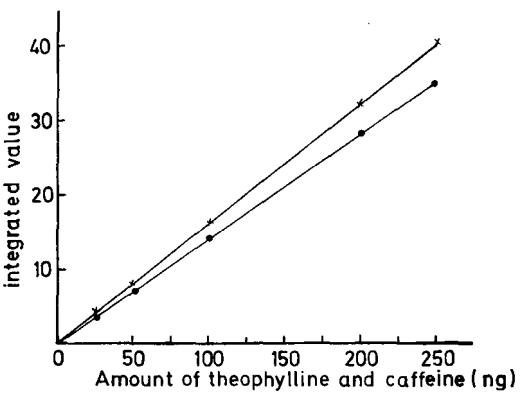

Fig. 1. Calibration curves for theophylline and caffeine. ×, caffeine (serum); •, theophylline (serum, saliva, urine) and caffeine (saliva, urine). 10 μl of biological fluid were applied to the TLC plate.

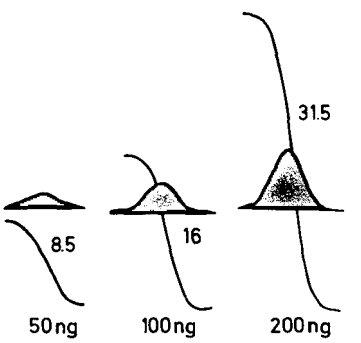

Fig. 2. Densitogram of 50, 100 and 200 ng caffeine after application of 10 μl serum with corresponding integral values of 8.5, 16 and 31.5.

In addition to the possibility of using micro-samples, the described method has the advantage of direct application of body fluids to TLC plates without prior extraction. Recently the determination of chinidine and salicylic acid, after direct application of serum and precipitation of proteins with ethanol on the TLC plate, has been described [16].

Theophylline and caffeine migrate completely from the serum and saliva after precipitation of the proteins, and are well separated. If the xanthines are applied in serum, somewhat lower R_F values are obtained compared with application in methanol, saliva or urine. Thus, for the quantitation of xanthines in serum, saliva or urine, the corresponding reference standards in the same bio-

logical fluid have to be run simultaneously. There was no interference by hemolysis. Hemoglobin was also precipitated by ethanol at the start point. The determination of xanthines was not influenced by higher bilirubin concentrations (e.g. 15 mg%). No interfering impurities, which could influence the measurement, were found after chromatography. Under a UV lamp (254 nm) a semiquantitative analysis of the serum concentrations can be carried out by fluorescence quenching of the xanthines (e.g. in the case of intoxication). The quantitation of the xanthines in plasma, saliva or urine is subsequently carried out with the appropriate calibration curves (Fig. 1).

The xanthines could be determined with good accuracy. The coefficient of variation of 10- and 20-μg samples of theophylline per ml of serum on different plates was 2% (n=10) and for the same serum concentrations of caffeine was 3% (n=10). For 10 μg caffeine and theophylline in 1 ml saliva or urine a coefficient of variation of 2—3% was found (n=10). The possibility of using the zigzag scanning method is an important advantage of the TLC scanner CS-910 (Shimadzu) as compared with other densitometers. The UV absorption of the xanthines is cumulatively recorded and integrated by a micro-beam of 1.25× 1.25 mm^2 during zigzag motion of the TLC plate, whereas in the case of linear scanning a time-consuming and often difficult adjustment of the split appropriate to the different form and size of the spots is necessary. Moreover, a better accuracy is achieved with the zigzag scanning method.

Possible interference of some substances with the xanthine determination was investigated (Table I). All the xanthine metabolites have lower R_F values than caffeine and theophylline. Theobromine, which occurs in cacao and chocolate, is not well separated from theophylline by the solvent chloroform—methanol (90:10). A good separation is achieved with the solvent ethyl acetate—methanol—25% ammonia (80:20:10).

Since no extraction is needed with the described method, the determination

TABLE I

SUBSTANCES WHICH DO NOT INTERFERE WITH THEOPHYLLINE AND CAFFEINE DETERMINATION

Solvent: chloroform—methanol (90:10).

Drugs	Phenobarbital	Furosemide
	Spironolactone	Cephalotin
	Gentamicin	Atropine
	Ampicillin	Diazepam
	Diphenhydramine	Codeine
	Ephedrine	
Xanthine metabolites	1-Methylxanthine	3-Methyluric acid
	3-Methylxanthine	1-Methyluric acid
	7-Methylxanthine	1,3-Dimethyluric acid
	1,7-Dimethylxanthine	
Endogenous substances	Bilirubin	Hypoxanthin
	Hemoglobin	Uric acid
	Xanthine	

of the xanthine derivatives can be quickly performed. About 25 samples can be quantitated in 3—4 h.

We use the described method clinically for the control of serum concentrations and for pharmacokinetic studies in prematures with apnea. As an example, the theophylline serum concentrations during the treatment of an apneic premature are shown (Fig. 3). The serum concentrations were determined just before the next oral dose and 2 h later at the time of peak serum concentrations. The serum concentrations of 6—9.5 μg/ml are well within the "therapeutic range" of 6—14 μg/ml [3, 17].

As a second example, illustrating the pharmacokinetics of theophylline and caffeine in prematures the elimination from serum is described (Fig. 4).

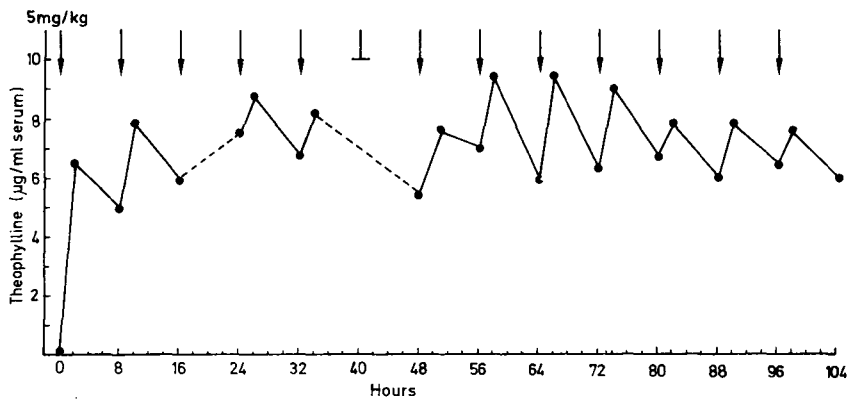

Fig. 3. Serum concentrations of theophylline in an apneic premature. Patient A: 1800 g, 0.5% theophylline solution, oral. Loading dose: 5 mg/kg. Maintenance dose: 2 mg/kg every 8 h (↓). One dose omitted (⊥).

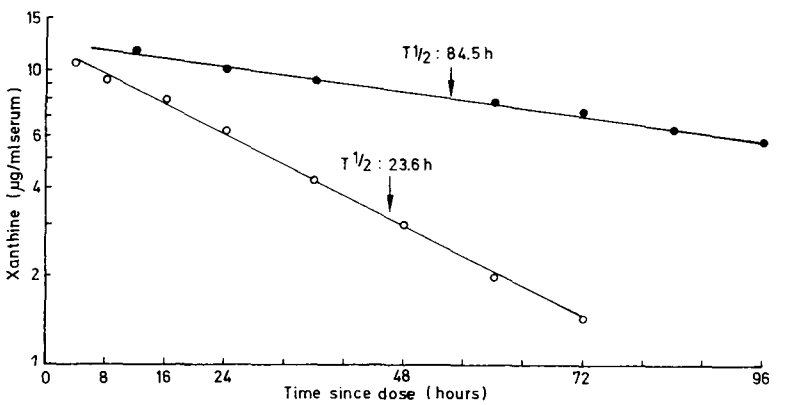

Fig. 4. Elimination of caffeine and theophylline from the serum of 2 prematures. Half-life ($T_{1/2}$) for theophylline and caffeine in adults: 3—5 hours. •, Patient P: 2000 g, single oral dose of 9 mg/kg caffeine (as caffeine citrate solution). ○, Patient P: 2000 g, 2 mg/kg theophylline (as 0.5% solution) oral every 6 h. Calculation of $T_{1/2}$ after steady-state-serum concentrations were attained and cessation of therapy.

Corresponding to a delayed elimination, the half-lives of theophylline (23.6 h) and caffeine (84.5 h) are markedly prolonged in comparison with adults. The extremely prolonged half-lives of theophylline and especially caffeine may be caused by immaturity of the demethylating liver enzymes, which metabolize the xanthine derivatives to ineffective methylxanthines and methyluric acids. Up to now the described micro-method has been successfully used by us for about 500 xanthine determinations in capillary blood, saliva or urine of prematures, children and adults.

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CHROMBIO.150

Book Review

Lipid chromatographic analysis, Vol. 3, edited by G.V. Marinetti, Marcel Dekker, New York, Basel, 2nd (revised) ed., 1976, IX + 289 pp., SFr. 115.00, ISBN 0-8247-6357-2.

Volume 3 of the second edition of Marinetti's *Lipid Chromatographic Analysis* contains the six chapters retained in essentially original form from the first edition published in 1967 and 1969. Although this fact is clearly stated in the Preface of each of the three volumes, it does not appear to have been sufficiently emphasized in the promotional material on the second edition. This oversight may be disappointing to those readers who may have anticipated all chapters to be revised and expanded in the new edition.

The contents of these chapters include procedures for column chromatographic separation of polar lipids, the thin-layer chromatography of neutral lipids, bile alcohols and acids, and for gas chromatography of sterols, fatty acids and long chain aldehydes. These accounts had been judged by the editor to be still of practical value at the time of publication. This editorial judgment, although basically sound, unfortunately denies the reader any reference to more recent applications or any improvements of the established routines. Since the first edition of the book is now out of print, these chapters provide the only source of this valuable information. Of especially great value are the chapters on column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids, on thin-layer chromatography of bile alcohols and bile acids, and on gas chromatography of the long chain aldehydes. More complete accounts of the strength and weaknesses of these methods are not presently available even in more up to date reviews. The other chapters are more modest in scope but also provide valuable outlines of practical chromatographic methods, which the authors have thoroughly tested in their own laboratories. All the contributors are experienced lipid chemists and biochemists who are well aware of both the theoretical possibilities and the practical needs of lipid resolution. Most readers will find their judgment helpful and up to date despite much progress made recently in related areas of chromatography.

Although the cost of the camera ready copy of about 290 pages including illustrations is high (US $ 35.00), the purchase of this volume is worthwhile especially since it contains the complete Author and Subject Index, which are not available with the purchase of Volumes 1 and 2.

Toronto (Canada) A. KUKSIS

Journal of Chromatography, 146 (1978) 182—183
Biomedical Applications

CHROMBIO. 188

Book Review

Analysis of drugs and metabolites by gas chromatography—mass spectrometry, Vol. 1, Respiratory gases, volatile anaesthetics, ethyl alcohol, and related toxicological materials, by B.J. Gudzinowicz and M.J. Gudzinowicz, Marcel Dekker, New York, Basel, 1977, XI + 223 pp., price SFr. 78.00, ISBN 0-8247-6576-1.

As the value of gas chromatographic (GC) and GC—mass spectrometric (MS) methods in biomedical and related fields is enormous, the Dekker series (Analysis of Drugs and Metabolites by Gas Chromatography—Mass Spectrometry) could be highly valuable to many workers. This would be especially true for those analysts relatively new to GC/GC—MS techniques. Unfortunately, this reviewer cannot be enthusiastic about Volume 1 (Respiratory Gases, Volatile Anesthetics, Ethyl Alcohol, and Related Toxicological Materials) by Gudzinowicz and Gudzinowicz. The knowledgeable scientist would not find sufficient new information to warrant reading this book, and the novice would probably not be able to separate the wheat from the chaff. Much useful information and many potentially useful references can be found in the book, but the authors exercised too little selectivity in choosing examples. Too many pages are devoted to methods from papers published in the 1960's or earlier, methods possibly not pertinent for the late 1970's. This is especially true of the first chapter: Respiratory gases, volatile anesthetics and related toxicological materials. Old methods and techniques should not be rejected out of hand; however, unless they are still the approaches of choice or illustrate a key point there is little reason for including them in a book on modern chromatographic analyses. Reading about some of this early GC work was for this reviewer a nostalgic experience, but served little other purpose. Further, the book mentions disappointingly little about mass spectrometry, which makes the name of this series misleading.

Occasionally the authors dwell on topics of peripheral interest. For example, the comments concerning: (a) the effect of ethanol upon the metabolism of other drugs and (b) the kinetics involved in the disposition of alcohol consumed at a cocktail party are interesting but not particularly germane to a discussion of the GC analysis of this compound. This same chapter (Ethyl alcohol and volatile trace components in breath, body fluids and body tissues) contains a discussion of the GC analysis of metronidazole, a distinctly non-volatile drug. The studies of Horning et al. on the need for converting non-

volatile urinary components to volatile species via derivatization are quoted in this chapter, and thus also appear to be misplaced.

Gudzinowicz and Gudzinowicz have gathered a great deal of information between the covers of their book. It is relatively free of structural and typographical errors; an incorrect structure is found on p. 48 (valence problem; too many bonds), but the authors have not, in this reviewer's opinion, employed sufficient resolving power in searching the literature to select and present only pertinent material. The purchasers of books, whose time and money are already spread thin, should not have to differentiate between relevant and irrelevant contents. Let us hope that forthcoming volumes in this series focus better on methods and articles true to the indicated topics. This is what distinguishes just another book from a really good one.

Rahway, N.J. (U.S.A.) W.J.A. VANDENHEUVEL

Journal of Chromatography, 146 (1978) 183—184
Biomedical Applications

CHROMBIO. 194

Book Review

Biomedical applications of immobilized enzymes and proteins, Vol. 2, edited by T.M.S. Chang, Plenum, New York, London, 1977, XX + 359 pp., price US$ 47.40, ISBN 0-306-34312-6.

The investigations of the uses of immobilized enzymes and proteins in analytical chemistry, medicine and industrial processes represent a new biochemical trend of ever increasing importance. This is evident from the steadily increasing number of original papers and monographs in this field (Immobilized Enzymes, Methods Enzymol., 44 (1976); Immobilized Enzyme Principles, edited by L.B. Wingard, Jr., E. Katchalski-Katzir and L. Goldstein, Academic Press, 1976; Biotechnological Application of Proteins and Enzymes, edited by Z. Bohak and N. Sharon, Academic Press, 1977). The monograph under review is the first one devoted exclusively to biomedical applications and it will certainly be very useful for specialists in this field.

Volume I was devoted to the classification and chemistries of immobilized enzymes and to experimental applications in therapy. Volume 2 now under review is a continuation with two sets of chapters: "Diagnostics and public health" and "Perspectives".

The potential of immobilized enzymes themselves, as analytical tools, is demonstrated by a number of automated and semiautomated systems of analysis, as for example the determination of serum glutamata oxaloacetate transaminase, glucose, inorganic phosphates and others (Campbell and Hornby). A variety of methods based on immobilized protein reagents or devices, that have been used for the detection and quantification of materials in urine,

were reviewed by Boguslaski and Smith. The extraordinary sensitivity of immobilized enzyme systems for environmental monitoring was demonstrated by a continuous monitor for the determination of organophosphates and carbamates (Goodson and Jacobs). The chapters on radioimmunoassay (Updike) and enzyme-linked immunosorbent assay (ELISA) (Engvall) are instructive. The immunoadsorption of hepatitis B antigen from blood plasma is described by Wong and Charm, visual detection of hepatitis B surface antigen and antibody by Laffin. Enzyme electrodes represent the most recent advance in analytical chemistry. The great progress achieved in this field is best evident from the table in which Guilbault reviews the enzyme electrodes developed for the determination of urea, glucose, L-amino acids (general), L-tyrosine, L-glutamine, L-glutamic acid, L-asparagine, D-amino acids (general), lactic acid, alcohols, penicillin, uric acid and amygdalin. The chapter on electrochemical preparation of enzyme-collagen membranes is also connected with enzyme electrodes (Suzuki et al.). Further, thermal enzyme probe utilizing the immobilized enzyme calorimeter for widespread biomedical applications (Weaver et al.) represents novel approach to chemical analysis, as does the volatile enzyme product method based on mass spectrometry and immobilized enzymes (Weaver).

In the second part of the book, dealing with the prospects of the use of immobilized enzymes and proteins, a number of very interesting examples are presented. Microcapsules as injectable prostheses, microvascular templates for the miniaturization of artificial organs, and further possibilities are discussed in the chapter on "Biomedical aspects of immobilized enzymes and proteins: a physiologist's view of the prospects" (MacIntosh). The advantages of the ultrasonic method of medical diagnostics will undoubtedly be increased by the introduction of a more sensitive detection by means of sound-sensitive enzymatic systems as chemical amplifiers of weak signals (Berezin et al.). Enzymes catalyzing sequences of reactions, attached to the same beads form immobilized multistep enzyme systems with an architecture in many aspects reminiscent of that of the naturally occurring multi-enzyme systems. Medical and biochemical applications of such multistep enzyme systems open up almost unimaginably wide possibilities (Mattiasson). In biochemical diagnostics and syntheses the sequential and cyclical actions of immobilized enzymes that require coenzymes for their function (Lenhoff et al., Campbell and Chang) can also be utilized. In the enzymatic therapy of thromboembolic diseases plasminogen activator preparations (Maciag et al.) can find considerable use in future; this is also true generally for enzyme therapy with soluble cross-linked enzyme polymers (Poznansky). Enzymes attached to nylon tubes (Sundaram) may also mean great progress in analysis. The book ends with a Subject Index.

The book reviewed here does not only contain a wealth of very valuable reviews on the new developing methods, but it also gives many a stimulus. It certainly will be a useful aid to all those working in the field of clinical and preventive medicine, protection of the environment, pharmacy, analysis, but also of biochemical and microbiological research.

Prague (Czechoslovakia) J. TURKOVÁ

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

APPARATUS

N-1109

SUGAR/AMINO ACID ANALYSIS SYSTEM

Available from Cenco is a descriptive leaflet of the Cenco S/A Analyser System which provides microprocessor controlled analyses of mono- and disaccharides, amino sugars, uronic acids and amino acids (short programme), and may be applied in the analysis of other carbohydrate/amino compounds such as sugar phosphates and methyl sugars. Features include stainless-steel pumping system (0–330 bar), electrically heated chromatographic column (40–95°), electrical reaction coil up to 130°, filter photometer detector, integrated data output over electrosensitive printer, processor controlled temperature/pressure sensing system, programmable integrator–calculator. Serum and urine samples can be injected without deproteinisation, and acid hydrolysates in small amounts without neutralisation.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1072

HPLC COLUMNS AND ACCESSORIES

Pharmacia Fine Chemicals presents a new range of high-performance columns and accessories for liquid chromatography in the life sciences. All components are solvent resistant and operate at pressures up to 10 bar. A complete range of accessories is available for column packing, recycling, upward flow elution and operation of columns in series. The columns (10 mm I.D.), with or without thermostat jacket, are supplied with flow adaptors as standard.

N-1155

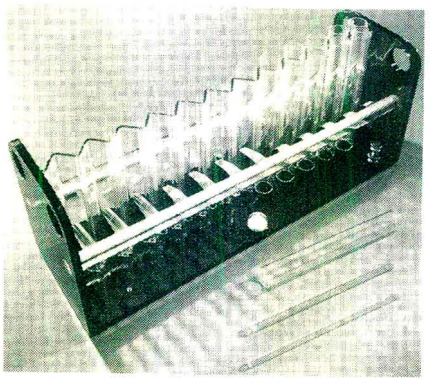
SERUM THEOPHYLLINE ANALYSIS

A 6-page application note from Hewlett-Packard describes a rapid procedure for the analysis of theophylline in serum. Only 50 µl serum are required and the method involves a protein precipitation step followed by separation and quantitation using a high-pressure liquid chromatograph. Graphs, tables and sample chromatograms are included in the note, which discusses column and mobile phase selection and gives complete information on the analytical conditions used.

N-1102

UNIVERSAL GEL PREPARATION RACK

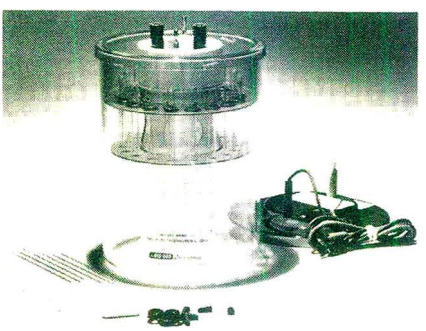
The Bio-Rad Model 215 gel preparation rack will hold a variety of tube sizes simultaneously: from 1 to 24 tubes in sizes from 5 to 18 mm O.D. and 60–250 mm in length. The bottom of each tube is sealed with Parafilm while spring steel fingers position each tube in a true vertical position for polymerization. Levelling screws and a bubble level are also built into the rack.

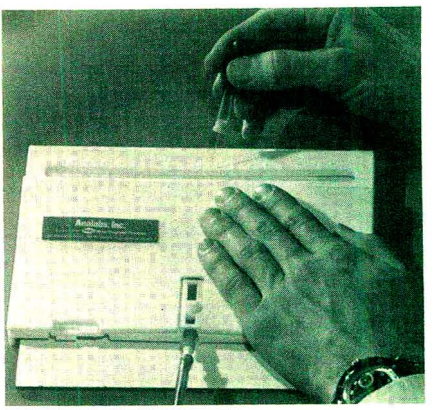


N-1097

GEL ELECTROPHORESIS CELL

Recently announced by Bio-Rad Laboratories is the versatile gel electrophoresis cell, Model 155, which is suitable for all high-resolution gel electrophoresis and isoelectric focusing applications. There are five interchangeable upper buffer chambers available. They are designed to hold up to 18 tubes each of the smaller 5, 7, or 8 mm O.D. tubes, or up to 12 tubes each of the 13 or 17 mm tubes. This range of tubes allows for protein loads of between 0.5 μg and 1500 μg.

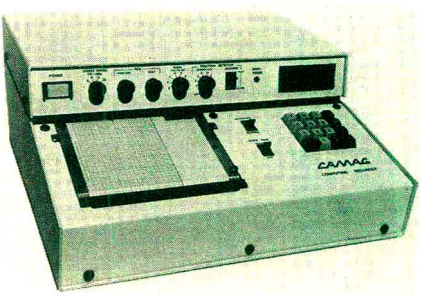




N-1134

TLC SPOTTER/DRIER

The new Analabs spotter/drier dries multiple samples on TLC plates by a gentle flow of gas across the full width of the plate. The flow is regulated by rolling a control wheel back and forth, and shut off by pressing down on the top plate. A slot with a scale marked in cm aids in the precise spotting of samples.



N-1128

CAMAG INTEGRATOR

New from CAMAG is a microprocessor-based integrator which has been developed primarily for the combined digital and analogue quantification of routine electrophoretic separations. It can also be used to evaluate thin-layer chromatograms and is compatible with a variety of densitometers. Scanning curve and digital results are printed out on the same chart.

N-1096

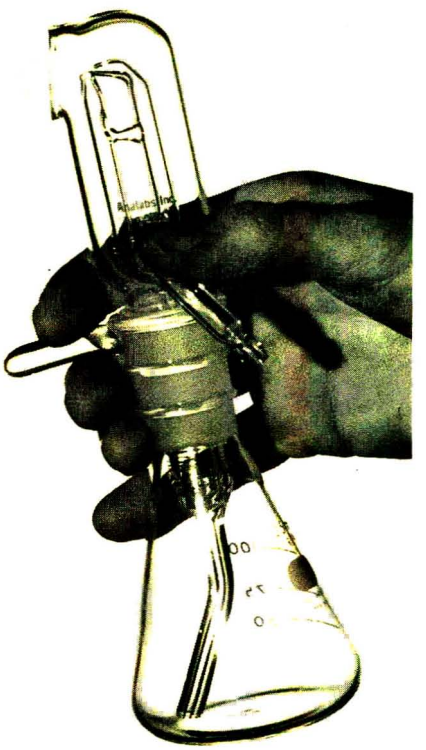
SEMI-MICRO ELECTROPHORESIS SYSTEM

Bulletin 337 from Gelman Instrument Co. describes the new semi-micro electrophoresis system designed to meet the needs of clinical and research laboratories. Using the semi-micro bridge, semi-micro chamber, and eight- or four-sample applicator, four different electrophoresis procedures can be performed simultaneously. The system readily accommodates tests for serum proteins, lipoproteins, haemoglobins, LDH isozymes, CPK isozymes, and alkaline phosphatase isozymes. As many as 32 tests can be done in one hour.

N-1145

SPRAY BOTTLE FOR TLC REAGENTS

Analabs has reintroduced the all-glass reusable spray bottle for TLC reagents. Use of air as propellant (with either a rubber pressure bulb or house air pressure) avoids the pollution problems of the aerosol sprayer.



CHEMICALS

N-1105

HARD-LAYER TLC PLATES

Now available from Analtech is a brochure describing their new hard-layer uniplates for classical TLC with polar and non-polar solvents, which have been specifically designed for high-volume use and rough handling. The GHL series plates have a hardened silica gel surface (suitable for writing on); included in the silica gel is a small amount of inorganic binder to eliminate the problem of interaction of adsorbent layer binder with the solvent system. The plates are available in standard sizes with or without UV-254 phosphor indicator, and/or pre-scored, and are considered to be particularly suitable for applications such as drug screening.

N-1157

C_{18} NANOGRAM TLC PLATE

The C_{18} nanogram TLC plate from Kontes is a chemically bonded, silica gel thin-layer plate for reversed-phase chromatography. The silica gel used allows for greater capacity and higher flow-rates than conventional types. Typical separations are polar amino acids and peptides, lipid profiles, vitamins, etc.

N-1107

AMPHOLINE PAGPLATES WITH NARROW pH RANGES

Now available from LKB-Produkter are three additional Ampholine PAGplates with narrow pH ranges (4–5, 4–6.5, 5.5–8.5) in addition to the previous pH 3.5–9.5. The new plates can be used for studying the structure of various kinds of proteins such as haemoglobin, α_1-antitrypsin and many enzymes, by thin-layer electrofocusing.

N-1136

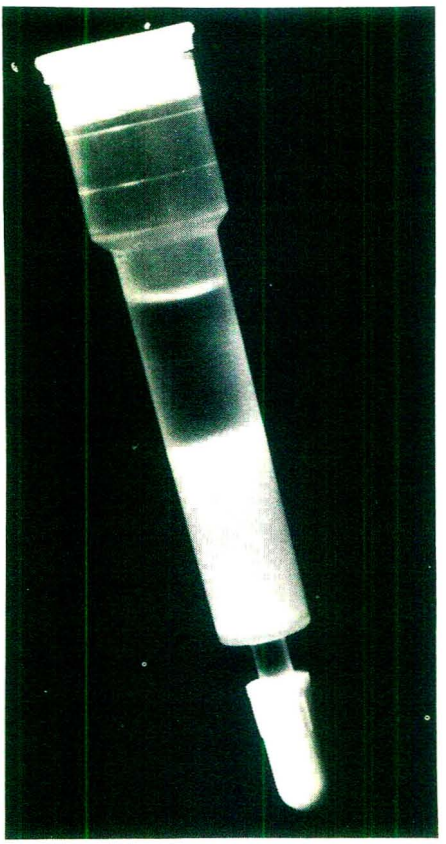
DRUG STANDARD KITS

Analabs has added two new Drug Enforcement Administration exempt drug standard kits to its existing line of analytical standards. These contain a selection of antipsychotic and antihistaminic drugs most commonly encountered. Each standard is dissolved in *tert.*-butanol (with a small amount of methanol in some cases) in a concentration of 1 mg/ml.

N-1142

PEPTIDE SEQUENCING COLUMNS

Now available from Isolab are new Quik-Sep columns prefilled with Dowex 50-X2 ion-exchange resin useful for the purification of PTH-amino acids which are produced in sequencing peptides and proteins by Edman degradation technique. The resin has been processed to eliminate methanol soluble and fluorescence producing impurities.



NEW BOOKS

Analytische und präparative Methoden der klinischen Biochemie, by N. Rehfeld and D. Reichelt, Akademie-Verlag, Berlin, 2nd (revised) ed., 1977, 496 pp., 188 figs., 45 tables, price M 62.00.

Molecular biology and pharmacology of cyclic nucleotides (Proc. NATO Advanced Study Institute on Cyclic Nucleotides, Tremezzo, September 19–30, 1977), edited by G. Folco and R. Paoletti, Elsevier/North-Holland, Amsterdam, Oxford, New York, 1978, X + 340 pp., price Dfl. 102.00, US $ 41.75, ISBN 0-444-80041-7.

Analysis of drugs and metabolites by gas chromatography–mass spectrometry, Vol. 4, Central nervous system stimulants, by B.J. Gudzinowicz and M.J. Gudzinowicz, Marcel Dekker, New York Basel, 1978, XI + 458 pp., price SFr. 125.00, ISBN 0-8247-6614-8.

An introduction to radioimmunoassay and related techniques (Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 6, Part II, edited by T.S. Work and E. Work), by T. Chard, Elsevier/North-Holland, Amsterdam, Oxford, New York, 1978, IV + 242 pp., price Dfl. 52.00, US $ 22.75, ISBN 0-7204-4220-6.

Laboratory techniques in biochemistry and molecular biology, Vol. 6 (Part I: Density gradient centrifugation, by R. Hinton and M. Dobrota; Part II: An introduction to radioimmunoassay and related techniques, by T. Chard), edited by T.S. Work and E. Work, Elsevier/North-Holland, Amsterdam, Oxford, New York, 1978, VIII + 534 pp., price Dfl. 160.00, US $ 69.75, ISBN 0-7204-4221-4.

Stable isotopes – Applications in pharmacology, toxicology and clinical research, edited by T.A. Baillie, Macmillan, London, 1978, XIII + 314 pp., price £ 15.00, ISBN 0-333-21747-0.

Plasma proteins – Analytical and preparative techniques, by P.C. Allen, E.A. Hill and A.M. Stokes, Blackwell, Oxford, London, Edinburgh, Melbourne, 1977, IX + 254 pp., price £ 11.75, ISBN 0-632-00279-4.

Disposition of toxic drugs and chemicals in man, Vol. 1, Centrally-acting drugs, by R.C. Baselt, Biomedical Publications, Canton, Conn., 1978, V + 306 pp., price US$ 22.50.

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

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

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

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford. New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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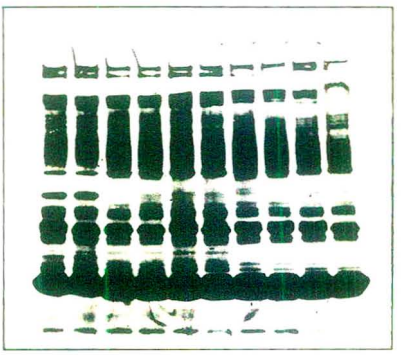
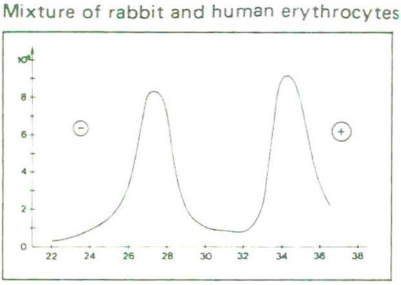
DESAGA

for Electrophoresis



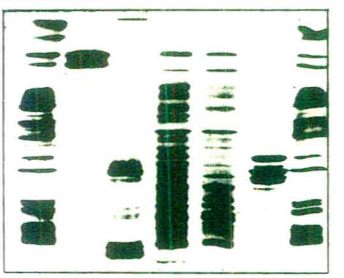
DESAGA Electrophoresis Systems DESAPHOR and MEDIPHOR combine all our experience in horizontal electrophoresis and electrofocusing.
DESAPHOR offers up to 265 × 400 mm for high resolution electrofocusing in polyacrylamide slabs or granulated gels. Any separation geometry may be realized using our well-known free movable, platinum electrodes. Two supersize buffer vessels allow long time runs on any medium. 400 mm and 2 × 2.5 l is too big? Choose another cooling plate of 125, 200 or 300 mm and use the buffer saving mode with 2 × 0.8 l.
MEDIPHOR offers the 125 mm cooling plate and 2 × 0.8 l buffer volume in a small size chamber. But it is made from polypropylene, too. And uses the same electrode, which is neither fixed to the chamber nor to the lid.
Ask for detailed information. We are producing chambers for horizontal electrophoresis for 15 years and want to share our experience.

Mixture of rabbit and human erythrocytes.



DESAGA Electrophoresis System HAVANA for all known techniques in polyacrylamide gel. This reliable instrument for each lab uses precision ground all-glass cells for optimum gel polymerisation. No grease or tape is needed as gasket; the gel contacts glass only. The glass cells also allow observation of the sample application – the most critical step.
The intensive cooling allows up to 40 W – high field strength and short running times. Human hemoglobins are fully separated in only 10 minutes.

It is a time saving instrument for routine operation, too. Multiple syringes transfer 12 samples simultaneously. Big populations are easily screened for genetic studies or medical surveys.
And it is a research instrument of high resolution. It allows electrofocusing in round gels and two-dimensional separations resolving more than 100 proteins from a sample. Gradient gels are easily cast and up to 30 mg protein can be separated in preparative cells and electrophoretically eluted and concentrated.
Any questions left? Give us the opportunity to provide an answer.



DESAGA FF 48 Apparatus for Free-flow Electrophoresis acc. to HANNIG is our top model.
The buffer flows vertically through a 0.5 mm gap between two cooled glass plates. At the bottom it is divided into 48 fractions. The sample is injected into the running buffer film and separated by a strong horizontal electric field. Components of different electrophoretic mobility are collected in different cooled glass tubes.
This continuous principle allows not only protein separation on a preparative scale up to 50 mg/h – but cell separation. Not only erythrocytes of different origines, but B and T lymphozytes and even their subclasses are separated without damage. Other scientists use it for membrane vesicles, mitochondria or ribosomes. All use small difference in surface charge to discriminate particles not differing in size or density.
Ask for detailed bibliography showing more than 100 entries for different topics.

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