ISSN 0378-4347

VOL. 162 NO. 4 APRIL 11, 1979
(Biomedical Applications, Vol. 4, No. 4)
THIS ISSUE COMPLETES VOL. 162

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1979

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	The publication schedule for the volumes 174–180 and for further *Chromatographic Reviews* issues (vol. 165) will be published later.						
Chromatographic Reviews				165/1									
Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 19 volumes in 1979. The subscription price for 1979 (Vols. 162–180) is Dfl. 2356.00 plus Dfl. 285.00 (postage) (total *ca.* US$ 1288.00). The subscription price for the *Biomedical Applications* section only (Vols. 162–164) is Dfl. 384.00 plus Dfl. 45.00 (postage) (total *ca.* US$ 209.00). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 161) are available at Dfl. 136.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional biobliographic information on this and other Elsevier journals, please contact Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

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Printed in The Netherlands

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(Continued overleaf)

Contents (continued)

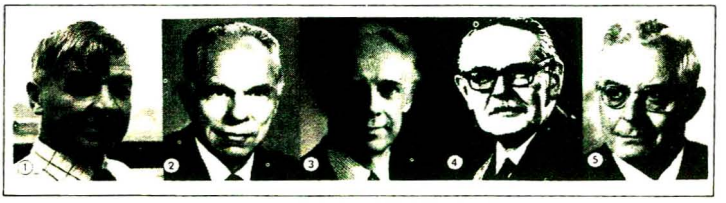
75 Years of Chromatography

A Historical Dialogue

L. S. ETTRE *and* A. ZLATKIS (Editors).

Journal of Chromatography Library - Volume 17

On the occasion of the 75th anniversary of the invention of chromatography, this book compile the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates). In their contributions t this volume, these pioneers review the events which influenced them t enter the field; explain the back ground of their inventions; sum marize their activities and result during their professional lives; and discuss their interactions with other scientists and other disciplines.



This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated.
The final chapter is devoted to "Those who are no longer with us"

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(*Nobel Prize laureates)

Feb. 1979 *526 pages* *US $49.75/Dfl. 112.00* *ISBN 0-444-41754-0* *LC 78-23917*

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Affinity Chromatography

JAROSLAVA TURKOVA, *Czechoslovak Academy of Sciences, Prague.*

JOURNAL OF CHROMATOGRAPHY LIBRARY - Volume 12

This book reviews the application of affinity chromatography for the isolation of various biologically active substances. The reviewing table comprises almost 1,400 references and is completed by data on use of solid supports and spacers. Great attention is given to the review of the most commonly used solid supports and to the method of attachment, together with the methods of characterization of both the carriers and the immobilized affinity ligands.

This extensive and up-to-date review is intended mainly for biochemists and biologists. It will be particularly useful to clinicians engaged in human or veterinary medicine, as well as to those dealing with chromatography and industrial chemistry.

CONTENTS: Chapters: 1. Introduction. 2. The principle, history and use of affinity chromatography. 3. Theory of affinity chromatography. 4. Application of affinity chromatography to the quantitative evaluation of specific complexes. 5. General considerations on affinant—sorbent bonding. 6. Choice of affinity ligands for attachment. 7. Hydrophobic chromatography, covalent affinity chromatography, affinity elution and related methods. 8. Solid matrix supports and the most used methods of binding. 9. Characterization of supports and immobilized affinity ligands. 10. General considerations on sorption, elution and non-specific binding. 11. Examples of the use of affinity chromatography. 12. Immobilized enzymes. Subject Index.

March 1978 x + 406 pages
US $69.75/Dfl. 167.00 0-444-41605-6

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Liquid Chromatography Detectors

by R. P. W. SCOTT, *Chemical Research Dept., Hoffmann-La Roche, Nutley, N.J.*

JOURNAL OF CHROMATOGRAPHY LIBRARY - Volume 11

The rapid development of liquid chromatography over the past decade has been due to the introduction of highly sensitive linear liquid chromatography detectors. This book provides a comprehensive treatment of the function and optimal working conditions of liquid chromatography detectors. Divided into four parts, the book gives detailed descriptions of the general characteristics of liquid chromatography, bulk property, and solute property detectors, as well as their use in liquid chromatography. The necessary detector specifications are defined which will permit a rational comparison of the performance of one detector with that of another.

CONTENTS: Introduction. **Parts: 1. General Characteristics of Liquid Chromatography Detectors.** History, function and classification of detectors. Performance criteria of LC detectors. Detector characteristics that affect column performance. Summary of detector criteria. Ancillary equipment. **2. Bulk Property Detectors.** General characteristics of bulk property detectors. The refractive index detector. The dielectric constant detector. The electrical conductivity detector. Additional bulk property detecting systems. **3. Solute Property Detectors.** Principles of detection. The ultraviolet absorption detector. The fluorometric detector. The polarographic detector. The heat of adsorption detector. The spray impact detector. The radioactivity detector. The electron capture detector. Transport detectors. **4. The Use of Detectors in Liquid Chromatography.** The selection of the appropriate detector. Quantitative and qualitative analysis. Practical hints on detector operation. Special detector techniques. Spectroscopic detectors. **Subject Index.**

1977 x + 248 pp. US $34.50/Dfl. 84.00
ISBN 0-444-41580-7

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7056

Journal of Chromatography, 162 (1979) 495—506
Biomedical Applications

CHROMBIO. 287

APPLICATION OF PATTERN RECOGNITION AND FEATURE EXTRACTION TECHNIQUES TO VOLATILE CONSTITUENT METABOLIC PROFILES OBTAINED BY CAPILLARY GAS CHROMATOGRAPHY

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(First received June 14th, 1978; revised manuscript received November 6th, 1978)

SUMMARY

The applicability of threshold logic units, a form of nonparametric pattern recognition, to the processing of metabolic profile data obtained by high-efficiency glass capillary column gas chromatography has been investigated. The test data included profiles of the volatile constituents of urine from normal individuals and from individuals with diabetes mellitus. A feature extraction algorithm allowed for dimensionality reduction and indicated the constituents most important in the normal versus pathological distinction. With an optimum number of dimensions, a normal versus pathological prediction rate of 93.75% was achieved. Gas chromatography—mass spectrometry was utilized to identify important profile constituents.

INTRODUCTION

Multicomponent chromatographic analyses have been developed for several of the classes of chemical constituents in various human physiological fluids [1—18]. Such analyses, generally termed metabolic profiles [1—2], provide qualitative and quantitative data that reflect the state of a variety of metabolic processes within the body. The primary utility of metabolic profiling is recognized as being in the study of the etiology of diseases through the biochemical elucidation of physiological and pathological processes. Eventually, selected profiling techniques may also serve as clinical screening or diagnostic aids.

Although the sampling and analytical aspects of certain profiling techniques are sufficiently refined for routine research use, biomedical correlations of pro-

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file data are rare. Several problems have limited the extraction of useful information from the complex profile data. Aside from pathological alterations, profiles vary considerably due to factors such as individual diet and genetic conformation. These individual variations can be of the same magnitude as the pathological variation. Ascertainment of the latter variations thus requires comparison of large sets of normal and pathological profiles, such that the nonpathological variations are effectively equalized in both pattern sets. Reliable manual comparison of large sets of profiles containing more than 200 constituents is simply not feasible. The alternative process of preselecting profile constituents for comparison, based on their probable metabolic activity, is not always desirable. Since much of metabolism is not well understood, preselection may discard potentially important information. Furthermore, preselection requires previous identification of essentially all profile constituents, for which the only practical technique is gas chromatography—mass spectrometry (GC—MS). Due to the small sample quantities involved, and the general lack of appropriate reference spectra, GC—MS identifications of volatile constituents are frequently arduous. Thus, a data analysis technique is required that must first be capable of distinguishing profiles of individuals in a particular diseased state from those of normal individuals. It must not require initial assumptions concerning the statistical distribution of the data. Secondly, but quite importantly, the analysis must indicate what components of the profile data are involved in the normal versus pathological distinction. The distinctive metabolites may then be identified, and their metabolic precursors can be determined with loading tests or other appropriate methodology.

In this report, we describe the development of threshold logic unit (TLU) techniques, a form of nonparametric pattern recognition [19], for the processing of the metabolic profile data. TLUs have been trained to distinguish normal and pathological patterns and, in combination with feature extraction algorithms, have designated the pattern components most significant in the distinction. Since TLU techniques function independent of assumptions of metabolic significance, preselection is not required, and identification need only be obtained for those compounds found to be significant in the normal versus pathological distinction.

Nonparametric pattern recognition techniques have been applied to several biomedical problems [20, 21] and their numerous applications in the chemical field have been recently reviewed [22, 23]. However, their prior utilization with multicomponent chromatographic data has been limited to studies of petroleum sample type identification [24], Wilcoxon-test correlations of human urinary amino acid analyses and initial correlations of volatile constituent analyses [6, 25].

In this study the TLU procedure has been tested with urinary volatile constituent profiles obtained by high-resolution (glass capillary) gas chromatography. Volatile constituent profiles are of interest [7—18] due to their complexity, frequently exceeding 200 detectable compounds, and due to their inclusion of by-products, intermediates, and terminal products of a wide variety of metabolic processes. The pathological condition chosen for testing purposes was diabetes mellitus, primarily because of sample availability. While the biochemical information extracted from the profiles in this study may be of some utili-

ty, the primary objective has been the development of a generally applicable methodology.

MATERIALS AND METHODS

Headspace sampling and chromatographic separation

The procedures and instrumentation utilized for the acquisition of the metabolic profiles have been previously described [16]. Volatiles in the heated (100°) urine headspace were adsorbed onto 2 mg of 2,6-diphenyl-*p*-phenylene oxide porous polymer (Tenax GC; Applied Science Labs., State College, Pa., U.S.A.). The porous polymer was contained in platinum microbaskets which were subsequently encapsulated for injection.

All chromatographic functions were automated [16]. Reproducibly prepared glass capillary columns [16] (60 m × 0.29 mm I.D.) coated with GE SF-96 methylsilicone fluid were employed. The column effluent was split between a flame ionization detector (FID) and a nitrogen-sensitive thermionic detector. Only the FID data were incorporated into the pattern recognition studies; the nitrogen-sensitive detector data were used in the identification of profile constituents.

In the only modification of prior procedures, a reference for relative retention time calculations was provided by the addition of two internal standards to the physiological fluid prior to headspace sampling. Full scale peaks (corresponding to approximately 25 ng by direct injection) resulted from the introduction of 0.3 μg of 6-undecanone and 20 μg of 10-nonadecanone in 5 μl acetone. The standards were added to the urine samples immediately prior to the transferring of them into the sampling vessel. As acetone elutes in the first peak of the profile and has a short retention time on the Tenax adsorbent, it had no adverse effect on the chromatograms.

Sample collection

Diabetic urine samples were collected from 29 individuals, including outpatients and patients at two hospitals. Of these, 15 were diagnosed as diabetics with further complications. Normal samples were obtained from 35 individuals, including those hospitalized for 24 h solely for the sample collection. Medical histories were maintained, but samples were not excluded on this basis. No diet regulation was involved.

Twenty-four hour urine samples were collected; interim samples were frozen over dry ice. The samples were then brought to room temperature, filtered, divided into 50 ml aliquots and refrozen at - 20°. No preservatives or diluents were added.

Data analysis

For use in TLU processing, each metabolic profile was represented as a pattern vector of the form:

$$\vec{X} = (x_1, x_2, \ldots\ldots x_n)$$

where each vector component, x_i, was calculated from peak areas in the profile

chromatogram. Using a training set of known patterns, representing normal and pathological profiles, an n-dimensional hyperplane, or classification surface, was developed which classified, or linearly separated, the patterns into the two known groups. The classification criteria were:

$\vec{W} \cdot \vec{X} < 0$ for $\vec{X}$ representing normal profiles

$\vec{W} \cdot \vec{X} > 0$ for $\vec{X}$ representing pathological profiles

where $\vec{W}$ is the weight vector, a normal vector from the classification surface. The TLU training procedures are quite simple and have been described elsewhere [19]. Once developed, the validity of the weight vector was tested by predicting, according to the above criteria, the classification of patterns not present in the training set.

In any pattern recognition process, it is important that the ratio (R) of the number of training patterns to the number of pattern dimensions be as large as possible; cases where $R \leqslant 2$ must be carefully interpreted [26]. As described below, a leave-out-one algorithm was employed in this study to allow operation with an initial $R \leqslant 2$. In addition, to control the pattern dimensionality, each pattern component was calculated as the sum of the peak areas in a small interval of the profile chromatogram. Such combining of peaks yields an apparent loss of information, in that differences in more than one peak in an interval may partially cancel, and observed differences cannot necessarily be assigned to a single peak. In fact, many intervals contained only one peak. Further, once an interval was found to be significant, it was subdivided and re-evaluated. This process can be continued until the significance can be assigned to a single constituent.

Initially, patterns of 100 dimensions were used. The section of the chromatogram between injection and the elution of the 6-undecanone internal standard was divided into 50 intervals of equal length in time. Similarily, the section between the elution of 6-undecanone and the 10-nonadecanone internal standard was divided into 50 uniform intervals. The 10-nonadecanone peak was the last to elute in nearly all profiles. The program located the two internal standards by examining an absolute retention time "window" (2 min for 6-undecanone and 3 min for 10-nonadecanone) and designating the largest peak in the "window" as the internal standard. For this study, the designation was also manually verified; no errors occurred.

Prior to the TLU training procedure, the pattern components were autoscaled [23] according to the formula:

$$x'_{ij} = \frac{x_{ij} - \mu_i}{\sigma_i}$$

where x'_{ij} is the autoscaled i^{th} component of the j^{th} pattern, x_{ij} is the initial component, μ_i the mean and σ_i the standard deviation of the i^{th} component in all patterns. The autoscaling operation equalizes the initial weighting of the intervals. Autoscaling improved the prediction rate of the subsequently trained

TLUs by as much as 15%. A normalization procedure, in which each component peak area was expressed as a fraction of the total chromatogram peak area according to:

$$x'_{ij} = \frac{x_{ij}}{\sum_{i=1}^{100} x_{ij}}$$

was found to reduce prediction rates, both with and without autoscaling [27].

Training and testing of TLUs was executed with the 100-dimensional patterns and reduced dimension patterns derived therefrom. In each case, training was performed with a leave-out-one algorithm [28] operated in a rotating basis. In the leave-out-one algorithm, all available patterns except one were used to train the TLU. The validity of the TLU was then tested by predicting the classification of the omitted pattern. The pattern set was then rotated to omit a different pattern from the training set, and the training and prediction were repeated. The rotation was repeated until each pattern had served as the prediction test pattern. The percentage of the test patterns that was correctly predicted was recorded for each cycle. A feature extraction algorithm, to designate pattern components most significant in the classification, was developed for the leave-out-one technique. Since the pattern components were autoscaled, the components of the weight vector were indicative of the significance of the respective components. An average weight vector was determined for a complete rotation cycle by calculating the vector sum of each of the individual weight vectors trained. At the condition of a rotation, the magnitude of the components of the average weight vector where thus indicative of the average significance of each pattern component. A preselected number of the least significant components was then discarded and another rotation cycle was initiated. The program was continued until the training process was unable (in 500 iterations) to find a hyperplane that linearly separated the profile classes.

The leave-out-one algorithm offers two significant advantages, as compared to the use of discrete training and prediction pattern sets. First, it yields a maximum value of R for any available data set. Second, it facilitates operation with $R \leqslant 2$, provided the ratio of the number of patterns to the number of intrinsic dimensions is greater than two. The intrinsic dimensions are defined [29] to be those dimensions that contain information significant in the classification process. In standard training studies with $R \leqslant 2$, a weight vector may be obtained that provides a linear separation of the pattern classes, but has no valid predictive capability. Such a separation may be based on the non-intrinsic dimensions. The non-intrinsic dimensions can be regarded as noise mixed with the intrinsic dimensions. The leave-out-one technique, in calculating an average weight vector, effectively filters out the non-intrinsic dimensions in a manner analogous to ensemble averaging, thus allowing the extraction of the intrinsic pattern components.

Peak areas and retention times were recorded with a commercial chromatographic data system (Model PEP-2; Perkin Elmer, Norwalk, Conn., U.S.A.). All subsequent processing was performed on a CDC 6600 computer. The training

and autoscaling programs were developed in part from the program set ARTHUR, created by Kowalski and Drewer [30]. Our programs are available upon request.

Gas chromatography—mass spectrometry

Volatile urine constituents were sampled as described above, except that the Tenax adsorbent was contained in a glass precolumn, rather than a platinum microcapsule. Details of this sampling technique have been previously reported [14]. Electron-impact spectra were obtained with a gas chromatograph-mass spectrometer (Hewlett-Packard Model 5980A). The glass capillary column was interfaced to the ion source with an all-glass, single-stage jet separator (Scientific Glass Engineering, Melbourne, Australia), maintained at 270°. Identifications were confirmed by comparison of spectra and retention indices of authentic samples recorded with the same instrument.

RESULTS AND DISCUSSION

Two training sets were prepared. One included the entire set of 35 normals and 29 diabetics (Data Set A), the other included the normals and only the 14 diabetics diagnosed to be free of complications (Data Set B). The results of TLU training and feature extraction with these two data sets are presented in Table I. Features were extracted at the rate of five or ten per cycle, as indicated in the Table.

At the optimum number of dimensions, 92.2% of the patterns in Data Set A and 83.7% of Data Set B were correctly predicted. These percentages are significantly greater than the 54.7% and 71.4%, respectively, that would be obtained by simply classifying each pattern as a normal. With the leave-out-one algorithm, satisfactory results were obtained despite the low ratio of patterns to initial dimensions. The increase in prediction with decreasing number of features substantiates the importance of the intrinsic dimensionality. As the insignificant dimensions were discarded, there was less chance of a change in an insignificant constituent adversely affecting the pattern classification. The improved prediction with increasing training set size was expected, as the weight vector was exposed to a more complete subset of the possible profiles and was thus more likely to be trained for the test data.

To utilize the information obtained by feature extraction, it is necessary to associate the retained dimensions with the original profiles. In Fig. 1, a diabetic profile (A) and a normal profile (B) are shown together with scale markings indicating the retained intervals in the test of 35 normals and 14 diabetics (Intervals Retained A) and in the test of 35 normals and 14 diabetics (Intervals Retained B). To describe the two chromatograms as typical of normals and diabetics would falsely imply that the pattern chromatograms can be generalized. However, the chromatograms shown are not unlike others obtained from such individuals.

The components of the average weight vector and the original pattern dimensions corresponding to the retained intervals are given in Table II. The initial assignment of patterns designated a negatively valued dot product of the pattern and weight vector to be normal, and a positive dot product to be

TABLE I

TLU PREDICTION RATE FOR DATA SETS A AND B WITH EITHER 10 OR 5 FEATURES EXTRACTED ON A ROTATION CYCLE

Data Set A		Data Set B	
No. dimensions retained	Correct (%)	No. dimensions retained	Correct (%)
100	67.2	100	57.1
90	70.1	90	57.1
80	71.9	80	57.1
70	71.6	70	69.4
60	79.7	60	71.4
50	81.3	50	73.5
40	79.7	40	77.6
35	84.4	35	81.6
30	79.7	30	79.6
25	84.4	25	81.6
20	82.8	20	83.7
15	92.2	15	83.7
10	DNC*	10	78.7
		5	DNC*

*DNC = Did not converge after 500 training iterations, i.e., could not linearly separate the training set data.

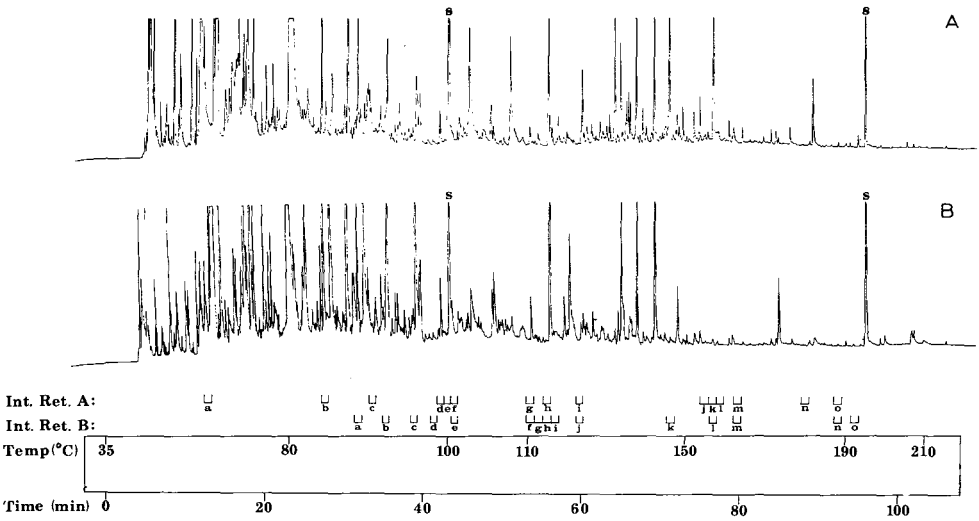

Fig. 1. (A) Chromatogram of the urinary volatiles of a diabetic male. (B) Chromatogram of the urinary volatiles of a normal female. Scale markings indicate intervals selected as significant in the distinction of normal and diabetic profiles, as reported in Tables III and IV. S= internal standard peaks.

TABLE II

COMPONENTS OF AVERAGE WEIGHT VECTOR AT 15 RETAINED DIMENSIONS AND IDENTIFIED COMPOUNDS FOR DATA SETS A AND B

Data Set A			Data Set B		
Interval*	Component	Identified component	Interval**	Component	Identified component
a—15	2.03	4-Heptanone	a—37	- 9.39	
b—32	- 9.68		b—41	- 0.24	
c—39	-12.96		c—45	5.51	Carvone
d—49	-13.27		d—48	7.96	
e—50	6.41	Indole	e—51	9.18	
f—51	6.56		f—60	4.90	
g—60	7.19		g—61	-10.82	
h—62	8.59		h—62	7.76	
i—66	- 5.78		i—63	4.29	
j—81	7.34		j—66	- 9.18	
k—82	11.56		k—77	9.18	
l—83	- 7.97		l—82	16.33	
m—85	7.19		m—85	6.12	
n—93	7.66		n—97	5.51	
o—97	7.03		o—99	- 8.57	

*Fig. 1 Int. Ret. A.

**Fig. 1 Int. Ret. B.

diabetic. Because the pattern components were autoscaled, a given pattern component may have either a negative or positive value. Thus, a peak of greater than average area that occurs in an interval with a negative weight vector component is indicative of a normal profile; a peak of less than average area is indicative of a diabetic profile. The converse is true for positive weight vector components. This consideration comprises an important aspect of autoscaling. Without this pre-processing, all pattern components would have positive values, and the presence of a given peak could indicate only one condition, regardless of the size of the peak. Thus, autoscaling is advantageous when significant peaks are present in both classes, but with differing areas. Autoscaling would be disadvantageous when the simple presence of certain peaks is indicative of the profile class.

In several intervals (15, 32, 39, 41, 49, 50, 63, 77, 82, 85, 93) a peak may be recognized in the chromatograms in Fig. 1 that varies in area as predicted by the sign of the weight vector components. Confirmation of the results in other intervals required examination of several more chromatograms. It is of interest that many of the significant intervals fall in the latter portion of the chromatogram and thus involve urinary constituents not observed in the previous volatile profile studies. Further, many of these intervals involve only small peaks which might be neglected in manual data analysis. The selection of several sets of adjacent intervals may simply reflect the locality of several important peaks, or may arise from certain significant peaks shifting between intervals due to

very slight variations in relative retention times.

Due to the interaction and frequent nonspecificity of metabolic processes, the information in certain pattern dimensions may be diagnostically equivalent. Such redundance may result in none of the dimensions achieving a high significance in the classification until one or more is discarded. Rapid feature extraction may discard a set of redundant dimensions in a single cycle, eliminating the information completely. This is demonstrated by the improved prediction rates obtained by slow feature extraction reported in Table III. Ten features were extracted per training cycle for five cycles, and one per cycle thereafter; results are given only for selected cycles. A trade-off between computation costs and classification results is also evident.

Another example of redundance may be observed in Fig. 1. Seven intervals (51, 60, 62, 66, 82, 85, 97) are clearly significant, in that they are retained in both weight vectors. The information content of the other intervals is likely to be redundant, with respect to either the intervals retained in both vectors or with respect to other periodically retained intervals. Because of this redundance, the significance of the retained and discarded dimensions must be carefully interpreted. While profile constituents in a retained dimension are clearly important in the pattern classification, a discarded dimension may also contain constituents involved in the aberrant metabolism.

To investigate further the redundance in the profile data and the effect of increasing the value of R, TLUs were trained and tested with abbreviated patterns. Data sets were prepared that contained only twenty intervals (1—20, 21—40, etc.). On the first training cycle, only the set composed of intervals 41—60 was linearly separable. Feature extraction resulted in a maximum pre-

TABLE III

TLU PREDICTION RATE FOR DATA SET A

With 10 features extracted each of first 5 rotations and 1 feature extracted each rotation thereafter.

No. dimensions retained	Cycle No.	Correct (%)
100	1	67.19
70	4	76.56
50	6	81.25
35	21	81.25
26	30	90.63
20	36	90.63
18	38	93.75
15	41	92.18
13	43	89.06
10	46	89.06
7	49	90.63
6	50	DNC*

*DNC= Did not converge after 500 training iterations.

diction rate of 82.8% with 13 of the original 20 dimensions retained. The seven most significant of these dimensions were then combined with the 13 most significant intervals (as determined from the test reported in Table I) of the remaining original 80 dimensions. Training and feature extraction with these 20 dimension patterns resulted in a maximum prediction of 93.8% correct with 11 retained dimensions. All eleven dimensions were included among those listed in Table I.

These results substantiate the previous conclusion that redundance exists within the profile information, but that several dimensions contain essential information. Further, within the range examined, the results are not significantly improved by manually selecting the initial dimensions and thus increasing the value of R.

Referring back to Fig. 1, several retained intervals are seen to contain more than one constituent peak. Thus, the significance of the interval cannot be assigned to a single constituent. To reduce this ambiguity, each of the 15 most significant intervals (Table II) was split into two equal intervals and the resulting 30 dimension patterns were used to train a new TLU. In the case of Data Set A, 17 of the 30 dimensions were retained at an optimum prediction rate of 92.2%, identical to the rate prior to the breakdown. A prediction rate of 91.8% was obtained with Data Set B with 13 of the dimensions retained. The signs of the weight vector components following the split invariably agreed with those corresponding to the same interval prior to the division, although the relative magnitude of the weight vector components did vary. A few of the subdivided intervals contained more than one constituent peak. It would be possible to continue the division until each interval contained only one peak or until small variations in relative retention times precluded the reliable assignment of a peak to a given interval.

The high prediction rates achieved by the TLUs are indicative of their potential as diagnostic or screening aids. However, their biomedical potential is more immediately discernable, and requires the identification of the significant constituents. Several of the constituents found to be significant in the pattern recognition studies have been tentatively identified, including both sulfur- and nitrogen-containing compounds. However, only three of these compounds have been confirmed by comparison to authentic spectra at present.

Once reference spectra can be obtained, the other identifications will be reported. The three identified and confirmed compounds and the corresponding intervals are listed in Table II. The weight vector components of each of these compounds was positive. Thus, increasing concentrations of these compounds were indicative of a diabetic classification. Increasing concentrations of 4-heptanone in the urine of diabetics has been previously reported by Liebich and Al-Babbili [7].

One reservation must be cited concerning the interpretation of the results of the TLU training and prediction. All but one of the diabetic patients were receiving various medications for diabetes and in some cases for other conditions. The one individual not receiving medication was misclassified in both TLU rotations. Contraceptives were the only medication known to have been taken by any of the normal individuals. Thus, the possibility that the pattern distinction was based on metabolites of the medication or compounds induced

by it rather than those related to the disease cannot be excluded. The analysis of more diet-controlled diabetics would clarify the interpretation of the pattern recognition results. However, the capability of the TLU technique to identify the profile components most significant in the class distinction is not diminished by this ambiguity.

CONCLUSIONS

The results of this study indicate the utility of threshold logic unit and feature extraction techniques in the determination of constituents important in the classification of urinary volatile constituent metabolic profiles. It is likely that these techniques can be readily extended to profiles of other complex fractions in which pathological changes cannot otherwise be distinguished from normal individual variations. By increasing the efficiency of the extraction of information from the profiles, pattern recognition techniques should enhance the utility of metabolic profiles in many biochemical studies.

ACKNOWLEDGEMENTS

We thank Drs. R. Robinson, J. Galloway, B. Obermeyer and R. Gurd for their help in obtaining urinary samples of various patients. Valuable comments of Dr. P.C. Jurs concerning the pattern recognition aspects are also acknowledged. This work was supported by the Grant No. PHS ROI GM 23668-01 from the NIGMS, U.S. Public Health Service. One of us (M.L. McConnell) was a recipient of the summer fellowship from the American Chemical Society, Division of Analytical Chemistry, sponsored by Carle Instruments, Inc.

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Journal of Chromatography, 162 (1979) 507—528
Biomedical Applications

CHROMBIO. 292

QUANTITATIVE GAS—LIQUID CHROMATOGRAPHY OF NEUTRAL SUGARS IN HUMAN SERUM GLYCOPROTEINS

FUCOSE, MANNOSE, AND GALACTOSE AS PREDICTORS IN OVARIAN AND SMALL CELL LUNG CARCINOMA*

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(First received August 30th, 1978; revised manuscript received November 7th, 1978)

SUMMARY

A precise and accurate gas—liquid chromatographic (GLC) method has been developed for the quantitative analysis of the neutral sugars L-fucose (6-deoxygalactose), mannose,

*Contribution from the Missouri Agricultural Experimental Station, Journal Series No. 8207. Approved by the Director.
**Experimental data taken in part from Master's thesis, University of Missouri, Columbia, Mo. 65211, U.S.A.

galactose, and glucose in ethanol precipitates of human serum proteins. The chromatographic conditions and sample preparation resulted in short analysis times (20 min per run) and made routine analyses practicable (twelve samples per day). The alditol acetate derivatization yielded single derivatives for each sugar. Complete separation was achieved on a 2.0 m × 2 mm I.D. column with 2.0% Silar-7CP on Chromosorb W AW 80—100 mesh. The results of hydrolysis showed that the release of fucose and galactose preceded the release of mannose. Hydrolysis with AG 50W-X8 (H^+) ion-exchange resin in 0.5 *N* HCl at 100°for 7 h optimized glycosidic bond cleavage with only minimal destruction of fucose, mannose and galactose. A combination of strong cation- and anion-exchange resin columns was used to remove chromatographic background of peptides, amino acids, amino sugars, and inorganic ions. An average R.S.D. of less than 4% with recovery of >86% for the three sugars was achieved. The homogeneity of the chromatographic peaks for the neutral sugars of normal human serum glycoproteins was confirmed by GLC—mass spectrometry. Significantly elevated ratios of fucose, galactose, and mannose to serum protein were observed for patients with small cell lung and ovarian carcinomas.

INTRODUCTION

Glycoproteins are macromolecules with one or more polypeptide chains to which sugars are covalently bonded. These sugars form branching chains where the terminal sugar appears to be limited to either sialic acid or L-fucose [1]. Glycoproteins comprise a large number of compounds. They occur biologically as enzymes, hormones, and immunoglobulins. The role of carbohydrates in glycoproteins of the hemagglutination system, as carriers, immunoglobulins, and in other biologically active glycoproteins, is not well understood [2]. However, studies show that carbohydrates in glycoproteins from cell surfaces may function in growth behavior of tissue cells [2] and thus be of importance in cancer cell division.

The levels in serum for protein-bound carbohydrates have been shown to be frequently elevated in patients with metastatic cancer [3—9]. Increases are not common, however, early in the course of malignant disease when the tumor is small, localized, and prior to any tissue invasion or metastases [6, 10—13]. Similar elevations of protein-bound carbohydrates have also been found in patients with active metabolic or acute inflammatory diseases [4, 6, 7, 12, 14]. In view of the latter findings, the study of these compounds for diagnostic purposes was largely abandoned. These shortcomings, nevertheless, do not eliminate the potential value of serum carbohydrates in the clinical management of patients with metastatic disease who are free of such disorders. The majority of previous investigations have involved serum fucose. Recently, studies by Rosato and co-workers [15—17], and Barlow and Dillard [18] showed that serum fucose levels could be used as an additional means of assessing the disease status in patients with breast and cervical carcinoma, respectively. Evans et al. [19] in 1974, measuring serum fucose in combination with neutral hexoses, reported that these materials had further application for determining the metastatic status of patients with cancer. This investigation included a variety of neoplastic diseases.

The methods for analysis of protein-bound carbohydrates [13—19, 20—23] in human sera generally have been non-specific colorimetric measurements. Consequently, these procedures have distinct disadvantages due to mutual

interferences by the individual carbohydrates although corrective factors can be applied to reduce this problem [19] and/or to spurious chromogens developed from protein degradation products. Utilizing the original method of Rosato [15—17] modified by Evans et al. [19], we have recently confirmed that serum fucose analysis can be useful as an aid in the clinical management of patients with advanced breast cancer [24]. In order to circumvent the methodological difficulties of the colorimetric procedures, Mrochek et al. [25] developed a high-resolution anion-exchange separation method. A sensitive cerate oxidimetric detector was coupled to the chromatographic column, and the eluted oxidizable constituents monitored by means of the fluorescence of Ce^{3+}. Serum protein-bound fucose and the neutral hexoses, mannose, and galactose were separated and measured by this means. Modification of the hydrolysis procedure prior to chromatography permitted the further analysis of sialic acid (N-acetylneuraminic acid). These methods and their preliminary application to patients with breast cancer have been reported [26]. Although providing specificity and sensitivity, this analytical technique tended to be too time-consuming for the routine analysis of a large number of samples obtained sequentially at frequent intervals. In order to develop a method that could be carried out rapidly and applied to large numbers of samples, yet retaining sensitivity and specificity, we have turned to gas—liquid chromatography (GLC).

GLC is desirable, due to its simplicity, speed, and sensitivity compared to other analytical methods. Cited in the literature are several methods for the derivatization and GLC analysis of neutral sugars [27—32], amino sugars [30, 31, 33—35], and sialic acids [36, 37]. Amino sugars and neuraminic acid (sialic acid) are most conveniently analyzed as the trimethylsilyl (TMS) ether derivative of the corresponding methyl glycosides or ketosides [34]. A disadvantage of methyl glycosides is that multiple derivatives for each component sugar are produced as a result of anomerization, thus making quantitation difficult. Serum amino sugars and neutral sugars may be determined as the alditol acetate derivatives [28—32, 35] while serum sialic acids have been determined separately as TMS derivatives [36, 37]. The advantage of the alditol acetate derivatives is that one derivative is formed for each sugar (neutral and amino), whereas the disadvantage is that the sialic acids cannot be determined as the alditol acetate derivatives [30].

Several methods have been developed for the identification and quantitation of sugars from glycoproteins [27—38], and all these methods were applied in the analysis of partially purified homogeneous glycoproteins. Our investigations showed that these alditol acetate derivatization GLC methods were not reproducible and low yields of neutral sugars were obtained when analyzing sugars from human serum protein.

Our method is a further development of the procedures used by Lehnhardt and Winzler [28]. We employed the same alditol acetate derivatization, but different hydrolysis conditions, more rigorous cleanup procedures, and improved GLC techniques in the analysis of neutral sugars from serum glycoproteins. Our GLC sample turnover rate for analysis is 20 min, and twelve samples can be processed and analyzed daily by one analyst.

The purpose of this paper is to present in detail the GLC method we have

developed, including alditol acetate derivatization, and mixed-bed ion-exchange cleanup, and to demonstrate its application to normal control subjects. Included as examples are preliminary results for patients with either small cell carcinoma of the lung or ovarian carcinoma in order to show changes from normal which may be found in patients with malignant diseases.

EXPERIMENTAL

Apparatus

A Bendix Series 2500 gas chromatograph equipped with hydrogen flame detectors was used (Bendix, Ronceverte, W.Va., U.S.A.).

This instrument was interfaced with a Hewlett-Packard 3352B Laboratory Data System with 24 K memory (Hewlett-Packard, Avondale, Pa., U.S.A.) for identification and quantitation of the neutral sugars.

Samples were analyzed on a 2.0 m × 2.0 mm I.D. borosilicate glass column packed with 2.0% (w/w) Silar-7CP on 80—100 mesh Chromosorb W AW. The injection port temperature was maintained at 220°, the detector at 250°. The column was temperature programmed from 160° to 220° at 12°/min. A carrier gas (nitrogen) flow-rate of 20 ml/min was used.

Chemicals

Alditol acetate standards were obtained from Regis (Morton Grove, Ill., U.S.A.). L-Fucose, 2-deoxyribose, 2-deoxyglucose, mannose, galactose, glucose, xylose, and arabinose were purchased from Sigma (St. Louis, Mo., U.S.A.).

Other chemicals were purchased from the following sources: chloroform, sodium hydroxide, methanol, and ethanol A.C.S. certified grade, from Fisher Scientific (St. Louis, Mo., U.S.A.); pyridine from J.T. Baker (Phillipsburg, N.J., U.S.A.); glacial acetic acid from Mallinckrodt (St. Louis, Mo., U.S.A.); acetic anhydride A.C.S. certified grade from Aldrich (Milwaukee, Wisc., U.S.A.); sodium borohydride from Sigma; anion-exchange resin AG 1-X8, 100—200 mesh (CH_3COO^-) and cation exchange resin AG 50W-X8, 100—200 mesh (H^+) from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). All-glass double-distilled deionized water (d.d. H_2O) was used for preparing aqueous solutions.

Calibration and internal standard solutions

A stock solution of neutral sugars was prepared to give concentrations of 0.400 mg/ml of L-fucose, 2.00 mg/ml of mannose, 2.00 mg/ml of galactose and 0.800 mg/ml of glucose in d.d. H_2O.

A standard solution was prepared from this stock solution by diluting 20.0 ml of stock solution to a final volume of 100 ml. Therefore, the working standard solution was composed of 80.0 μg/ml of L-fucose, 400 μg/ml of mannose, 400 μg/ml of galactose, and 160 μg/ml of glucose. Thus, 1.0 ml of the working solution contains similar levels of the protein-bound neutral sugars in 0.5 ml of normal serum. Three 1-ml aliquots of the standard working solution were derivatized and used for the calibration of the GLC system.

Single internal-standard stock solutions of 2-deoxyglucose and 2-deoxyribose were prepared to yield concentrations of 2.00 mg/ml and 4.00 mg/ml, respectively, in d.d. H_2O. The working internal standard solution was prepared by

diluting 10.0 ml of each stock solution to a final volume of 100 ml.

The stock solutions were stored at −20°, and the working solutions were held at 4° and prepared freshly every two weeks.

Representative serum samples for analytical determinations

Blood was obtained from 22 normal control male and female subjects free of any known disorders in order to establish the normal mean and range [22] (serum carbohydrate/protein), and from patients with histologically documented small cell carcinoma of the lung, and from patients with carcinoma of the ovary. The patients with cancer were included on the basis of their known disease status or response category as determined by clinical and laboratory parameters. The selection of disease status or response categories was made initially in order to show both maximal differences from normal if these occurred, and to demonstrate the subsequent changes associated with response to therapy. All patients were free of other diseases and had normal renal function. Patients with abnormal liver function were excluded unless secondary to proven liver involvement with tumor.

Samples were obtained from three categories of patients with small cell carcinoma of the lung: (1) prior to any therapy; (2) patients with measurable disease who were in partial or complete remission after treatment; and (3) patients who after treatment had recurrent disease or progressive disease. Partial remission was defined as a reduction by at least 50% of the product of the longest perpendicular diameters of the most clearly measurable mass lesion with no increase (of 25% or more) in any other indicator lesion, no new clinically apparent areas of malignant disease, and no significant deterioration in weight (<10%), symptoms or performance status. For classification as a complete response, all clinically detectable tumors had disappeared. Progressive disease included any of the following criteria: increase in any measurable lesion by more than 50%, or appearance of new areas of malignant disease, along with significant deterioration in symptoms, decrease in weight (>10%), or decrease of one level in performance status. Those patients whose measurable tumor mass disappeared following treatment and who had no evidence of disease elsewhere were considered in complete remission. Patients with carcinoma of the ovary who were in complete remission, as defined for small cell carcinoma of the lung, and those who had recurrent disease after therapy or progressive disease before or after treatment were included. All patients who fulfilled the criteria for admission to therapeutic protocol studies were considered as candidates for determination of serum protein-bound carbohydrates if qualified on the basis of the disease categories under investigation. All patients with ovarian carcinoma, prior to any treatment were staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification. Of those with progressive disease, six had been originally classified as Stage IV, fourteen as Stage III, and three as Stage II; of those in complete remission, nine had been originally Stage III, one Stage II, and Three Stage I. Anti-tumor chemotherapy was used to treat both the patients with small cell carcinoma of the lung and those with ovarian carcinoma. In general, this consisted of multiple drugs given in courses at specific intervals. All blood samples were drawn prior to therapy, and at least 14 to 28 days following the previous course of treat-

ment. After clotting occurred, serum was separated by centrifugation in a refrigerated centrifuge and aliquot samples frozen at −50° to −70° until analysis.

Preparation of ion-exchange resins

Cation-exchange resin

(1) Place the resin, about 500 g of AG 50W-X8, 100—200 mesh (H^+), in a 2000-ml Erlenmeyer flask. Wash the resin by addition of 750 ml of methanol and swirling. Discard the methanol solution and repeat the methanol wash. After discarding the methanol solution, wash the resin with 1 l of d.d. H_2O followed by discarding the aqueous solution and repeating the H_2O wash. (This step is for new resin only.)

(2) Exhaust the resin with 1 l of 2 *N* NaOH for 1 h in a water-bath, at 70°.

(3) Discard the NaOH solution, add 1 l of 2 *N* NaOH and hold for 2 h at 70°. Then discard the supernatant NaOH solution.

(4) Add 1 l of 2 *N* NaOH containing 2% EDTA in acid form and maintain for 3 h at 70°. (Note: steps 2, 3, and 4 should be carried out at 70° with gentle swirling every 15—30 min.)

(5) Discard the NaOH solution and rinse resin three times with excess d.d. H_2O. This can be done in the 2 l Erlenmeyer flask or in a 2—4 l graduated cylinder.

(6) Add to the resin 1 l of 6 *N* HCl, place in a 100° water-bath, swirl every 15 min for 1 h, discard HCl solution, and add 1 l of 6 *N* HCl for 3 h at 100°. Discard HCl solution. Add 1 l of 6 *N* HCl and hold for 12 h (overnight) at 100°.

(7) Rinse resin with d.d. H_2O until neutral.

Anion-exchange resin

(1) Same as step 1 for cation-exchange resin, except anion-exchange resin, AG 1-X8, 100—200 mesh (Cl^-), is used.

(2) Exhaust resin with 2 *N* NaOH at room temperature for 1, 2 and 3 h, as for cation-exchange resin. Then rinse four times with d.d. H_2O.

(3) Add 1 l of 2 *N* acetic acid for 1, 3, and 6 h, respectively, with gentle swirling occasionally during the regeneration. (Note: all anion-exchange steps were performed at room temperature.)

Storage of regenerated resins

Keep regenerated resins at 4°, cation-exchange resin in 2 *N* HCl, anion-exchange resin in 2 *N* acetic acid. Before use place resin in regeneration column and rinse with d.d. H_2O until neutral.

Storage of exhausted resin

Store both types of resin at 4°, the cation-exchange resin under 3 *N* HCl and the anion-exchange resin under 1 *N* HCl. Never allow resins to remain in the laboratory at room temperature for long periods of time.

Rotary evaporation system

All rotary evaporations were performed on a system assembled by our laboratory for this method. The system consists of a Model KLRC-3 Compound Liquid Ring Vacuum Pump (Kinney, Boston, Mass., U.S.A.), six CaLab rotary evaporators (CaLab, Oakland, Calif., U.S.A.), six 100-ml glass evaporator traps with vacuum-tight PTFE plugs (Kontes Glass Co., Vineland, N.J., U.S.A.), and two Model 1225 RS water heating baths (William Boekel, Philadelphia, Pa., U.S.A.).

Included in the system are six pyrex Kjeldahl connecting bulbs, 100 × 45 mm O.D. (Corning 2020), which prevent cross-contamination between samples, a vacuum gauge, and an automatic shut-down control, which allows the pump to run for 3 min after switching the pump to the off position, thus flushing out corrosive material.

Analytical procedure

Sample preparation for serum protein-bound neutral sugars

(1) Remove the serum sample and thaw in running warm water and allow to come to ambient temperature.

(2) Mix sample on a vortex mixer for 15 sec. Pipet 500 μl of the sample with a 500 μl Eppendorf pipet into a 16 × 100 mm pyrex screw-cap tube.

(3) Add 5.0 ml of 95% ethanol and mix on vortex mixer. Allow sample to stand at ambient temperature for 15 min.

(4) Centrifuge the sample for 15 min at 1500 *g* with a clinical centrifuge. Carefully decant the supernatant avoiding loss of precipitate.

(5) Invert the pyrex tube on tissue paper and allow precipitate to drain dry for 15 min at ambient temperature.

(6) Add 2.0 ml of 0.1 *N* NaOH to the precipitate. Let stand at ambient temperature with periodic vortex mixing until the precipitate is completely dissolved (usually 1—2 h).

Hydrolysis

(7) Add 2.0 ml of dry AG 50W-X8 (H^+) 100—200 mesh resin to the sample solution. Pipet 400 μl of 3.53 *N* HCl with a 200 μl Eppendorf pipet into the sample solution. Solution is 0.50 *N*.

(8) Tightly cap the tube with a PTFE-lined screw-cap and mix sample on a vortex mixer for 15 sec.

(9) Hydrolyze the sample for 7 h in a heating block at 100±1° (Scientific Instruments Shop, University of Missouri, Columbia, Mo., U.S.A.). Vortex sample for 15 sec every 30 min.

Cleanup of hydrolysate

(10) Allow hydrolysate to come to ambient temperature. Quantitatively transfer the hydrolysate onto a column (30.0 × 1.0 cm I.D. pyrex glass fitted with PTFE stopcock) packed with 15.0 ml of AG 50W-X8 (H^+) 100—200 mesh resin, which is in series with another glass column packed with 10.0 ml of AG 1-X8 (CH_3OO^-) 100—200 mesh resin.

(11) Wash column series with 35 ml of 50% (v/v) methanol—H_2O and collect

effluent in a 125 ml $ 24/40 pyrex-glass flat-bottomed rotary evaporator flask (Corning 4100). Allow solutions to drain by gravity flow. The flow-rate was about 1 ml/min.

Derivatization

(12) Pipet 1.00 ml of 2-deoxyribose solution, 200 μg/ml, (used as an internal standard to monitor the recovery) into the rotary evaporator flask containing the effluent. Take contents of flask to dryness on rotary evaporator (water-bath at 60°).

(13) Pipet 1.00 ml of 2-deoxyglucose, 200 μg/ml (internal standard) into the flask. Add 1.0 ml of d.d. H_2O and mix flask contents well by swirling to contact walls.

(14) Reduce the sugars by adding 2.0 ml of 0.22 *M* $NaBH_4$ and mix by swirling. Allow to stand for 30 min at ambient temperature [39].

(15) Add 1.0 ml of glacial acetic acid and evaporate to dryness on rotary evaporator.

(16) Remove borate by adding 2.0 ml of HCl—methanol (1:1000, v/v) to the dry flask, swirling, and taking to dryness by rotary evaporation. Repeat this step two times.

(17) Acetylate the sample by adding 1.0 ml of acetic anhydride and 1.0 ml of pyridine. With a glass stopper on the flask, mix by swirling. Heat flask in a 100° oil-bath for 30 min.

(18) Remove flask from oil-bath and transfer contents with a disposable pipet into a disposable pipet fitted with a glass-wool plug (1/4") in the constricted tip end and collect the sample in a 16 × 75 mm pyrex tube.

(19) Evaporate the sample just to dryness under a gentle stream of dry nitrogen while in a heating block at 60°.

(20) Redissolve the sample by pipetting 1.00 ml of $CHCl_3$ with a 1000 μl Eppendorf pipet into the tube. Cap the tube with a PTFE-lined screw-cap and mix sample on a vortex mixer for 15 sec.

(21) Inject 5.0 μl into the gas—liquid chromatograph for analysis.

Protein analyses

The procedure used for protein determination was a semi-automated Technicon System for nitrogen determination described by Wall and Gehrke [40].

RESULTS AND DISCUSSION

GLC of alditol acetates

Fig. 1 shows the separation of six alditol acetates achieved in 12 min on a glass column packed with 2.0% (w/w) Silar-7CP on Chromosorb W AW, 80—100 mesh. An internal standard, 2-deoxyglucose, was included for accurate quantitation of the neutral sugars.

Precision of GLC analysis with standards

Repeated injections of 5.0 μl of a standard solution (0.4—2.0 μg each) of six neutral sugars gave relative standard deviations of 0.3—1.9% (Table I). These data show excellent precision of the GLC analysis for standards over the range 80—400 μg of sugar per ml.

TABLE I

PRECISION OF GLC ANALYSIS OF NEUTRAL SUGAR STANDARDS USING AN INTERNAL STANDARD METHOD

	Sugar (μg/ml)*					
	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
$\bar{x}$	80	82	81	396	405	186
σ	1.5	0.6	0.6	1.2	2.1	0.6
R.S.D. (%)	1.9	0.7	0.7	0.3	0.5	0.3

*Reference standard analyzed independently three times.

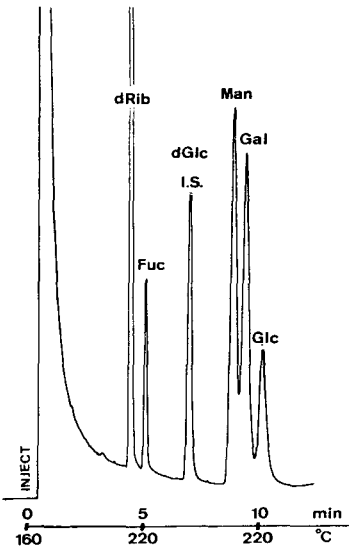


Fig. 1. GLC separation of alditol acetates of neutral sugars. Sample: 5 μl (≈1 μg) each. Program: from 160° at 12°/min to 220°. Attenuation: 1 × 10^{-10} A.f.s. Column: 2.0% (w/w) Silar-7CP on Chromosorb W AW, 80—100 mesh, 2 m × 2 mm glass.

Calculation of sugar concentration

Quantitation of the neutral sugars was based upon an internal standard method using 2-deoxyglucose as internal standard (I.S.). The relative weight response (RWR) values compared to 2-deoxyglucose were as follows: $RWR_{Fuc/I.S.}$ = 1.01; $RWR_{Gal/I.S.}$ = 0.91; $RWR_{Man/I.S.}$ = 0.96; $RWR_{Glc/I.S.}$ = 1.11. The RWR values for the four neutral sugars were determined by at least three independent analyses of calibration standards, and remained constant over a three-month period.

Hydrolysis of serum proteins

Hydrolysis with 1.0 *N* HCl at 100° for 10 h was compared to hydrolysis with 1.0 *N* HCl plus 2.0 g AG 50W-X8 (H^+) resin at 100° for 10 h. Both studies were performed in triplicate.

2-Deoxyribose was added as an internal standard after ion-exchange cleanup to each sample and its percentage recovery was monitored. In general, higher values for the neutral sugars mannose and galactose were obtained when hydrolyzing with AG 50W-X8 (H^+) resin than without. Better precision, lower R.S.D. (%) values, and improved recoveries for 2-deoxyribose (mean 94%) and higher recovery values for the neutral sugars were also obtained when hydrolyzing with the aid of AG 50W-X8 (H^+) resin (Table II).

The use of AG 50W-X8 (H^+) in hydrolysis resulted in higher yields with less neutral-sugar destruction, and removed chromatographic interferences which resulted in better precision. Thus, hydrolysis results with this resin gave better precision and neutral sugar recoveries than hydrolysis without it.

The hydrolysis of ethanol precipitates of serum proteins was investigated to determine the best conditions for maximum glycosidic bond cleavage and minimal destruction of the neutral sugars. Aliquots of a pooled control serum were used for this study. Different acid concentrations and times of hydrolysis at 100° were investigated. Samples were hydrolyzed with 1.0 *N* HCl for 2—14 h with 0.5 *N* HCl for 4—15 h, and with 0.05 *N* HCl for 15—60 h. Except for these different hydrolysis conditions, the procedure followed was identical to the analytical procedure previously described.

The results of hydrolysis (Fig. 2) show that the release of fucose and galactose preceded the release of mannose at each hydrolysis acid concentration. A slight loss of fucose can be observed with each acid concentration at longer hydrolysis times. The 0.5 *N* HCl condition gives as high or higher values for all three sugars than does the 0.05 or 1.0 *N* HCl. Therefore, for convenience samples were hydrolyzed for 7 h and routine analyses used hydrolysis with 0.5 *N* HCl and 2.0 g AG 50W-X8 (H^+) 100—200 mesh at 100° for 7 h.

Method linearity

Duplicate analyses of 0.10—58.0 mg ovomucoid (egg white; Sigma) hydrolysates were performed to establish the working range of the method. A linear relationship for mannose and galactose was obtained (Fig. 3). Precision between duplicates was quite good, as shown by the range given on the graph.

TABLE II

COMPARISON OF HYDROLYSIS WITH CATION-EXCHANGE RESIN AND 1.0 *N* HCl

A and B were different pooled control sera.

10 h at 100° with 2.0 g AG 50W-X8 (H^+) 100—200 mesh and 1.0 *N* HCl					10 h at 100° with 1.0 *N* HCl				
Serum A	Neutral sugars (μg/ml)				Serum B	Neutral sugars (μg/ml)			
	dRib	Fuc	Man	Gal		dRib	Fuc	Man	Gal
x	748	38.3	365	317		615	43.5	352	310
σ	23	2.1	2.9	3.5		86	2.9	18	19
R.S.D. (%)	3.2	5.5	0.8	1.1		14	6.7	5.2	6.2
Recovery of spike (Mean %)	94	80	89	90		77	82	76	79

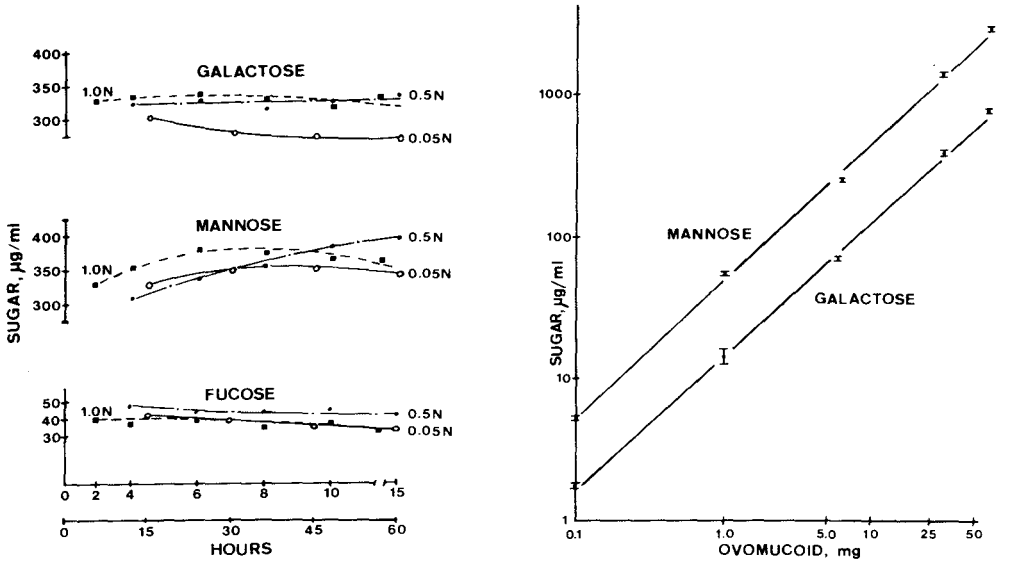


Fig. 2. Hydrolysis of serum protein with 2.0 g of AG 50W-X8 (H^+) at 100°.

Fig. 3. Linearity of GLC method.

Precision of analysis for neutral sugars in serum — Matrix-independent and matrix-dependent

Neutral sugars were determined using 0.5 ml serum of individual samples (matrix-dependent) and pooled control serum (matrix-independent). A chromatogram for a matrix-dependent analysis is shown in Fig. 4.

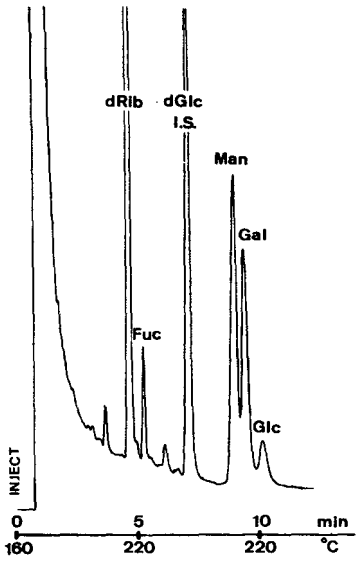
The matrix-independent and matrix-dependent precisions for the GLC analysis of three neutral sugars in serum protein are given in Table III. The data for the matrix-independent samples were obtained by analyzing the same sample of pooled control serum independently 16 times, whereas the data for the matrix-dependent samples were obtained from 30 different serum samples each analyzed independently twice over three months. Excellent precision of analysis was achieved.

Recovery of neutral sugars from serum

Recoveries of neutral sugars were determined by spiking serum samples with standards. Chromatograms for non-spiked and spiked serum samples are shown in Figs. 5 and 6, respectively. The recoveries obtained from 30 different serum samples are given in Table IV.

Precision and recovery of protein analyses from serum of cancer patients

The matrix-dependent precision and recovery data for the protein analyses are given in Tables V and VI. The precision values for the matrix-dependent



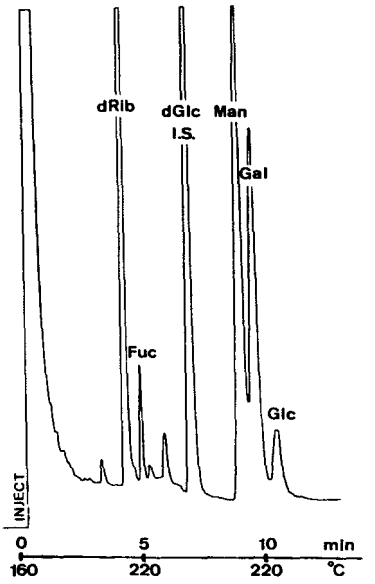


Fig. 4. GLC of neutral sugars in serum protein from breast cancer patient. Conditions as in Fig. 1.

Fig. 5. GLC of neutral sugars in serum protein from breast cancer patient. Conditions as in Fig. 1.

TABLE III

PRECISION OF GLC ANALYSIS OF SERUM PROTEIN-BOUND NEUTRAL SUGARS

	Sugar (μg/ml)		
	Fucose	Mannose	Galactose
Matrix-independent (n = 16)			
$\bar{x}$	42.9	374	340
σ	2.73	13.0	11.7
R.S.D. (%)	6.4	3.5	3.4
Matrix-dependent (n = 30)*			
$\bar{x}$	68	577	551
σ**	2.5	16.5	11.6
R.S.D. (%)	3.6	2.9	2.1

*Precision for different serum samples over three months.

**$\sigma = \sqrt{\frac{\Sigma(x_2 - x_1)^2}{2P}}$ where P is the number of pairs (P = 30); each pair was analyzed on the same day.

TABLE IV

RECOVERY OF ADDED NEUTRAL SUGARS FROM CANCER SERUM

Recoveries were from different serum samples over three months (n = 30). Added at the following levels of μg per 0.5 ml serum were fucose 80, mannose 400, and galactose 400.

	Recovery (%)		
	Fucose	Mannose	Galactose
$\bar{x}$	86	85	91
σ	6.0	3.9	3.5
R.S.D. (%)	6.9	4.6	3.9

TABLE V

PRECISION OF PROTEIN ANALYSES FROM CANCER SERUM

Precision is for different serum samples over three months.

Matrix-dependent	Protein (mg/ml serum)
n = 38	
$\bar{x}$	73
$\sigma^{\star}$	1.4
R.S.D. (%)	1.9

$^{\star}\sigma = \sqrt{\frac{\Sigma(x_1 - x_2)^2}{2P}}$ where P = 38.

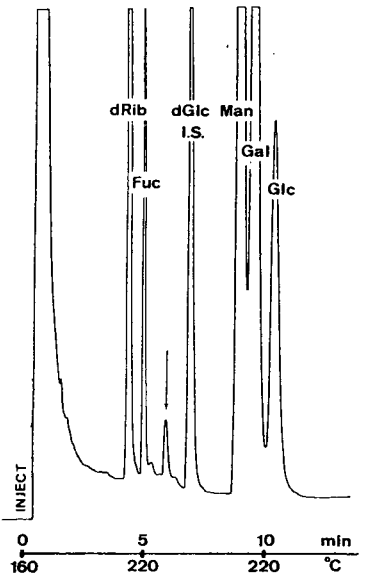


Fig. 6. GLC of neutral sugars in serum protein from breast cancer patient spiked with neutral sugars. Conditions as in Fig. 1. Spiked sugars (μg added per ml serum): Fuc, 160; Man, 800; Gal, 800; Glc, 320.

TABLE VI

RECOVERY OF BOVINE SERUM ALBUMIN ADDED TO CANCER SERUM PROTEIN

Recovery is calculated from different serum samples over a period of three months. Ten mg of bovine serum albumin were added to each serum sample.

	Recovery (%)
$n = 34$	
$\bar{x}$	101
σ	3.54
R.S.D. (%)	3.5

samples were obtained from 38 different serum samples analyzed independently twice over three months, whereas the data for the recoveries for matrix-dependent samples were obtained by analyzing 34 spiked and non-spiked individual serum samples. Excellent precision and recoveries were obtained.

Effect of storage temperature on the serum protein-bound sugar levels

The influence of serum storage temperature on the serum protein-bound sugar levels was investigated. The serum was from a freshly prepared pooled normal sample from our laboratory personnel. Fresh whole blood was taken from two of the Experiment Station Chemical Laboratories' staff. The blood was transferred to 30 ml Corex centrifuge tubes and allowed to clot for 15 min. The blood was centrifuged at 5000 *g* for 15 min, then the serum was decanted into a 125 ml Erlenmeyer flask. With continuous stirring, 0.5-ml aliquots of serum were pipetted into 13 × 75 mm culture tubes. The serum was divided and aliquots were stored at room temperature, 4° (refrigerator temperature), -20°, and -70°.

Three aliquots of the freshly prepared serum were analyzed to establish the original values for the sugars. The stored serum samples were analyzed in triplicate at the following specified storage times and temperatures: room temperature, 2, 4, 8, and 24h; +4°, 1, 3, 7, and 28 days; -20°, 1, 3, 7, and 28 days; —70°, 1, 3, 7, and 28 days.

The serum-bound sugar levels in serum stored at room temperature for up to 24 h and at +4°, —20°, and —70° for up to 28 days were found to have essentially the same levels as freshly prepared serum.

Effects of successive freezing and thawing on the total protein and protein-bound sugar levels in serum

A pooled control serum sample was used. The frozen sample was thawed at room temperature, and aliquots were taken for the determination of sugars and protein. The serum sample was then frozen again for 24 h and the same process was repeated. For each of the four freezing and thawing cycles independent duplicate analyses were made for total protein and sugars. The average values are presented in Table VII. The data indicate no change in the levels of fucose, mannose, galactose and total serum protein after repeated freezing and thawing. This information is helpful in knowing the integrity of the sample during storage.

TABLE VII

EFFECT OF FREEZING AND THAWING ON STABILITY OF PROTEIN-BOUND NEUTRAL SUGARS AND SERUM PROTEIN

Freezing and thawing cycle	Sugar (μg/ml)			Protein (mg/ml)
	Fucose	Mannose	Galactose	
1	41.9	361	339	60
2	40.5	340	323	59
3	42.4	361	337	61
4	40.0	356	325	61
$\bar{x}$	41.2	355	331	60
σ	1.1	10	8.2	0.96
R.S.D. (%)	2.8	2.8	2.5	1.6

Analysis for free and bound neutral sugars

Investigations were then made on the levels and nature of the covalently bound neutral sugars in serum glycoproteins, to verify and differentiate the free and covalently bound sugars. Also, experiments were made on lipid bound neutral sugars in the ethanol precipitates of the proteins.

Precipitation of glycoprotein with ethanol

The protein in duplicate aliquots of a control pooled serum sample was precipitated with ethanol, analyzed, and additional aliquots were precipitated with ethanol and the precipitates washed once with ethanol. Other precipitates were washed with ethanol two and four times, then analyzed. The results are given in Table VIII.

The data show that fucose, mannose, and galactose are protein-bound. The level of these neutral sugars in the protein does not significantly change after four washings with ethanol; a decrease of about 10% was noted for mannose and galactose. However, the level of glucose does change significantly after two washings (>50%). Most of the glucose can be removed by washing with ethanol and most of it is present as the free neutral sugar. Thus, during precipitation of glycoprotein with ethanol, glucose coprecipitates or is occluded.

Dialysis of neutral sugars from serum

A pooled control serum sample was used (different from that used for the ethanol-washing experiment). The serum was dialyzed using seamless cellulose tubing with an average pore size of 24Å. The cellulose tubing was permeable to water and compounds of low molecular weight, but not to proteins. Serum (15.0 ml) was pipetted into a dialysis tube (1 in. flat width × 8 in.), tied at one end and dialyzed at 4° against 1.0 l of d.d. H_2O for over 48 h. The dialysis water was replaced with fresh d.d. H_2O after 2, 4, 6, 8, 24 and 48 h. Then, aliquots of serum were taken from the dialysis tube after each designated time period, precipitated with ethanol, and analyzed for protein-bound neutral sugars by GLC (Table IX).

TABLE VIII

PRECIPITATION OF SERUM PROTEINS WITH ETHANOL: EFFECT OF MULTIPLE ETHANOL WASHINGS

Five ml of ethanol were added to 0.5 ml of serum, shaken vigorously, allowed to stand 15 min, then centrifuged at 1500 *g* for 30 min. Each value is the average of two independent analyses on a pooled control serum.

No. of ethanol washings	Neutral sugars (μg/ml)			
	Fucose	Mannose	Galactose	Glucose
1	44.0	265	294	111
2	47.9	266	272	56.3
4	48.5	237	264	44.7

Dialysis of isolated serum glycoprotein for neutral sugars

The glycoprotein was dialyzed in seamless cellulose tubing with an average pore size of 24 Å. A pooled serum aliquot (0.5 ml) was precipitated with ethanol, then suspended in 2.0 ml of d.d. H_2O with sonication, and dialyzed (dialysis tube: 1 in. flat width × 8 in., tied at one end) at 4° against 1.0 l of d.d. H_2O over 24 h with occasional agitation. The dialysis water was replaced with fresh d.d. H_2O after 2, 4, 6 and 8 h. Then the dialysate containing the protein was transferred to a Virtis 150-ml flask and lyophilized to near dryness. The lyophilized sample solution was transferred into a 16 × 100 mm culture tube, 2.0 ml of 0.1 *N* NaOH were added, and the dialyzed protein was analyzed for neutral sugars by GLC.

The results of this experiment are given in Table IX together with data from experiments for neutral sugars in the ethanol-washed precipitates, and dialyzed serum. The data from multiple ethanol washings of the glycoprotein, dialysis of pooled serum, and dialysis of isolated glycoprotein are all comparable; that

TABLE IX

NEUTRAL SUGARS IN SERUM PROTEIN

Each control a different pooled serum; 0 = control.

Sugar	Ethanol-precipitated serum proteins washed with ethanol		Dialysis of neutral sugars from serum		Dialysis of isolated serum glycoproteins	
	0 Washings	4 Washings	0 h	24 h	0 h	24 h
Fucose (μg/ml)	44.0	48.5	42.9	43.3	45.8	42.8
Mannose (μg/ml)	265	237	374	378	345	335
Galactose (μg/ml)	294	264	340	340	330	332
Glucose (μg/ml)	111	44.7	257	55.0	263	76.0

is, all of the fucose, mannose, galactose, and some of the glucose, in ethanol precipitates of serum are covalently bound to serum proteins.

The levels of fucose, mannose, and galactose were not changed on dialysis of serum or serum proteins, but the level of glucose changed significantly, and so confirmed that about 25% of the glucose in serum is covalently bound in serum glycoprotein.

Lipid-bound neutral sugars

Ethanol precipitates of pooled control serum were dissolved in 0.1 *N* NaOH and extracted for lipids according to the Folch method [41] with chloroform—methanol (2:1, v/v). The lipid extract was analyzed for covalently bound neutral sugars, but none were found above the background level. Thus, the neutral sugars are not present in serum in the form of glycolipids.

Analysis of alditol acetates by mass spectrometry

The mass spectra of high purity alditol acetates of 2-deoxyribose, L-fucose, arabinose, xylose, 2-deoxyglucose, mannose, galactose, and glucose were obtained by GLC—electron-impact mass spectrometry (MS). Alditol acetates of sugar standards and sugars from glycoproteins of serum from cancer patients were prepared according to the previously described analytical method. The mass spectral fragmentation pattern of the derivatized standards and samples from cancer patients were compared with those of commercially available (Regis) high purity neutral sugar alditol acetates. Comparison of these sets of spectra based on spectra published by Lonngren and Svensson [42] showed very good agreement, thus confirming the identity of the peaks of interest and their homogeneity. A small unknown peak in glycoprotein from cancer samples (indicated by an arrow in Fig. 6) was analyzed by GLC—MS and the elucidation of its structure was attempted. The unknown compound showed deviations from the characteristic fragmentation pattern of alditol acetates [42] with the largest detectable fragment at *m/e* 331. High-resolution GLC—MS of the *m/e* 331 ion indicates a nitrogen-containing compound with the formula $C_{17}H_{17}NO_6$.

Normal levels of protein-bound neutral sugars in sera

The protein and neutral sugar levels for normal non-fasting males, normal non-fasting females, and normal fasting females were determined (Table X). The $\bar{x}$, σ, and R.S.D. (%) values for a population of n are also presented in the table. On comparing a group of normals analyzed by Mrochek's high-performance liquid chromatographic (HPLC) method [25] with data for our group of normals by GLC (Table XI), no significant differences were observed. The GLC method gave slightly higher values for all the neutral sugars. This may be attributed to the difference in hydrolysis conditions.

Sugar:protein ratios in serum from ovarian and small cell lung carcinoma

In Fig. 7 are given the serum fucose:protein ratios for patients with small cell carcinoma of the lung. Each patient is distributed in one of three disease or response categories: (1) pre-treatment, progressive disease; (2) complete or partial response following chemotherapy; and (3) post-treatment, recurrent, or

TABLE X

NORMAL LEVELS OF PROTEIN-BOUND NEUTRAL SUGARS IN SERA

The R.S.D. (%) values are for the respective population.

	Protein (mg/ml)	Sugar (μg/mg of protein)		
		Fucose	Mannose	Galactose
Non-fasting males (n = 11)				
$\bar{x}$	76	0.71	6.17	5.64
σ	6.8	0.07	0.56	0.63
R.S.D. (%)	8.9	10	9.1	11
Non-fasting females (n = 11)				
$\bar{x}$	72	0.73	7.1	6.6
σ	2.6	0.08	0.76	0.81
R.S.D. (%)	3.6	11	11	12
Fasting females (n = 9)				
$\bar{x}$	73	0.75	7.0	6.5
σ	3.4	0.18	0.73	0.67
R.S.D. (%)	4.6	24	10	10

TABLE XI

COMPARISON OF DATA BY HPLC AND GLC FOR PROTEIN-BOUND NEUTRAL SUGARS

The R.S.D. (%) values are for a population of n.

	Sugar (μg/ml of serum)*					
	Fucose		Mannose		Galactose	
	HPLC**	GLC***	HPLC	GLC	HPLC	GLC
Non-fasting females						
n	12	11	12	11	12	11
$\bar{x}$	48	52	444	511	436	477
R.S.D. (%)	25.7	9.9	17.6	9.4	19.5	11.2
Fasting females						
n	4	9	4	9	4	9
$\bar{x}$	40	55	392	514	414	473
R.S.D. (%)	20.2	25.0	16.2	10.9	15.7	10.2

*Each value is an average for n in a population.
**Determined by Mrochek's HPLC Method [25].
***Determined by our GLC method.

progressive disease. As shown, the majority of patients with progressive disease whether before or after treatment had elevated values. In contrast, the majority (13/16) of those patients in complete or partial remission had fucose:protein ratios within the normal range. Three patients had somewhat elevated values. Whether at the time blood was drawn these patients had occult disease not detected by the usual clinical means is not known. Similar results were found for patients with ovarian carcinoma. Both fucose:protein and galactose:protein

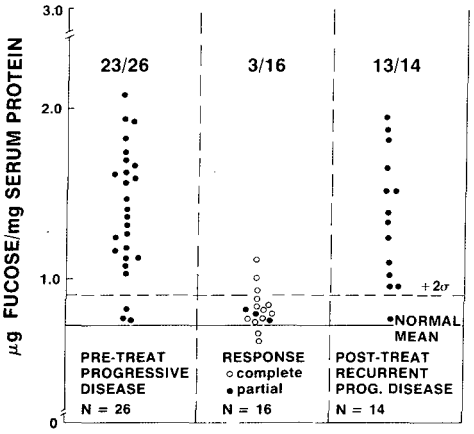


Fig. 7. Fucose levels in small cell carcinoma of lung.

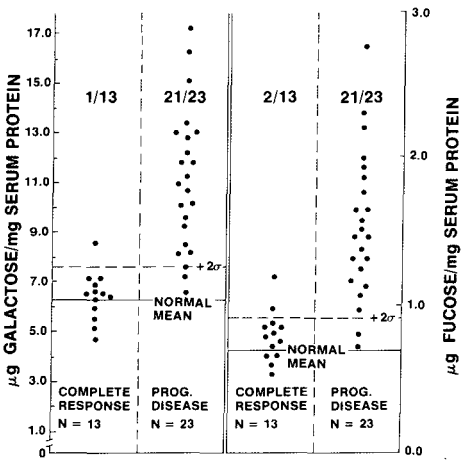


Fig. 8. Galactose and fucose levels in ovarian carcinoma.

values are plotted in Fig. 8 for 23 patients with progressive disease and 13 patients considered to be in complete clinical remission.

In Table XII the results for galactose:protein ratios are compared for serum and malignant effusions, ascites and/or pleural fluid, obtained from five patients with progressive ovarian cancer. As noted, the serum, ascites, and pleural fluid tend to parallel the value for the serum for each respective patient.

In our previous study of serum fucose:protein ratios involving patients with breast cancer [24] we confirmed that the ratio was elevated in acute metabolic and inflammatory diseases other than cancer, and demonstrated that (a) diet (i.e. fasting or non-fasting) had no effect on the ratio; (b) the ratio was elevated in close to 90% of the patients with metastatic breast disease; and (c) the ratio paralleled changes in tumor mass, or progression or regression after therapy.

TABLE XII

GALACTOSE:PROTEIN RATIO IN SERUM AND MALIGNANT EFFUSION FOR PATIENTS WITH OVARIAN CARCINOMA

Patient	μg Galactose per mg protein		
	Serum*	Ascites effusion	Pleural effusion
A	15.8	16.7	16.6
B	10.7	10.6	—
C	11.5	—	10.8
D	12.2	12.4	—
E	17.0	14.0	—

* $\bar{x}$ for normal females = 6.2 (range 4.8—7.6).

Studies to date indicate that in general both galactose:protein and mannose:protein ratios are lower and less frequently elevated for patients with either breast cancer or small cell carcinoma of the lung, in contrast to the fucose:protein ratios. However, for patients with ovarian cancer whose disease is progressing, in general we have found that both galactose:protein and mannose:protein ratios also were elevated if the fucose:protein ratios were raised.

Small cell carcinoma of the lung spreads to other organs or tissues early in the course of disease, with frequent involvement of liver, bone marrow and brain. However, this carcinoma responds rapidly to chemotherapy and remission rates of 50—70% have been reported. Nevertheless, the carcinoma is prone to recur and the possibility of occult disease even when the patient is considered in complete remission may be great. Carcinoma of the ovary tends to remain confined to the abdominal cavity without distant metastases. Patients, however, are most frequently present initially with advanced disease (stage III and IV), and it is very difficult to assess disease status early in its course or to determine if residual tumor remains after treatment.

It is evident that additional diagnostic information is needed in the clinical management of patients with small cell carcinoma of the lung and ovarian cancer. These preliminary results show the great potential of the fucose, mannose, and galactose:protein ratios in following the course of the disease.

Comments on the method

(1) The optimum times for complete reduction and acetylation of the neutral sugars were investigated and confirmed with literature conditions [13, 27, 35].

(2) 2-Deoxyglucose was added as internal standard for calculating the neutral sugar concentrations.

(3) 2-Deoxyribose was added to monitor the effectiveness of sample cleanup, and losses on evaporation of acetylating reagents.

(4) A strong cation exchange resin, AG 50W-X8 (H^+), was used to remove substances that interfered in derivatization.

(5) A strong anion-exchange resin, AG 1-X8 (CH_3COO^-), removed chromatographic interferences.

(6) To assure accuracy and precision of this method, thoroughly mixed samples for homogeneity and addition of exact amounts of internal standard are mandatory.

(7) A pooled normal control sample with pre-determined neutral sugar levels was prepared and analyzed with each group of samples (about 20) to establish and monitor the performance of day-to-day analyses.

(8) The temperature for drying samples on the rotary evaporator and in heating blocks should not exceed 60°. After samples are taken to dryness in the heating block they should be removed immediately. Prolonged heating causes some loss of the neutral sugars.

(9) Each day the instrument settings, RWRs, and performance are checked and compared by injecting a standard solution.

(10) In our laboratory the Silar-7CP column has been used for more than 400 analyses without loss of separation efficiency.

CONCLUSIONS

The potential of the neutral sugar:protein ratios (fucose, mannose, and galactose) as markers for clinical cancer management has been demonstrated. Initial results for small cell lung and ovarian carcinoma showed that more than 88% of the patients with progressive disease had elevated fucose:protein values. In contrast, following chemotherapy more than 81% of those patients in complete or partial remission of disease had fucose:protein ratios within the normal range. In post-treatment small cell lung cancer patients with recurrent progressive disease more than 93% had elevated fucose:protein values. Thus, our studies to date indicate that the fucose:protein ratio is a good index for following changes in tumor burden in small cell lung and ovarian carcinoma. In general both the galactose:protein and mannose:protein ratios are lower and less-frequently elevated for patients with either breast cancer or small cell carcinoma of the lung, in contrast to the fucose:protein ratio. However, for following the progression and remission of ovarian carcinoma the sugar:protein ratios for fucose, mannose, and galactose serve as reliable markers. This is extremely helpful to the physician in following response to chemotherapy; as for patients with ovarian carcinoma it is very difficult to know the disease status early in its course or to determine if residual tumor remains after treatment.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. James Hueser, Medical Oncologist, for obtaining the normal female control samples through the local Chapter of the American Cancer Society. Special thanks are extended to Linda Friedrich and Debi Whisenand for their expert technical assistance.

Sincere appreciation is also expressed to the professional staff and graduate students of the Experiment Station Chemical Laboratories for their help and encouragement.

The work was supported in part by a contract from the National Cancer Institute, Bethesda, Md., U.S.A. (Contract No. NIH NO1 CM 12323).

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Journal of Chromatography, 162 (1979) 529—537
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DETERMINATION OF PLASMA TESTOSTERONE BY MASS FRAGMENTOGRAPHY USING TESTOSTERONE-19-d_3 AS AN INTERNAL STANDARD

COMPARISON WITH RADIOIMMUNOASSAY

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(Received August 28th, 1978)

SUMMARY

Analytical procedures for the measurement of testosterone by mass fragmentography (MF) using trideuterated testosterone (testosterone-19,19,19-d_3) are described. For the calculation of plasma testosterone, peak height ratios were measured by MF performed on the molecular ions of the TFA derivative of testosterone (*m/e* 480) and testosterone-19, 19,19-d_3 (*m/e* 483). The sensitivity of the method was judged from the lower limit of detection of the mass spectrometer which was at 10 pg. For the measurement of the precision, the inter- and intra-assay coefficients of variation (C.V.) were calculated by using a pooled plasma sample; they were 3.15% and 1.79%, respectively. The specificity was investigated by the use of 5α-dihydrotestosterone and the MF method was found to afford a highly selective technique. These results obtained by MF have been compared with the results obtained by a radioimmunoassay method.

INTRODUCTION

The assay of steroid hormones in body tissues or fluids has been greatly simplified by the introduction of analytical methods involving radioimmunoassay (RIA) [1—5], enzyme immunoassay [6—8], and the competitive protein binding technique [9—11]. There are, however, often serious restrictions to the use of these methods in that the preparation of the appropriate antisera or binding proteins is relatively complicated. Cross-reactivity with other steroids is also a serious problem in achieving acceptable specificity [12—14]. Moreover, these methods have the inherent drawback of the non-tracer technique in that the correction for losses of a particular substance in various biological samples in the extraction and purification procedure can not be easily made.

The usefulness of mass fragmentography (MF) in which gas chromatography—mass spectrometry with a multiple ion detector (GC—MS—MID) is

used in conjunction with stable isotope labeling has recently been of great interest in the measurement of trace amounts of substance in biological materials because of its high sensitivity and high selectivity [15—18]. In this technique stable isotope (SI)-labeled carriers serve as the ideal internal standard to correct for losses of a substance under study in the initial isolation procedures. This MF technique provides a sensitive, specific, and reliable method for biological assays.

The MF technique reported in this paper was developed from our need for a simple, reliable and rapid method for the determination of plasma testosterone levels in a biological sample. As the internal standard, we used testosterone-19,19,19-d_3 (testosterone-19-d_3), synthesized in our laboratory [19]. We compared the MF technique for the determination of male plasma testosterone with a radioimmunoassay method used in routine work.

MATERIALS AND METHODS

Chemicals

Testosterone-19,19,19-d_3 (testosterone-19-d_3) was synthesized in our laboratory as described previously [19]. The isotopic composition was 99.0 atom% deuterium (d_3, 97.8%; d_2, 2.2%; d_1, 0.0%). Non-labeled testosterone was purchased from Tokyo Kasei Kogyo, Tokyo, Japan (reagent grade) and was recrystallized from *n*-hexane—acetone before use.

Mass fragmentography

MF measurements were made with a Shimadzu LKB-9000B gas chromatograph—mass spectrometer equipped with a Shimadzu high-speed multiple ion detector—peak matcher 9060S. The electron energy was set to 20 eV and the trap current to 60 μA. The MID was focused on the ions at *m/e* 480 and *m/e* 483 and the peak height ratio was determined. GC was performed on a glass column (2 m × 3 mm I.D.) packed with 1.5% OV-1 on Shimalate W (80—100 mesh). The column temperature was 230° and the temperature of the flash heater and the separator was 250°. The temperature of the ion source was 270°. Helium carrier gas flow-rate was about 30 ml/min. The recordings were made on a Shimadzu two-pen recorder R-12M, the chart speed being 10 mm/min.

Preparation of calibration curve

To each of four standards containing 2—20 ng of testosterone in 20 μl of ethanol, 10 ng of testosterone-19-d_3 dissolved in 20 μl of ethanol were added. After evaporation of the solvent, each standard was dried in an evacuated desiccator. To each sample was added 200 μl of trifluoroacetic anhydride (reagent grade: Wako, Osaka, Japan). After standing for 30 min at room temperature, excess trifluoroacetic anhydride was removed under a stream of nitrogen and the residue was dissolved in 50 μl of *n*-hexane. A 2—5 μl volume of the above *n*-hexane solution was analyzed by GC—MS.

in 20 μl ethanol and the plasma was allowed to stand for 30 min at room temperature. After adding 40 μl of 3 *N* NaOH, the plasma sample was extracted with 3 × 3 ml of diethyl ether. The diethyl ether phase was collected, washed with 1 ml of 5% acetic acid and then with 1 ml of water, and dried over anhydrous sodium sulphate. After evaporating the solvent, the residue of the diethyl ether extract was dissolved in 5 ml of 70% aqueous methanol and stored at −15° for 1 h. After centrifugation to remove plasma lipids, the upper layer was decanted and evaporated to dryness. The residue was then dissolved in chloroform and subjected to thin-layer chromatography (TLC) on Kieselgel $60F_{254}$ plates (0.25 mm thickness; Merck, Darmstadt, G.F.R.). The TLC plate was developed with chloroform—ethyl acetate (4:1, v/v) and the UV positive zone corresponding to standard testosterone with an R_F value of 0.3 was scraped off. Testosterone was eluted with 5 ml of chloroform and 5 ml of ethyl acetate in turn and the organic solvents were evaporated. The residue was dried in an evacuated desiccator. The trifluoroacetate (TFA) derivative was formed by reacting the residue with trifluoroacetic anhydride as described above and 2—5 μl of the sample was subjected to GC—MS.

Determination of accuracy

Testosterone in amounts of 1.93, 4.83 and 9.66 ng dissolved in 20 μl of ethanol was added to 1.0 ml aliquots of pooled male plasma. The testosterone content of the pooled plasma was 5.86 ± 0.02 (S.D.) ng/ml in triplicate determinations. After preparation of the sample for MF as described above, the observed peak height ratio of *m/e* 480 to *m/e* 483 was determined in triplicate.

Radioimmunoassay

Plasma testosterone was determined by an RIA method using a commercial kit from Cea Ire Sorin (Gif-Sur-Yvette, France). For the extraction of testosterone from plasma samples, 0.05 ml of plasma was used for each sample preparation.

RESULTS AND DISCUSSION

The availability of MF and of testosterone-19-d_3 synthesized in our laboratory enabled us to develop a method for measuring human plasma testosterone.

Considerations for MF analysis

Preparation of TFA derivative. Fig. 1 shows the mass spectra of testosterone-19-d_3 and testosterone-19-d_3-diTFA. In the mass spectrum of testosterone-19-d_3 (Fig. 1A) the relative abundance of the molecular ion (*m/e* 291) was about 6.5%. In the mass spectrum of testosterone-19-d_3-diTFA (Fig. 1B), on the other hand, the relative abundance of the molecular ion (*m/e* 483) was as high as 33%. Therefore it would be an advantage to make the TFA derivative for the MF analysis to obtain higher sensitivity. The preparation of testosterone-diTFA requires only a simple derivatization step. There was only a single peak derived from the TFA derivative and no other peaks were seen in the gas chromatogram. Furthermore as pointed out by Vestergaard, et al. [20], the specificity of the assay for testosterone was such that TFA deriva-

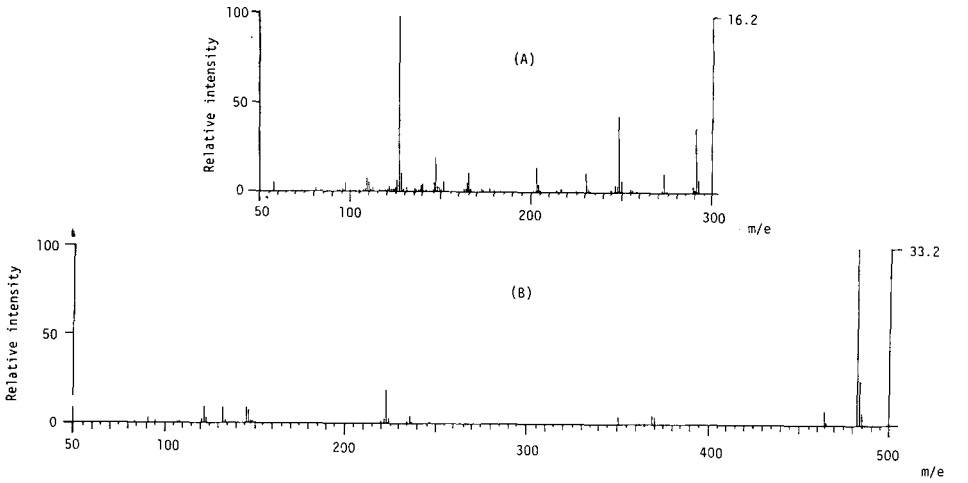

Fig. 1. Mass spectra of: (A) testosterone-19-d_3; (B) diTFA derivative of testosterone-19-d_3.

tives of possible interfering steroids such as 17-oxo steroids, dihydrotestosterone, and epitestosterone did not interfere in the assay under the conditions of the present assay.

For the quantitative analysis of testosterone in plasma, the MF analysis was performed using the TFA derivatives by measuring the abundant molecular ions of *m/e* 480 for testosterone-diTFA and of *m/e* 483 for testosterone-19-d_3-diTFA. The peak height ratio was then measured.

Extraction and purification. In the extraction procedure of blood samples it was necessary to eliminate lipids present in plasma, since these interfered with the MF analysis. This required the storage of a 70% methanol solution of the ether extract at −15° for 1 h. Under the GC conditions employed, the retention time of cholesterol was about 30 min. A purification step by TLC was useful in eliminating cholesterol from the blood sample and it allowed us to reduce the MF analysis time.

Fig. 2 shows the mass fragmentogram from the plasma extract with recordings of the peak intensities of the molecular ions for testosterone-diTFA (*m/e* 480) and testosterone-19-d_3-diTFA (*m/e* 483). The assay was performed by adding about 10 ng of the deuterated internal standard (testosterone-19-d_3) to the plasma sample. The retention times of testosterone-diTFA and testosterone-19-d_3 diTFA were the same (about 4 min) and there was no interference in the molecular ion peaks by contributions from other materials in the plasma extract at these masses.

Sensitivity. The sensitivity of the determination procedures described here was judged based on the signal-to-noise ratio. The lower limit of detection of the mass spectrometer was 10 pg for testosterone as shown in Fig. 3.

Calibration curve. Known mixtures of testosterone and testosterone-19-d_3 were prepared so that the sample size (2—5 μl) injected into the gas chromatograph—mass spectrometer covered the testosterone range of 200 pg—2 ng with a fixed amount (1 ng) of testosterone-19-d_3. Each mixture was then analyzed as the TFA derivative, focussing on the molecular ions at *m/e* 480

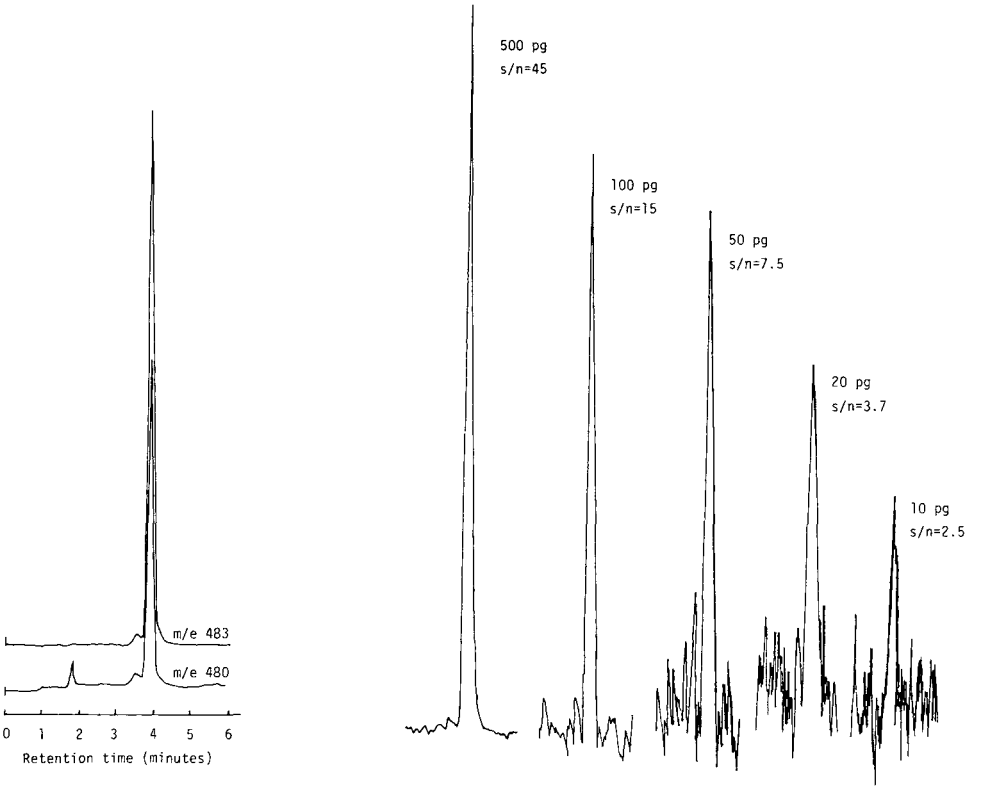

Fig. 2. Mass fragmentograms of testosterone-diTFA and testosterone-19-d_3-diTFA after processing from plasma sample.

Fig. 3. Sensitivity.

for testosterone and *m/e* 483 for testosterone-19-d_3. There was a good correlation between the mixed molar ratio and the observed peak height ratio as shown in Table I.

The peak height ratio was plotted against the molar ratio of testosterone to testosterone-19-d_3 and a calibration curve was obtained. The curve was rectilinear for molar ratios 0.2—2.0. Least-square analysis of the observed ratio gave a regression line with a slope coefficient of 0.9999.

As internal standards in the MF analysis of plasma or serum testosterone levels, isotopically-labeled testosterones such as deuterated testosterone prepared by the exchange reaction with deuterium oxide [21], testosterone-4-^{14}C [22], and testosterone-1,2,6,7-^{3}H [20] have been used. However, the presence of non-labeled testosterone in these labeled compounds requires relatively complicated correction techniques for the observed peak height ratio. The deuterated testosterone used in the present study, on the other hand, served as the ideal internal standard for quantitative determinations because of the absence of non-deuterated testosterone (the isotopic composition of testosterone-19-d_3 was 97.8% for d_3, 2.2% for d_2, and 0.0% for d_1). Therefore, the use of our deuterated testosterone has the advantage over the

deuterated internal standard with lower isotopic purity or the commercially available radioisotope-labeled testosterones.

The difference in mass number between the non-labeled and the labeled testosterone (testosterone-19-d_3) was three and this was appropriate for the precise assay of plasma testosterone.

Accuracy. Testosterone in amounts of 1.93, 4.83, and 9.66 ng was added to 1.0-ml aliquots of pooled male plasma. The amount of testosterone in these samples was then measured by the present method. The testosterone content in the pooled plasma measured in triplicate was 5.86 ± 0.02 ng/ml. The results presented in Table II show that the amounts of testosterone added were in good agreement with the amounts of testosterone measured, the relative error being less than 2.0%.

Comparison of results by MF and RIA

The reliability of the present MF method was determined by measuring the testosterone content in a pooled plasma sample divided into 17 aliquots of 1.0 ml. The results were then compared with those from an RIA method used in routine work, as shown in Table III. The mean testosterone concentrations of the pooled plasma from a healthy male were 6.22 ± 0.19 ng/ml for the MF method and 6.34 ± 0.84 ng/ml for the RIA method. These values were essentially identical and were within the reported "normal" range of plasma testosterone levels (3.6—11.7 ng/ml) [23]. The inter-assay coefficients of variation (C.V.) obtained with pooled plasma divided into 17 samples were

TABLE I

RELATIONSHIP BETWEEN MIXED MOLAR RATIO ADDED TO PLASMA SAMPLE AND OBSERVED RATIO DETERMINED BY MASS FRAGMENTOGRAPHY

Mixed ratio*	Observed ratio**
0.1954	0.1998 ± 0.0021
0.4886	0.4912 ± 0.0085
0.9772	0.9884 ± 0.0197
1.9544	1.9793 ± 0.0274

*Molar ratio of testosterone to testosterone-19-d_3.
**Mean ± S.D. of triplicate measurements.

TABLE II

ACCURACY OF MASS FRAGMENTOGRAPHY ANALYSIS OF TESTOSTERONE IN PLASMA

Added (ng)	Expected (ng)	Found (ng)				Relative error (%)
		Individual values*			Mean ± S.D.	
—	—	5.88	5.86	5.83	5.86 ± 0.02	—
1.93	7.79	7.63	7.76	7.57	7.65 ± 0.08	—1.84
4.83	10.69	10.78	10.35	10.48	10.53 ± 0.18	—1.49
9.66	15.52	15.19	15.51	15.72	15.47 ± 0.21	—0.32

*Each individual value represents the mean of triplicate measurements.

TABLE III

MEASUREMENT OF TESTOSTERONE IN POOLED MALE PLASMA BY MF AND RIA

No.	MF* (ng/ml)	RIA** (ng/ml)
1	6.50 ± 0.28	5.22
2	6.07 ± 0.11	8.01
3	6.45 ± 0.06	7.44
4	5.99 ± 0.11	6.99
5	6.51 ± 0.08	5.70
6	6.14 ± 0.16	7.29
7	6.14 ± 0.03	6.93
8	6.12 ± 0.08	5.70
9	6.32 ± 0.12	6.72
10	6.06 ± 0.09	7.44
11	5.94 ± 0.07	6.54
12	6.02 ± 0.12	5.85
13	6.59 ± 0.13	5.43
14	6.13 ± 0.21	5.64
15	6.17 ± 0.09	5.37
16	6.42 ± 0.06	5.88
17	6.15 ± 0.02	5.70
Mean	6.22	6.34
inter-C.V. (%)	3.15	13.38

*Each value represents the mean ± S.D. of triplicate measurements: the mean intra-assay C.V. was 1.79% in 17 triplicate measurements.
**Each assay was made by duplicate measurements.

3.15% for the MF method and 13.38% for the RIA method. The intra-assay precision for the MF method, as calculated from triplicate analyses of each plasma sample, was excellent (C.V. = 1.79%). These results confirmed the assay reliability of the present MF method.

The inter-assay C.V. for the RIA method was about 10% higher than that for the MF method. The main reasons for this difference may be that in the RIA method: (1) a check on the purification yield for each sample can not be made; (2) errors involved in the more frequent pipetting are significant; and (3) the reproducibility of the curvilinear standard curves on repetitive assays is relatively poor.

The usefulness of the present MF techniques is that it was not necessary to take into account the correction for the purification yield, since deuterium-labeled testosterone was used as the internal standard. In addition, it was also advantageous that the calibration curve was linear and easily constructed. The reproducibility of the curve was excellent. Moreover, identity of the compound under study can be confirmed, not only by selecting the molecular ion, but also by selecting other suitable fragment ions.

The accuracy of analytical procedures must depend largely on the specificity of the assay. Anti-testosterone sera normally display a marked degree of crossreactivity toward 5α-dihydrotestosterone [2–4]. The specificity of the assay for testosterone was investigated by adding known amounts of 5α-dihy-

drotestosterone (0.5, 2.5, 5.0, 10.0 ng) to pooled plasma. The testosterone content in the plasma samples was then measured by the MF and RIA methods. The results are shown in Fig. 4. It is obvious that the MF method was highly specific in measuring plasma testosterone. In the RIA method, however, cross-reactivity toward 5α-dihydrotestosterone was significant.

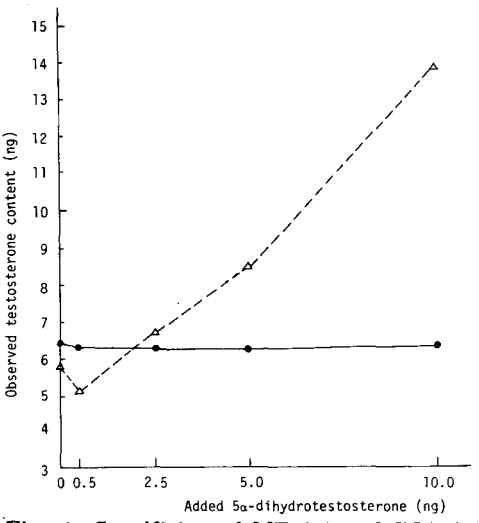

Fig. 4. Specificity of MF (•) and RIA (△). Each value represents the mean of triplicate measurements.

To summarize, the MF method described here afforded a sensitive and reliable technique to measure plasma testosterone. Specificity was high because the GC–MS system was used to separate and to detect specifically the compound in question. To analyze 20 plasma samples, the entire assay could be completed in 2½ working days, requiring one working day more than in the RIA method reported by Furuyama et al. [2]. However, pipetting is necessary only twice in the MF method described here and thus the practicability of this method is comparable to that of RIA.

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Journal of Chromatography, 162 (1979) 539—546
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IMPROVED SELECTIVE ION MONITORING MASS-SPECTROMETRIC ASSAY FOR THE DETERMINATION OF N,N-DIMETHYLTRYPTAMINE IN HUMAN BLOOD UTILIZING CAPILLARY COLUMN GAS CHROMATOGRAPHY

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(Received October 23rd, 1978)

SUMMARY

A gas—liquid chromatographic—mass spectrometric procedure is described for the assay of dimethyltryptamine (DMT) in whole blood. The use of a glass capillary column in combination with selective ion monitoring results in an assay with a high degree of specificity and sensitivity. 5-Methoxy-DMT is used as an internal standard and carrier in the isolation procedure. The superior resolving characteristics of the capillary column (as compared to previously employed packed columns) allows monitoring of the intense *m/e* 58 ion arising from the DMT side-chain. A sensitivity limit of 10 pg/ml blood is realized with a 10-ml blood sample.

INTRODUCTION

Gas—liquid chromatography—mass spectrometry (GLC—MS) is a specific, sensitive analytical technique for determining concentrations of drugs [1, 2] and endogenous compounds [3] in various states of health and disease [4]. The use of GLC—MS in psychiatric disorders is particularly important as endogenous compounds of interest are frequently present in low concentrations and specificity is of considerable concern. This is particularly true for dimethyl-

tryptamine (DMT) and its potential role in schizophrenia. The indoleamine DMT is a known psychotomimetic agent [5—7], and has been reported to be present in human blood and urine [8—12]. Moreover, an enzyme system capable of forming DMT has been demonstrated to be present in man [13—16].

In 1973 we reported on a GLC—MS isotope dilution assay with a sensitivity of 1 ng/ml for measuring DMT in human plasma [12]. Using this assay, we found no differences in DMT plasma concentrations between groups of normals, chronic schizophrenics, acute schizophrenics, and psychotic depressives [17]. In fact, we were unable to detect DMT in plasma at all except in one normal and one psychotic depressive. Subsequent studies by Bidder et al. [18] and Lipinski et al. [19] reported finding DMT in blood and urine in a limited number of patients with psychotic illnesses. Kaplan et al. [20] observed that DMT blood concentrations following administration of a psychoactive DMT dose (0.7 mg/kg) to human subjects were low, peaking at less than 100 ng/ml at ten minutes post-dose (the time of the greatest subjective response) and dropping off rapidly thereafter.

One possible explanation for the failure to find a correlation between blood concentrations of DMT and schizophrenia may be that endogenous DMT concentrations are exceedingly small. Any differences in DMT concentrations between normals and schizophrenics would be missed if the higher level were still below the detection limit of the assay employed. Several refinements in the original assay procedure improved the detection limit to 0.05 ng/ml whole blood. Using this modified assay procedure Angrist et al. [21] found no statistically significant differences in DMT concentrations between psychiatric patients and control subjects. In this study 50% of the patients and 40% of the controls gave measurable DMT concentrations. In the work described here, the assay procedure has been modified extensively, resulting in a practical sensitivity limit for DMT of 10 pg/ml whole blood.

EXPERIMENTAL

Internal standard

5-Methoxy-N,N-dimethyltryptamine (5-OCH_3-DMT, 99% pure; Aldrich, Milwaukee, Wisc., U.S.A.) was used as an internal standard. A solution containing 100 ng of 5-OCH_3-DMT per 10 μl methanol was prepared for introduction of the internal standard.

Gas—liquid chromatography—mass spectrometry

A Finnigan Model 3200 GC—MS instrument equipped with a 6110 computer system was used. The chromatographic conditions were as follows: glass capillary column, 18 m × 0.33 mm, coated with SE-30; oven temperature, 200°; injection port temperature, 260°; carrier gas (helium) flow-rate ca. 2 ml/min; retention time of trimethylsilyl (TMS) derivative of DMT, 6.8 min, and that of 5-OCH_3-DMT TMS derivative, 13.7 min. The mass spectrometer was operated using the following conditions: ionizing potential, 70 eV; emission current, 0.8 mA; electron multiplier, 1800 V. Measurements were performed by selective ion monitoring, focusing the spectrometer upon the ions *m/e* 58 for DMT and *m/e* 232 for 5-OCH_3-DMT.

Extraction procedure and derivative formation

The internal standard (100 ng) was added to 10 ml of whole blood and equilibrated with a Vibra-stirrer. Extraction with chloroform (2 × 15 ml) was then carried out after pH adjustment with 2.5 ml of 1 *N* ammonium hydroxide. The combined chloroform phases were filtered (Whatman No. 2 paper), reduced to 0.5 ml with a stream of nitrogen, and extracted with 1 ml of 0.5 *N* HCl. The aqueous phase was then washed with chloroform (3 × 1 ml), made alkaline with 0.4 ml of 2.5 *N* sodium hydroxide, and extracted with chloroform (2 × 1.0 ml). The chloroform was evaporated and the residue transferred with methanol to a micro derivatization vessel (Pierce micro reacti-vial or equivalent). Derivatization was effected by treating the isolate with 10 μl of bis-trimethylsilyltrifluoroacetamide (BSTFA) and pyridine (4:1, v/v) at 70° for 10 min.

Calibration standards

Calibration mixtures were prepared, each containing 100 ng of 5-OCH_3-DMT and DMT levels up to 1000 pg. A working curve was constructed by plotting the ratio of the peak height intensities of the two ions of interest, I_{58}/I_{232}, vs. the amount of DMT present in the mixture.

RESULTS AND DISCUSSION

The mass spectrum of DMT-TMS (Fig. 1) is dominated by the base peak, $CH_2{=}\overset{+}{N}(CH_3)_2$, *m/e* 58. Other pertinent but much less intense ions are M—58 (*m/e* 202) and M (*m/e* 260). The internal standard for this assay is 5-OCH_3-DMT, which has a retention time (as the TMS derivative) twice that of DMT-TMS. The mass spectrum of the internal standard (Fig. 2) is characterized by an intense *m/e* 58 ion (base peak) and ions at *m/e* 232 (M—58) and 290 (M). The internal standard is added in relatively large amount (100 ng) to serve also as a carrier in the isolation procedure. Monitoring of the *m/e* 58 ion for both DMT and the internal standard is not possible, as the effective dynamic range of the computer is not sufficiently great. Rather, the *m/e* 232 (M—58)

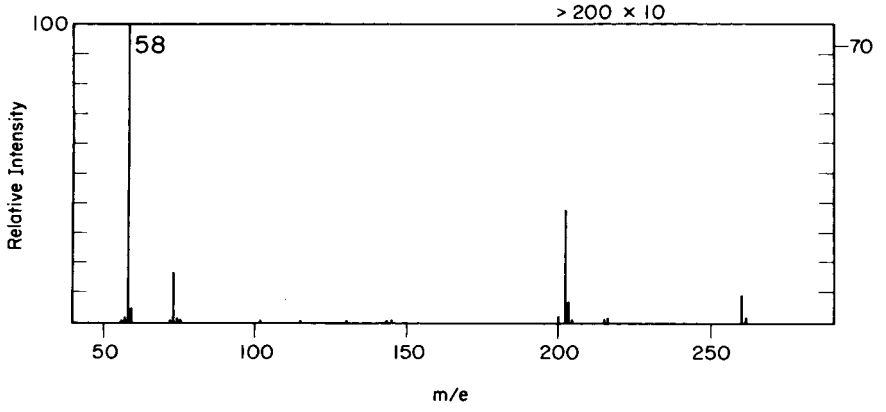

Fig. 1. Mass spectrum of DMT-TMS.

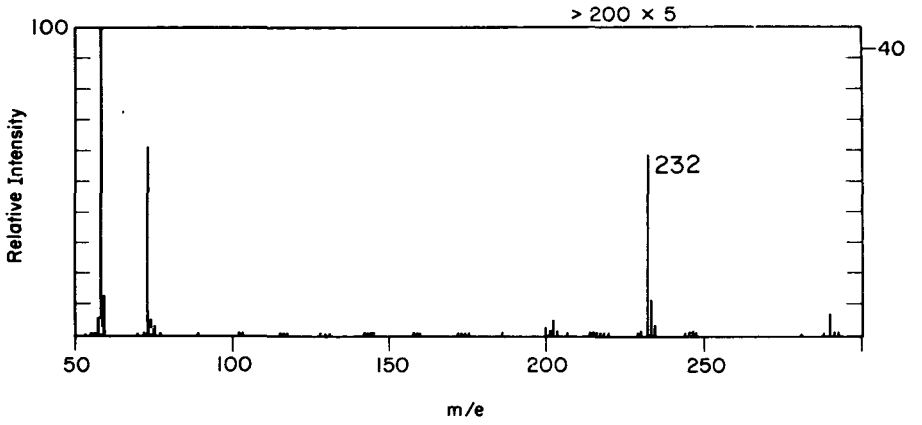

Fig. 2. Mass spectrum of 5-OCH_3-DMT-TMS.

ion from 5-OCH_3-DMT is used for this purpose. We found that, unlike packed column chromatography, adsorption losses with capillary columns are minimal. As little as 4 pg of DMT can be detected as its TMS derivative when monitoring the *m/e* 58 ion, wheras with packed column injection, at least 50 pg is required. Splitless injection is used for sample application to the capillary column so that the entire sample is analyzed. Aliquots of derivatized isolates containing approximately 5—10 ng of internal standard are injected into the capillary column for each measurement; a 30—40% total isolation efficiency allows multiple injections of each isolate for replicate analyses.

Ion response plots for the internal standard and a mixture of DMT and internal standard are shown in Fig. 3. The *m/e* 58 ion response is monitored

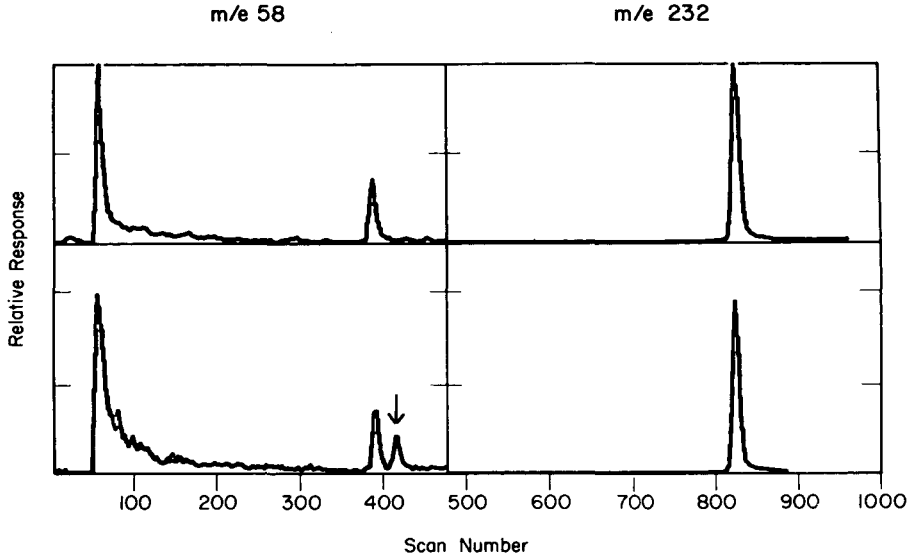

Fig. 3. Ion response plots of internal standard (top panel) and a 500 pg/100 ng mixture of DMT and internal standard (bottom panel).

during the first half of the analysis (0—8 min). The computer then switches to the *m/e* 232 ion for the latter half of the analysis to record the response from the internal standard. The retention time for DMT-TMS (6.8 min) corresponds to scan number 415; for the internal standard the corresponding values are 13.7 min and scan number 820. The response at scan number 390 observed with these standard mixtures results from a reagent component which does not interfere with the analysis. A linear response was obtained for up to 1000 pg DMT with the prepared standards (see Experimental section). Replicate values on the 50-pg standard gave a coefficient of variation of 16.2% (n = 5).

To evaluate the possibility of interfering substances yielding the *m/e* 58 ion, several 10-ml aliquots of control blood were carried through the isolation procedure and analyzed for DMT. Essentially no response was observed at the DMT retention time (Fig. 4). Similar experiments with control blood spiked with DMT gave positive results (see Fig. 5). In a related experiment, 40 ml of control blood were spiked with 50 pg DMT per ml blood. The assay was carried out on four 10-ml aliquots. Results are shown in Table I.

The assay procedure has been used to evaluate DMT levels in whole blood (both arterial and venous) from schizophrenic patients. The results of this study for arterial and venous blood from the same patients are listed in Table

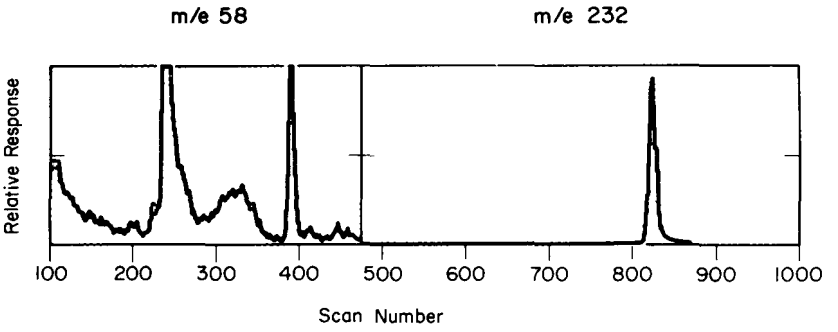

Fig. 4. Ion response plot of control blood isolate.

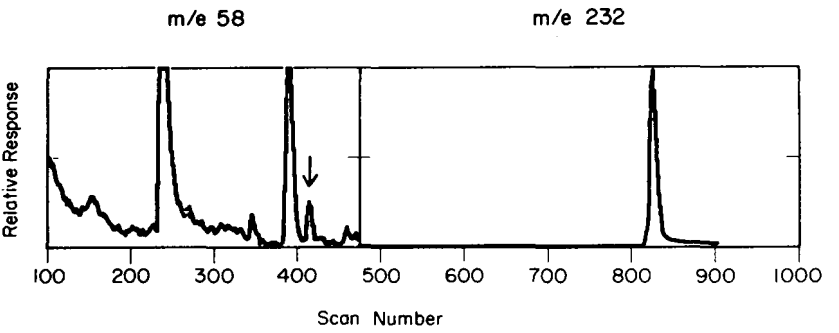

Fig. 5. Ion response plot of an isolate of control blood spiked with 50 pg DMT per ml blood; the response corresponds to 42 pg/ml blood.

TABLE I

ANALYSIS OF CONTROL BLOOD SPIKED WITH DMT (50 pg/ml)

The DMT found is the average result from two injections in each case.

Sample No.	DMT found (pg/ml)
Control (unspiked)	0*
1	48
2	48
3	44
4	56
Mean	49
σ	5.0
C.V. (%)	10.3

*Found < 10 pg/ml.

II. Absolute values ranged from a high of 118 pg/ml to undetectable amounts (< 10 pg/ml) in three patients. Entry 9-A shows the results obtained with blood samples from patient number 9 to which DMT had been added at 60 pg/ml (blind spike). The observed values are in excellent agreement with theory. Fig. 6 illustrates a positive result obtained with this study; for this patient a DMT concentration of 18 pg/ml was found.

The increased sensitivity (10 pg/ml using a 10-ml blood sample) of this assay compared with the sensitivities (1 ng/ml and 50 pg/ml) using 10-ml blood samples) of those previously published [12,21] arises from a number of factors. Use of a capillary column with its inherently greater resolving power when compared with that of a packed column reduces the possibility of im-

TABLE II

ANALYSIS FOR DMT IN BLOOD FROM SCHIZOPHRENIC PATIENTS

DMT found is the average result from two injections in each case.

Patient No.	DMT found (pg/ml)	
	Arterial	Venous
1	24	40
2	84	70
3	118	103
4	0*	0
5	24	21
6	29	0
7	23	18
8	0	0
9	0	0
9-A**	61	65

*Found <10 pg/ml.
**Blood from patient No. 9 to which DMT was added (blind spike) at 60 pg/ml.

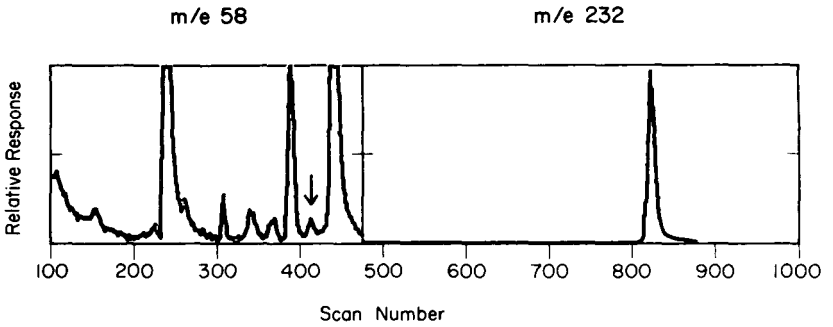

Fig. 6. Ion response plot of an isolate from a schizophrenic patient; the response corresponds to 18 pg/ml blood.

purity interference. Suñol and Gelpi [22], for example, have employed capillary column GLC in the analysis of several indoles. A factor of 100 is observed between the intensities of the base peak (*m/e* 58) and the molecular ion (*m/e* 260) in the mass spectrum of DMT-TMS (Fig. 1). The former is thus, from a sensitivity standpoint, a much more attractive ion than the latter, which is the ion monitored in our earlier packed column assays [12, 21]. The greater separating power of a capillary column allows the monitoring with confidence of the intense *m/e* 58 ion (arising from the DMT side-chain fragmentation), even though this low mass ion could arise from a variety of contaminants. GLC peaks from a capillary column are sharper because of greater column efficiency; increased sensitivity is thus realized with this greater peak height per unit mass. The combination of all these factors results in a practical sensitivity of up to 100 times greater than those assays previously reported [12, 21].

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Biomedical Applications

CHROMBIO. 293

AN IMPROVED AND EASY TECHNIQUE FOR POLYAMINE DETERMINATION IN BIOLOGICAL SAMPLES

APPLICATION TO CELL-FREE SYSTEM FROM HYPERTROPHIED RAT HEART

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(First received June 27th, 1978; revised manuscript received November 10th, 1978)

SUMMARY

An accurate, improved cation-exchange chromatographic method using *o*-phthalaldehyde and ultraviolet detection at 280 nm for the determination of free polyamines (putrescine, spermidine, spermine) has been developed. Different samples, such as the 105,000 *g* supernatant of reticulocyte or heart muscle, and KCl ribosomal wash containing initiation factors, can be analysed. The minor modification of reagents results in a good precision and sensitivity, which is demonstrated by a relative standard deviation of 5—9% and recoveries of 98%. This technique is of particular interest because it allows polyamine determination in biological samples with high concentrations of salt.

INTRODUCTION

In many situations such as heart hypertrophy, cell growth, or regenerating rat liver, the polyamine pool increases within a few hours of either chemical or pharmacological treatment [1, 2].

Polyamines are involved in many steps of the replication and transcription processes. Because of their polyanionic nature they interact with DNA and RNA and increase protein synthesis in cell-free systems [3—6]. The stimulatory effects occurring under such conditions could be due to a rapid accumulation of newly synthesized free polyamines [7].

Therefore the relationship between these molecules and the events involved in the regulation of cell growth or differentiation requires an improvement of the sensitivity of the analytical and quantitative techniques for polyamine determination in biological samples.

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High-speed amino-acid autoanalysers coupled with continuous fluorescence monitoring allow a good quantitative separation [8—11]. Other methods such as gas chromatography, mass spectrometry or radioimmunoassay [12] are fast and sensitive. However, all these techniques require sophisticated equipment which might not be available in all laboratories for routine analysis. Moreover, common quantitation techniques involving butanol extraction before high-voltage paper electrophoresis or thin-layer chromatography, followed by specific staining, are very dependent on ionic strength and might result in an underestimation of the amount of polyamine [13].

This paper describes a simple specific method which requires neither expensive instrumentation nor many extraction steps. We have developed an easy, efficient procedure which can be applied to biological samples of high ionic strength, utilizing a cation-exchange chromatographic method for separation, and *o*-phthalaldehyde (OPA) for detection (because of its good aqueous solubility and high sensitivity), followed by detection of the reaction by their absorbance at 280 nm.

The sensitivity of this method was sufficient to enable us to establish a relationship between induction of cardiac hypertrophy induced by L-triiodothyronine (T_3) treatment and the amount of free polyamines in different cell compartments.

EXPERIMENTAL

Reagents

Putrescine, sarcosine, spermidine and spermine from Sigma (St. Louis, Mo., U.S.A.) were dissolved in water to make 10 m*M* stock solutions which were kept at 4°. More dilute solutions were not stable. All reagents used for the buffers were from Merck (Darmstadt, G.F.R.), except *o*-phthalaldehyde (OPA), which was obtained from Sigma or from Fluka (Buchs, Switzerland), and fluorescamine which was a gift from Roche (Basel, Switzerland). Buffers for the fluorescamine solution and reaction were prepared according to the description of Felix et al. [14]. The method and buffer when OPA was used, were as described in refs. 15 and 16 with some modifications.

For the assays with different buffers and pH conditions the reagent was prepared with 1 *M* boric acid, adjusted to pH 10.3 with 2 *M* potassium hydroxide, then β-mercaptoethanol was added to 1:1000, and Brij 30% to 1.5:1000. The OPA, dissolved in a few millilitres of methanol, was diluted in the buffer to a final concentration of 40 mg/ml. Under these conditions the pH is kept at 9.8—10.

The reaction mixture has a final volume of 2 ml; 1 ml of the reagent solution is added to the samples of polyamines. Phosphate or pyridine buffer is, in some cases, added to obtain the desired pH. The volume of the sample never exceeds 0.5 ml.

For chromatography and assays with KCl, OPA is at a concentration of 125 mg/ml in 0.5 *M* boric acid dissolved in 2 *M* potassium hydroxide to give pH 10.2. Brij 30% is then added at 1.5:1000 and β-mercaptoethanol at 1:1000, then 0.16 *M* pyridine—acetic acid buffer from a 2 *M* (pH 5.5) stock solution is added to obtain a final pH of 8.65—8.7. The stability under nitrogen is very

good for many days. After 45 min reaction, the loss of absorbancy was 2% lower than after 25 min.

Tissue extracts, preparation of polysomes and initiation factors

Polysome and reticulocyte initiation factors were prepared as described previously [17]. The ribosomes and soluble fractions from normal and treated rat hearts (daily intraperitoneal injection of T_3 at the dose of 15 μg per 100 g) were prepared as previously described [18], except that phosphate buffer (pH 7.6) was used instead of Hepes buffer.

The initiation factors were prepared according to the method of Heywood and Rich [19]; however, the ribosomes were washed for 1 h at 4°.

Polyamine extraction

The supernatant and the crude initiation factors were dialysed for 24 h at 4° against 20 volumes of distilled water. The dialysate was lyophilized and redissolved in water. KCl was precipitated with 1 *M* perchloric acid while the pH was kept above 4.0, and removed by centrifugation at 2500 *g* for 10 min. An aliquot of the supernatant was applied to a Dowex column.

Chromatography

A 0.5 × 9 cm column was packed with Dowex 50W-X4, 200—400 mesh, from Bio-Rad Labs. (Richmond, Calif., U.S.A.). The first step of the analysis of the polyamines on this cation-exchange column is as described by Katz and Comb [20] for the separation of nucleosides. The experimental procedure is given in Fig. 1A and B. After regeneration of the column, the sample is applied onto the column in 0.05 *M* HCl. The column is washed for 0.5 h with water then with 20 m*M* phosphate (pH 8.0) for 0.5 h, and finally equilibrated with 20 m*M* phosphate—0.5 *M* KCl buffer for 1 h. The polyamines are eluted with 30 ml of a linear KCl gradient from 0.5 to 3.0 *M* in 20 m*M* phosphate buffer. Putrescine is eluted at 1.3 *M* KCl, spermidine at 2.25 *M* KCl and spermine at 2.75 *M* KCl (Fig. 1B).

Automatic monitoring was obtained, as shown in Fig. 1A, using an Isco spectrophotometer (absorbance monitor U A_4) with a standard flow-cell of 76 μl illuminated volume and an Isco 950 digital integrator.

RESULTS AND DISCUSSION

Recovery of polyamines and accuracy

Known amounts of polyamines were loaded on the Dowex column and eluted. The areas under the peaks were determined with the 950 Isco integrator. Areas and concentrations are related in Fig. 2. The standard plots in Fig. 2 were used afterwards in all the biological experiments. The relative standard deviation is less than 9% for putrescine, 7% for spermidine and 5% for spermine. Recovery was between 90 and 98% and was determined by adding known amounts of polyamines to the biological samples. Reproducibility is good when the ionic concentration does not exceed 1.0 *M*, whatever the biological fluid. Other amines such as lysine, arginine or sarcosine do not interfere.

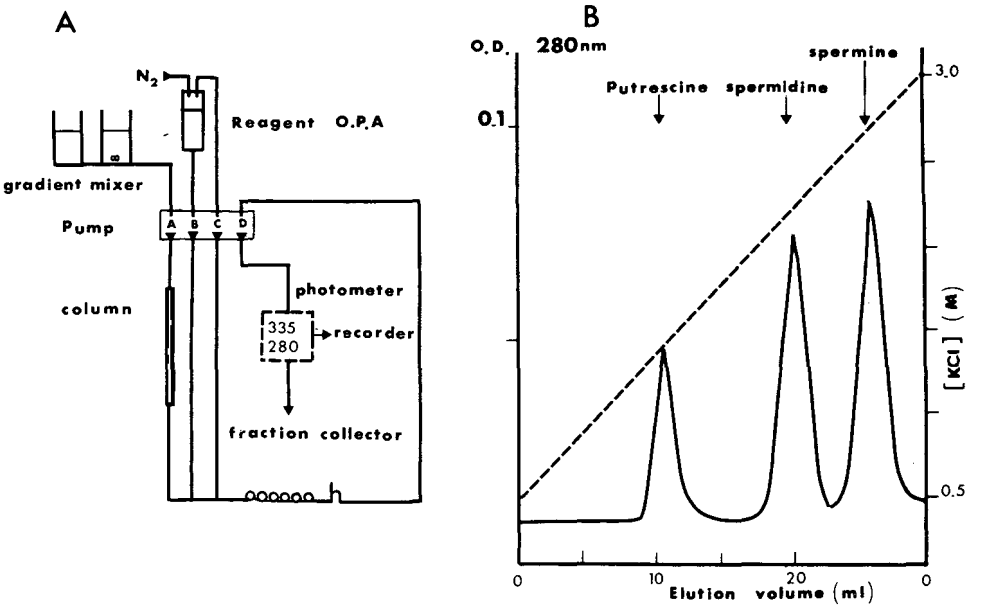

Fig. 1. Calibration experiments: chromatography on Dowex 50W-X4 with automatic monitoring at 280 or 335 nm. (A) Experimental procedure. Flow-rates: gradient mixer (A), 9.54 ml/h; reagent (B), 19.2 ml/h; nitrogen (C), 9.54 ml/h; final effluent (D), 25.2 ml/h. (B) Elution curve obtained by cation-exchange liquid chromatography of a calibration mixture of 62.5 nmoles of each polyamine. Elution gradient was 0.5—3.0 *M* KCl in 20 m*M* phosphate.

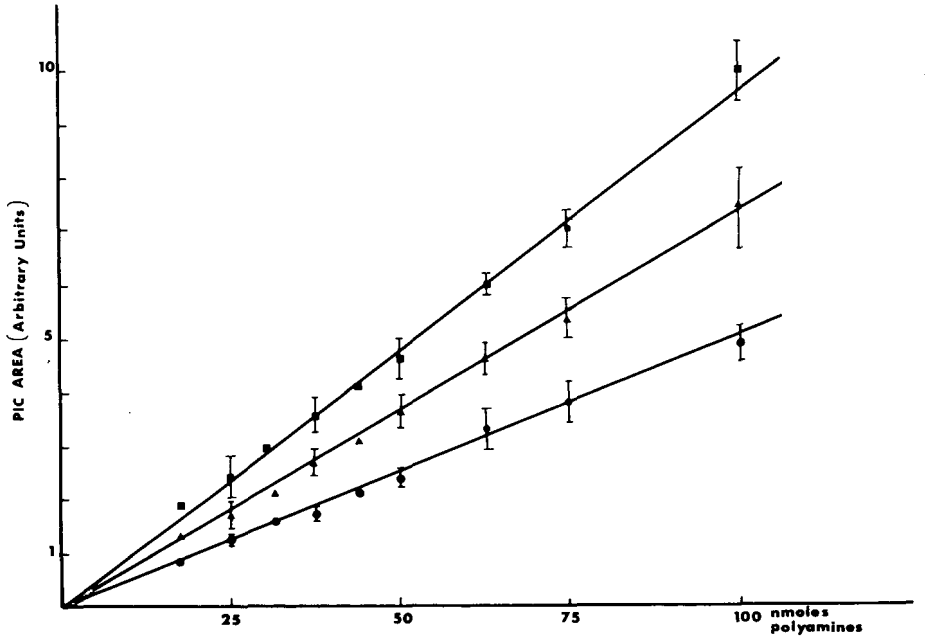

Fig. 2. Standard curves for determination of polyamines: quantitation of putrescine (•); spermidine (▲); and spermine (■). Peak areas (expressed in arbitrary units) were plotted against known amounts of polyamines (in nmoles).

Choice of reagents and wavelength

According to Benson and Hare [21], OPA in the presence of 2-mercaptoethanol reacts with primary amines to form highly fluorescent products, the nature of which has not yet been determined. In contrast, neither OPA nor fluorescamine forms fluorophors with secondary amines.

We have examined the reaction of fluorescamine and OPA with three polyamines of biological interest; putrescine, spermidine and spermine. The two reagents have been compared for sensitivity and reaction rates when used in tenfold excess: OPA is seven and five times more sensitive than fluorescamine with spermidine and spermine, respectively (Fig. 3). Similar results have been published by other authors [9, 21, 22].

Fig. 4 shows that spermidine and spermine have two significant absorbance levels, at 335 nm, used for fluorescence detection, and at 280 nm. The molar

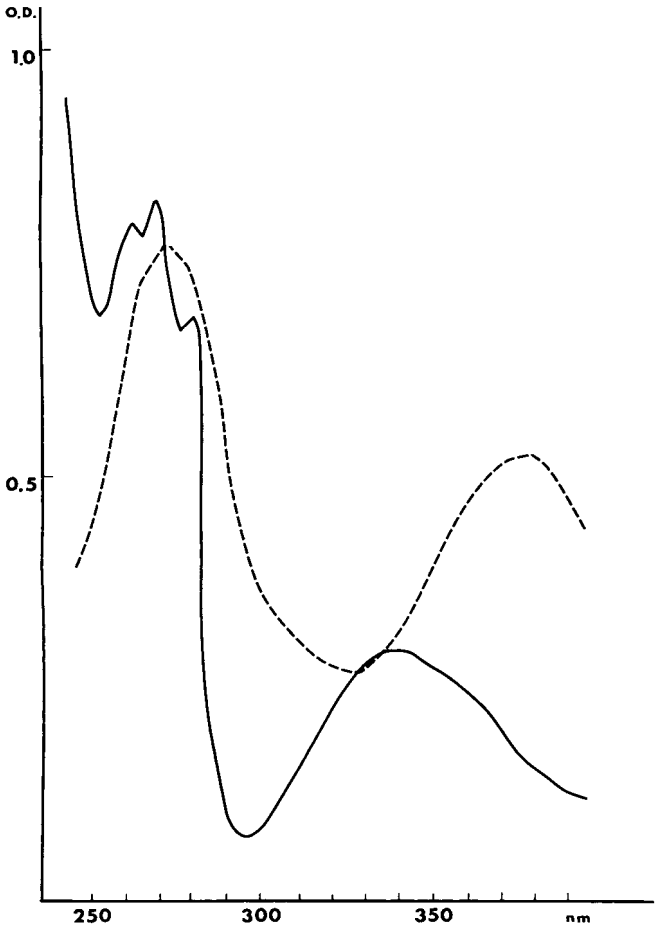

Fig. 3. Ultraviolet and visible spectra of reaction products of spermine $5.0 \times 10^{-4} M$ with fluorescamine (- - - -) and spermine $1 \times 10^{-4} M$ with *o*-phthalaldehyde (——). Fluorescamine and OPA are in tenfold excess. Spectra are recorded against blanks containing reagents alone. Equilibrium is reached immediately with fluorescamine and after 15 min with OPA.

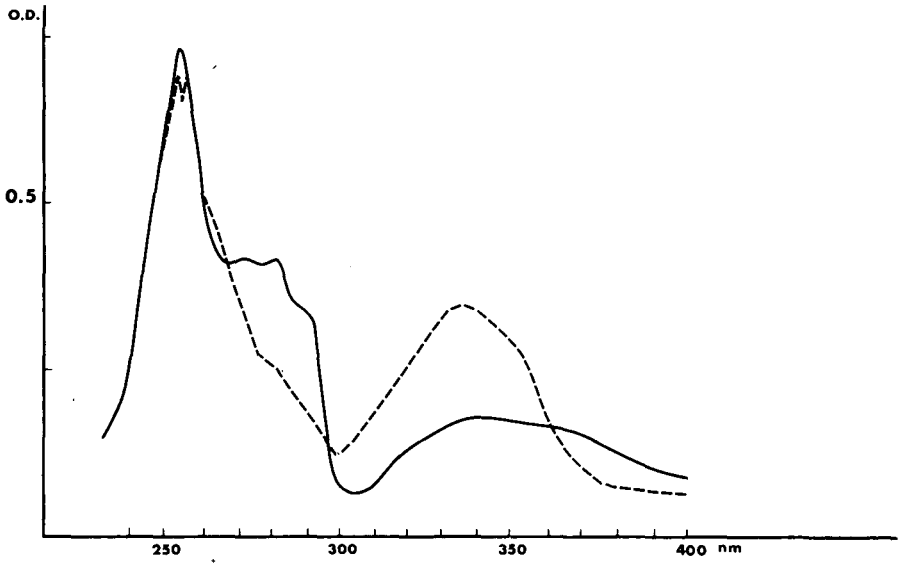

Fig. 4. Ultraviolet and visible spectra of the reaction products of *o*-phthalaldehyde with spermine 1×10^{-4} *M* (- - - - -) and spermidine 1×10^{-4} *M* (———).

extinction coefficient after 20-min reaction of OPA and the three polyamines (Table I), indicates that for spermine in particular, the sensitivity is higher at 280 nm than at 335 nm.

Kinetics of the reaction in phosphate buffer

In 50 m*M* phosphate (pH 8.0, which gave a pH of 9.8 in the final reaction mixture), with each of the three polyamines examined we observed a hypochromicity at 280 nm and at 335 nm, both reaching a plateau after 20—25 min (Figs. 5—7).

Kinetics of the reaction in pyridine buffer

As polyamines were isolated on a CM-cellulose column, eluted with a 0—0.5 *M* pyridine—acetic acid gradient, it was necessary to determine the influence of this buffer on the OPA reaction [9]. There is still hypochromicity at 335 nm with spermine and spermidine (Figs. 5 and 6), but a hyperchromic effect appears with putrescine at this wavelength (Fig. 7). A plateau is reached faster than in phosphate buffer.

All these results, summarized in Table I, show that the best sensitivity for the determination of spermine in pyridine buffer is obtained at 280 nm, whereas for spermidine and putrescine there is little difference compared with determination in phosphate buffer.

The major advantage is the better sensitivity in spermine determination, which is the more interesting problem from a biological point of view.

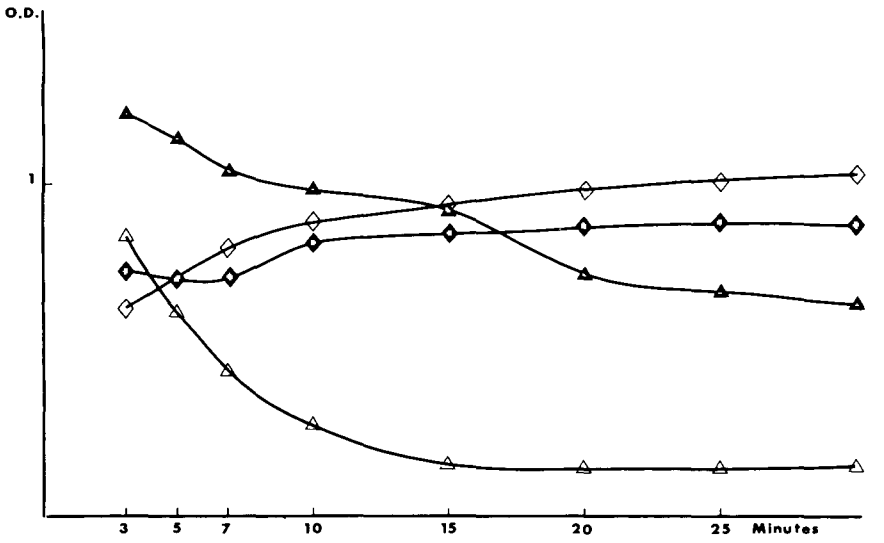

Fig. 5. Kinetics of the reaction of spermidine 1×10^{-4} *M* with tenfold excess of OPA. Phosphate buffer (50 m*M*, pH 8.0; final pH 9.8) at 335 nm (▲) and 280 nm (◆). Pyridine—acetic acid buffer (250 m*M*, pH 5.0; final pH 8.6) at 335 nm (△) and at 280 nm (◇).

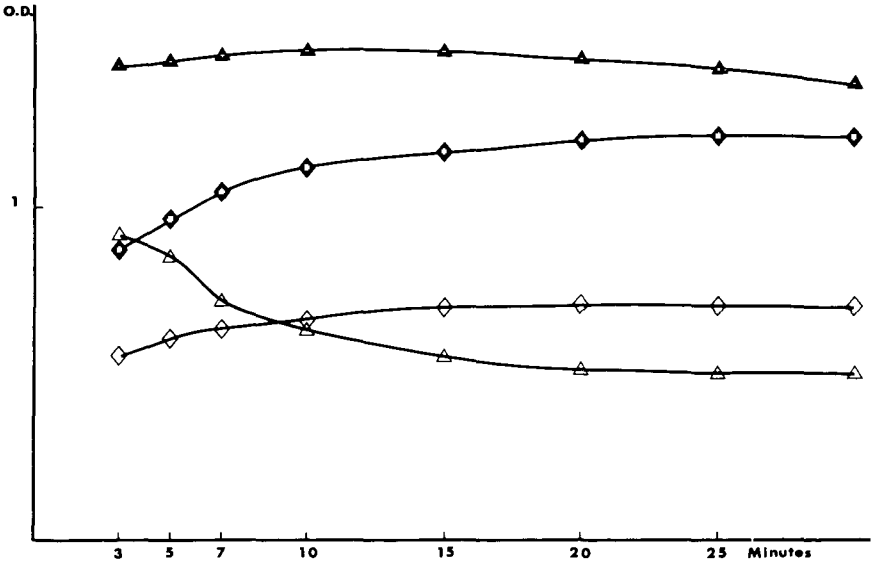

Fig. 6. Kinetics of the reaction of spermidine 1×10^{-4} *M* with OPA. Phosphate buffer (50 m*M*, pH 8.0; final pH 9.8) at 335 nm (▲) and at 280 nm (◆). Pyridine—acetic acid buffer (250 m*M*, pH 5.0; final pH 8.6) at 335 nm (△) and at 280 nm (◇).

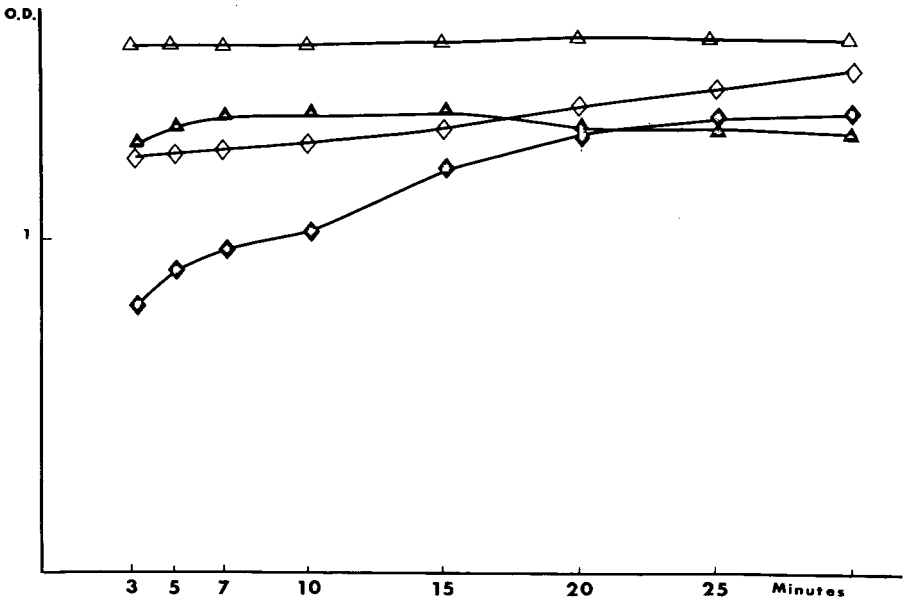

Fig. 7. Kinetics of the reaction of putrescine 1×10^{-4} *M* with OPA. Phosphate buffer (50 m*M*, pH 8.0; final pH 9.8) at 335 nm (▲) and at 280 nm (◆). Pyridine—acetic acid buffer (250 m*M*, pH 5.0; final pH 8.6) at 335 nm (△) and at 280 nm (◇).

TABLE I

COMPARISON OF OPA REACTION PRODUCTS WITH POLYAMINES IN TWO BUFFERS AND AT TWO WAVELENGTHS

Reaction time, 20 min. Buffers: 50 m*M* phosphate (final pH 9.8); pyridine—acetic acid, 250 m*M* (final pH 8.6).

	Molar absorbance index ($M^{-1} \cdot cm^{-1} \cdot 10^{-5}$)			
	Phosphate buffer		Pyridine buffer	
	335 nm	280 nm	335 nm	280 nm
Putrescine	63.5	70	78.5	60
Spermidine	59	48	24.5	31
Spermine	35	37.5	14.5	50.5

Influence of ionic strength, pH, and nature of the ions

The ionic strength conditions are those of the chromatographic conditions on Dowex 50W-X4, and elution by a linear 0.5—3.0 *M* KCl gradient (Fig. 1B). Actually, this chromatographic procedure was chosen because it was not possible to use CM-cellulose without butanol extraction when analysing biological samples.

20 mM phosphate buffer with KCl. It can be seen from Fig. 8 and Fig. 9A that strong hypochromicity at 280 nm is accompanied by hyperchromicity at 335 nm for spermine and spermidine.

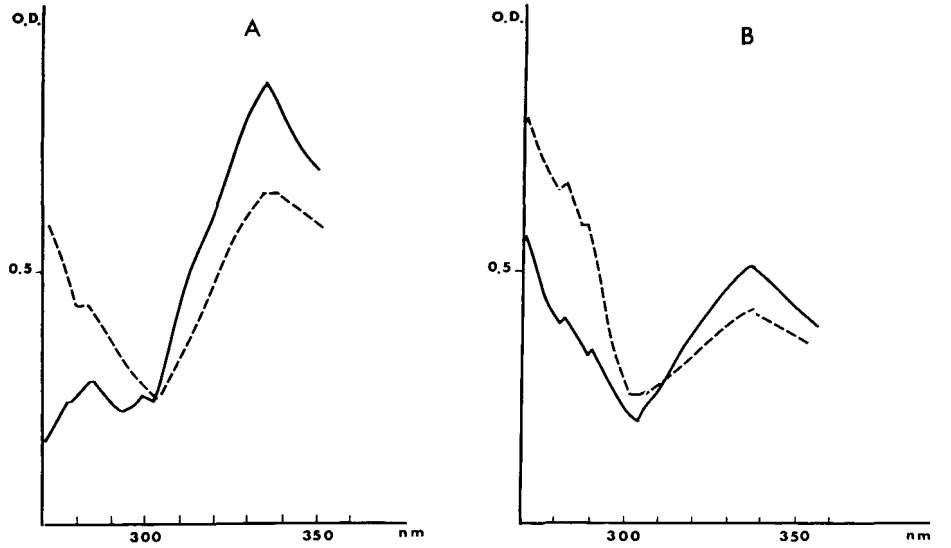

Fig. 8. Ultraviolet and visible spectra in 20 m*M* phosphate buffer, of reaction products of OPA with: (A) spermidine 1×10^{-4} *M* with 2.0 *M* KCl (———), without KCl (- - - - -); and (B) spermine 1×10^{-4} *M* with 3.0 *M* KCl (———), without KCl (- - - - -).

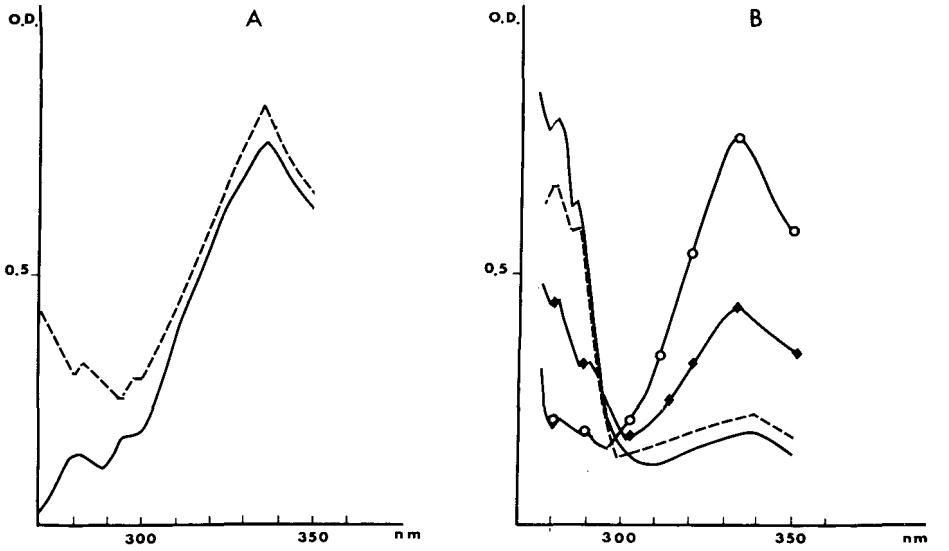

Fig. 9. Ultraviolet and visible spectra of reaction products of OPA with: (A) putrescine, 1×10^{-4} *M* in 20 m*M* phosphate buffer, with 1.5 *M* KCl (———), without KCl (- - - - -); (B) pyridine—acetic acid 160 m*M* (final pH 8.5) (polyamine concentration 1×10^{-4} *M*). (○), Putrescine with or without KCl, 1.5 *M*; (■), spermidine with or without KCl, 2.0 *M*; (- - -), spermine with 3.0 *M* KCl; (———), spermine without KCl.

When varying the pH with acetic acid in the above conditions, putrescine has a higher absorbancy at a slightly alkaline pH, whereas spermine and spermidine absorb more at a higher pH (Fig. 10A).

As the data presented above showed better results with the use of pyridine buffer, we examined the effect of the addition of pyridine to the 20 m*M* phosphate—KCl buffer.

Action of 20 mM phosphate—160 mM pyridine buffer. At pH 10.0 better reactivity was obtained at 160 m*M* pyridine with a good stability. When the pH is varied in the presence of 160 m*M* pyridine, the maximum absorbance at 280 nm is obtained at pH 8.5 for spermine and spermidine, whereas no change occurs with putrescine (Fig. 10B).

Under these conditions (pH 8.5, 20 m*M* phosphate, 160 m*M* pyridine—acetic acid) large concentrations of KCl have almost no influence on the spectra, as shown in Fig. 9B.

The decrease in molar extinction coefficient at 335 nm for the reaction of spermine with OPA in phosphate buffer alone could be due to an influence of secondary amino groups on the reaction with primary amino groups. The new reaction products will be more stable in the presence of pyridine buffer at 280 nm.

Therefore, while the three polyamines are eluted with different KCl concentrations, the conditions determined above allowed quantitative analysis after a simple and reproducible separation on a column of Dowex 50W-X4 ion-exchange resin.

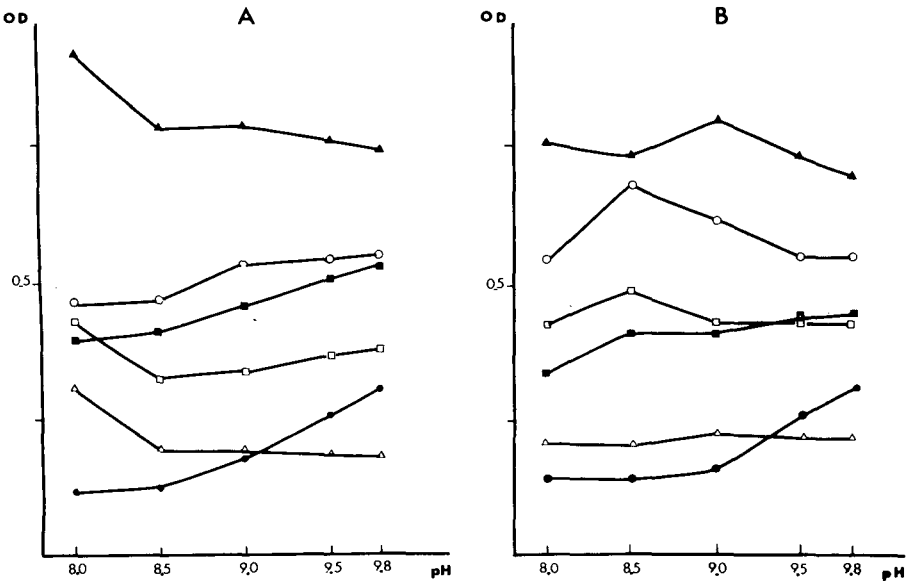

Fig. 10. Effect of pH on absorbance of the reaction products of OPA (at 335 nm closed symbols, at 280 nm open symbols) with putrescine (▲), spermidine (■), and spermine (●), (A) in the presence of 20 m*M* phosphate buffer, (the pH was adjusted with acetic acid) and (B) in the presence of 160 m*M* pyridine (the pH was adjusted with acetic acid or potassium hydroxide).

Application to the determination of free polyamine levels in three cases

The procedure described is particularly useful for biological samples containing a high salt concentration as, for example, a 0.5 *M* KCl ribosomal wash, in which the polyamine concentrations have been determined successfully by this rapid ion-exchange technique.

Reticulocyte polyamines. No free putrescine was detected in the different cell fractions with the experimental procedure. This confirms the results of other authors [23] (Table II). The extraction of free polyamines by dialysis becomes quantitative when the ribosomal wash protein concentration is lower than 20 mg/ml. If the concentration is higher, the spermidine—spermine ratio in the dialysate is modified.

The amount of polyamines found in the initiation factors corresponds to 35 nmoles per mg of ribosomes. The results have been confirmed with biological tests in a cell-free system: the amount of polyamines needed for maximal activity in a cell-free protein synthesis assay [6] corresponds exactly to the amount found after dialysis of the crude initiation factors (unpublished results).

This confirms that the simple technique described here is well adapted to the determination, with a good reproducibility, of free cytoplasmic polyamines that play a fundamental role in the control of protein synthesis.

Rat heart polyamines. No free polyamines were detectable in the crude initiation factors, perhaps because the amount of myosin present in our preparations retains some polyamines in a non-specific way. However, accumulation of newly synthesized polyamines can be detected by this experimental procedure. The level of each polyamine is comparable to other published data [24] (Table II). Spermidine—spermine ratios in the supernatants of reticulocytes and muscle show great differences.

Polyamine levels during T_3-induced rat heart hypertrophy in high-speed supernatants. Just after the first injection, the level of free polyamines synthesized increases, then it decreases over a period of six days (Fig. 11); but the spermidine—spermine ratio shows that the synthesis rates are different. During treatment there is no accumulation of putrescine, and particularly at 6 and 12 h.

TABLE II

SPERMIDINE AND SPERMINE CONCENTRATIONS IN DIFFERENT CELLULAR COMPARTMENTS

	Spermidine	Spermine
Reticulocyte		
Initiation factors (nmoles/mg protein)	35.2	3.85
Supernatant (nmoles/mg protein)	1.5	0.12
Cardiac muscle supernatant		
(nmoles/g)	91	50.5
(nmoles/mg protein)	3.8	1.9

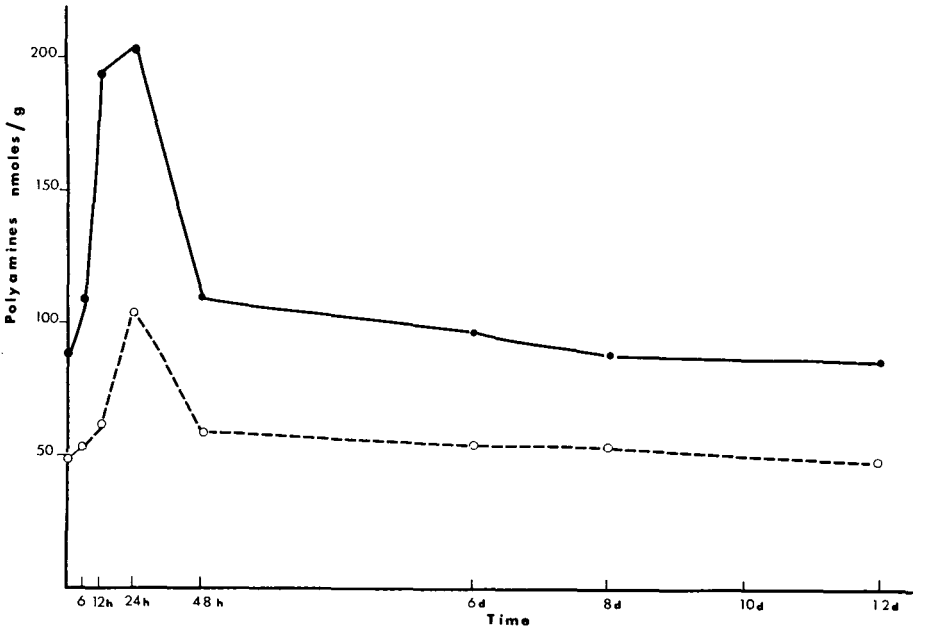

Fig. 11. Polyamine concentration in rat heart post-ribosomal supernatant during triiodothyronine treatment. (●——●), spermidine, (o---o), spermine.

It has been shown that after any cellular modifications following administration of drugs the spermidine level increases more than the spermine level, and maximal activity of the amine biosynthesis pathway is quickly reached [25, 26].

For example, T_3 treatment produces a rapid increase of heart ornithine decarboxylase activity and this first enzyme in polyamine synthesis has a short half-life [27].

CONCLUSION

The procedure developed to determine polyamines is an easy, efficient routine cation-exchange chromatographic method, highly sensitive and reproducible, using a common spectrophotometer (absorbance at 280 nm) and a common reagent, *o*-phthalaldehyde.

This improved technique is particularly useful in the measurement of free polyamines from dialysed salt extracts, as for example the 0.5 *M* KCl ribosomal wash and high-speed supernatant. It does not involve several tedious isolation steps and enzymes can be studied with the same preparation.

The concentration of free polyamines in reticulocytes, cells and heart muscle have been determined successfully by this rapid chromatographic procedure and demonstrates the utility of the method for any biological samples, even in the presence of high ionic-strength buffer.

ACKNOWLEDGEMENTS

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Délégation Générale à la Recherche Scientifique et Technique.

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Journal of Chromatography, 162 (1979) 561—572
Biomedical Applications

CHROMBIO. 288

RAPID METHOD FOR THE ASSAY OF 4-AMINOBUTYRIC ACID (GABA), GLUTAMIC ACID AND ASPARTIC ACID IN BRAIN TISSUE AND SUBCELLULAR FRACTIONS

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(Received October 5th, 1978)

SUMMARY

The thin-layer electrophoretic separation at pH 4.8 of brain extracts and a procedure for fluorescent staining of the plates with fluorescamine are described for the rapid routine determination of 4-aminobutyric acid (GABA), glutamic acid and aspartic acid in brain extracts and in particulate fractions of brain tissue. Automated sample application, electrophoretic separation using two chambers, and quantitation by in situ fluorescence scanning allows the assay of 280 samples within three working days. The method is reproducible (S.D. $\leqslant$ 8% of the mean) within the range of 0.2—2 nmole per spot. The staining procedure can be applied to a variety of related analytical problems. The method has proved useful for the determination of the specific radioactivities of GABA, glutamic acid and aspartic acid in metabolic studies.

INTRODUCTION

Flat bed chromatographic methods have attracted less attention since sensitive automated column chromatographic methods were developed for the routine determination of amino acids. However, the possibility of separating many samples in parallel, together with the improvement of sensitivity by using fluorescamine [1, 2] instead of ninhydrin as the staining reagent continues to make flat bed chromatographic methods attractive for application which demand numerous determinations of selected acids or related compounds.

Our study of subcellular pools of 4-aminobutyric acid (GABA) required the assay of this amino acid in many samples of subcellular fractions, which were

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obtained by density gradient centrifugation. Automated ion-exchange column chromatography was not sufficiently available for the analysis of so many samples, and this prompted us to develop the present method.

Thin-layer electrophoresis at pH 4.8 proved to be a rapid and reliable method for the separation of GABA, aspartic acid and glutamic acid from all other primary amino group-containing compounds which are present in brain extracts in comparable amounts to those of the non-essential amino acid. The staining of thin-layer plates with fluorescamine was improved. Low background fluorescence was achieved together with good reproducibility of staining of the amino acid spots. These methods are described in the present work.

MATERIALS AND METHODS

All usual laboratory chemicals were from E. Merck, Darmstadt, G.F.R. Fluorescamine (Fluram) was purchased from Pierce. Rockford, Ill., U.S.A. Sodium diatrizoate was a gift from Winthrop Labs., Newcastle-upon-Tyne, Great Britain.

The laboratory animals (male Sprague-Dawley rats and male CD_1 albino mice; Charles River, St. Aubin-les-Elbeuf, France) were kept under standardised conditions, having access to standard diet and water ad libitum.

Sample preparation

(a) The animals were decapitated and the heads immediately immersed in liquid nitrogen. The still-frozen brains were homogenized with 10 volumes of 0.2 *N* perchloric acid. Aliquots of the supernatants were diluted with 10 volumes of dioxane. 100-μl volumes of these solutions were directly applied to thin-layer plates in 12 mm long streaks. Alternatively, 5-μl aliquots of the extracts were applied as round spots.

(b) Synaptosomal fractions were isolated according to described methods [3—5]. The sucrose- or Ficoll-containing suspensions were mixed with one tenth of the volume of the suspension of a 40% (w/v) solution of trichloroacetic acid and the precipitates removed by centrifugation.

The supernatants were extracted four times with the same volume of water-saturated diethyl ether. The residual diethyl ether was removed from the aqueous phase in a stream of air. The samples, up to 5 ml, were then applied to a 1 ml column of Dowex 50W-X8 (200 mesh, H^+ form). The columns were washed with 10 ml of distilled water. The amino acids were eluted using 6 ml of a 40% (w/v) trichloroacetic acid solution. The trichloroacetic acid was removed from these eluants by extraction with diethyl ether, as described above.

Pre-separation on Dowex columns of up to 60 samples can be easily achieved in parallel using the multiple column chromatographic device described previously [6].

The samples were dried by lyophilization and the residues dissolved in 0.1 ml of water and 2.9 ml of ethanol or dioxane. Usually 100 μl of this solution were applied to a thin-layer plate for GABA determination in a synaptosomal fraction.

The synaptosomal fraction of the sodium diatrizoate gradient was diluted to 10 ml with water. 0.5-ml aliquots were mixed with 1 ml of ethanol. The precipitate was sedimented completely by centrifugation at 1000 *g* for 10 min.

100-μl volumes of the supernatant were applied on thin-layer plates for separation.

Protein determination

The acid precipitates of the homogenates and the sucrose and Ficoll gradients were washed twice with 0.2 *N* perchloric acid and dissolved in 4 ml of 1 *N* NaOH at 37° (2 days). The alkaline solutions were appropriately diluted with water; aliquots were used for protein determination using the method of Hartree [7].

Samples (0.5 ml) of the sodium diatrizoate gradients were precipitated with a solution of perchloric acid in methanol (0.2 *N*) instead of aqueous perchloric acid, in order to dissolve diatrizoate. The precipitates were washed twice with the methanolic perchloric acid, before they were processed further in the same manner as described for the other samples.

Sample application and electrophoretic separation

For the electrophoretic separation 20 × 20 cm commercial silica gel covered glass plates (silica gel 60, E. Merck; silica gel 1500, Schleicher & Schüll, Dassel, G.F.R.) were used. Ten samples (two or three standards and seven or eight tissue samples) were applied in 12-mm streaks at a distance of 8 cm from one plate edge. If a suitable thin-layer scanner for in situ fluorometry is available, samples can also be applied as spots of a diameter less than 3 mm.

The sample applicator used in this work allowed the automated application of ten spots or lines at the same time. It was the prototype of the Autodoser (Desaga, Heidelberg, G.F.R.), which was developed by one of us at the Max-Planck-Institute for Brain Research, Frankfurt/M., G.F.R. The automated sample application with this apparatus allows the concentration of volumes up to 0.8 ml on a spot of the thin-layer surface, without affecting the quality of the separations.

The plates were sprayed with about 30 ml pyridine acetic acid buffer pH 4.8 until they were evenly wetted.

Buffer composition: 100 ml pyridine; 75 ml glacial acetic acid, 30 g citric acid, 2300 ml water [8]. The cooled plate thin-layer electrophoresis equipment according to Pastuska (Camag, Muttenz, Switzerland) was used. The buffer reservoirs were filled for each separation with 35 ml of fresh buffer. The plates were cooled, with circulating methanol at 0°. At 600 V a current of about 70 mA was observed under these conditions. For the separation of GABA, glutamic acid and aspartic acid of brain extracts 60 min runs were suitable.

Staining with fluorescamine

After completion of the electrophoretic separation the plates were dried for 10 min at 110° in an oven with circulating air. The dried plates can be stored for two days, before they are stained with fluorescamine and evaluated by in situ fluorescence scanning.

Before staining, the plates were heated again to about 50°. Then they were dipped rapidly into a bath containing an alkaline solution, which was prepared as follows: 100 ml of saturated solution of NaOH in methanol was mixed with 100 ml of *n*-butanol. This solution was gradually diluted with 600 ml of

toluene. After 30 sec the plate was removed from the bath and dried in a horizontal position for a few minutes at room temperature and then completely for 10 min at 110°.

The staining solution contained 10 mg of fluorescamine in 100 ml of a mixture of acetone—*n*-butanol (1:1; v/v). A stainless-steel tank (22 × 20 × 0.7 cm) was used for dipping, containing approximately 200 ml of the fluorescamine solution.

The plates were cooled to room temperature. They were then dipped twice into the fluorescamine solution, with a drying period of 1—2 min. Alternatively, the plates can be sprayed with 20 ml of the same fluorescamine solution. However, it should be pointed out that even spraying requires experience in order to obtain staining as homogenous as that obtained by dipping. The plates were stored for at least 2 h at room temperature, before they were quantitatively evaluated by in situ fluorescence scanning. If the plates were stored (protected from dust and light) they could be evaluated even two days after staining. However, fluorescence staining with fluorescamine is not absolutely stable. A constant time schedule between separation, staining and in situ fluorescence measurement is advisable.

In situ fluorescence scanning

Any commercial scanning attachment to a spectrofluorometer should be suitable for the quantitative evaluation of the fluorescamine-stained plates. In the present work an Aminco-Bowman spectrofluorometer with the thin-film scanning attachment has been used. Activation of fluorescence was achieved at 390 nm; fluoroscence was measured at 490 nm. The recorded fluorescence intensity was evaluated by peak height measurement.

RESULTS

Electrophoretic separation of amino acids at pH 4.8

At pH 4.8 neutral amino acids remain near the origin, acidic amino acids and peptides move towards the anode and basic amino acids and peptides move cathodically. As is shown in Fig. 1 none of the 25 usual amino acids co-migrate with GABA, glutamic acid or aspartic acid. Electrophoretic separation for 60 min on a silica gel thin-layer plate, using a pyridine acetate buffer (pH 4.8) allows an adequate separation of these three amino acids from all other fluorescamine-stainable constituents present in comparable amounts in brain tissue.

The 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA-T) inhibitors, 4-aminohex-5-ynoic acid (RMI 71645) and 4-aminohex-5-enoic acid (RMI 71754) [9—11], are not separated from each other, but they are sufficiently separated from GABA to allow GABA determinations in their presence. β-Alanine and homocarnosine do not interfere either. Minor, but unidentified, constituents might co-migrate with GABA or at least may yield overlapping spots. The method is therefore not necessarily adequate for GABA-determinations in tissues with low GABA concentrations such as liver, muscle or kidney.

Staining with fluorescamine

Staining with fluorescamine of identical amounts of electrophoretically

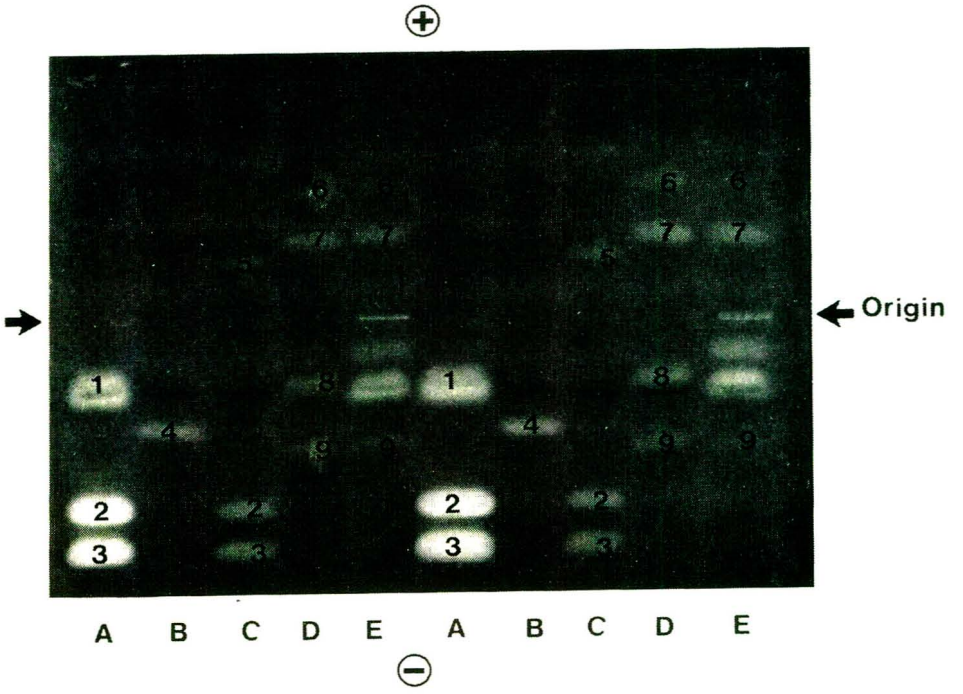

Fig. 1. Thin-layer electrophoretically-separated amino acids and brain extracts (pyridine—acetic acid buffer pH 4.8; 600 V; 60 min) stained with fluorescamine. (Activation of fluorescence at 350 nm). A = Mixture of the following L-amino acids: Ala, Arg, Cys, Gly, Ile, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Trp, Val (about 2 nmole of each). 1 = Neutral amino acids; 2 = His + Arg; 3 = Lys. B = 4-Aminohex-5-ynoic acid plus 4-aminohex-5-enoic acid (4). C = Arg (2); Lys (3); glutathion (5). D = Asp (6); Glu (7); $GluNH_2$ (8); GABA (9). E = Rat brain extract (corresponding to 1 mg of fresh tissue); Asp (6); Glu (7); GABA (9).

separated amino acids under the conditions described in detail in the methods section results in reproducibly fluorescing spots. These show a linear relationship between the amount of amino-acid and peak height (or peak area) of the recorded fluorescence intensity in the range from 200 to 2000 pmole per spot (Fig. 2). The linear correlation coefficients for the amounts of the four amino acids and the fluorescence intensity are $\geqslant 0.996$. Amounts of an amino acid exceeding 2000 pmole per spot should be avoided, unless the amount of the standard is closely similar and only a narrow concentration range is considered for the measurement.

The reproducibility of the method, including pre-separation of the samples, was tested by applying 10-nmole amounts of each amino acid on Dowex 50W-X8 columns, and by their subsequent measurement in aliquots of the column eluates. Table I summarizes the results. It can be seen from this table that recovery was over 90% for GABA, glutamic acid, glutamine and aspartic acid. The standard deviation for the whole procedure was better than 8% of the mean value. It should be noted that under the conditions of sample preparation and sample separation glutamine is not hydrolyzed to glutamic acid.

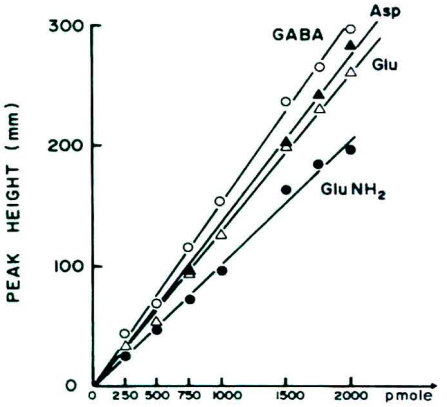

Fig. 2. Relationship between the amount of amino acid and peak height of the recorded fluorescent spots of GABA, aspartic acid, glutamic acid and glutamine. After thin-layer electrophoretic separation of standard mixtures of these amino acids, the plates were stained with fluorescamine, as described in the Materials and methods section; the fluorescent spots were evaluated by in situ scanning of fluorescence.

TABLE I

RECOVERY OF GABA, GLUTAMATE, ASPARTATE AND GLUTAMINE FROM DOWEX 50W-X8 COLUMNS AND REPRODUCIBILITY OF THE ASSAY

10-nmole amounts of each amino acid were applied to the columns. One tenth of each column eluate was separated by electrophoresis. The figures in the Table are the means of duplicate determinations.

Sample No.	Recovery (nmole)			
	GABA	Glutamate	Glutamine	Aspartate
1	9.0	9.2	8.7	10.0
2	9.4	9.3	8.7	9.7
3	10.0	9.5	10.0	10.6
4	10.0	8.2	8.9	8.3
5	10.3	8.4	9.7	9.1
6	8.3	8.1	9.0	8.9
Mean ± S.D.	9.5 ± 0.8	9.0 ± 0.6	9.2 ± 0.6	9.3 ± 0.7

Comparison of GABA determinations in brain by electrophoresis and ion-exchange column chromatography

In order to test the practicality of the electrophoretic method, determinations in brains of rats with varying GABA concentrations were carried out. The same tissue samples were also analyzed in duplicate using a Labotron amino-acid analyzer (Liquimat 2). Buffer: lithium citrate (23 g/l); lithium chloride (18 g/l), pH 4.6. The *o*-phthaldehyde method was utilized for detection.

The increase of GABA levels in the rat brains was achieved by pretreatment

of the animals with4-aminohex-5-enoic acid, the enzyme-activated irreversible inhibitor of GABA-T [11] mentioned previously. Table II shows the results of this comparison. From these figures it appears that the two methods give results which are in good agreement: 98 ± 13% (S.D.) of the GABA amount obtained with the ion-exchange column chromatographic method was found with the thin-layer electrophoretic method and the two sets of data were well correlated (R^2 = 0.94). The average standard deviation of duplicate determinations of brain GABA was for the electrophoretic method ± 9% of the means, and ± 1% for the ion-exchange column chromatographic method.

Distribution of GABA, glutamic acid and aspartic acid in particulate fractions of mouse brain cortex

The electrophoretic separation method combined with fluorescence staining using fluorescamine was applied to the determination of GABA, glutamic acid and aspartic acid in fractions of density gradient centrifugations. The results obtained for the distribution of these amino acids in sub-fractions of mouse brain cortex homogenates are summarized in Tables III and IV.

Table III shows the results for fractionation in sucrose solutions. It appears that the sum of GABA found in the supernatant (S_2) and the crude synaptosomal fraction (= crude mitochondrial fraction) (P_2) is somewhat larger than the GABA amount in the equivalent amount of homogenate, but this difference is not statistically significant. In the case of aspartic acid a similar relationship holds, but concentrations of free glutamic acid seem to be unchanged during tissue processing. From Table IV it appears that the concentrations of GABA and glutamic acid in synaptosomal preparations obtained by different procedures are of comparable magnitude. However, at least in our hands, the yield of synaptosomes was significantly smaller if sucrose—Ficoll gradients [4] were used instead of sucrose [3] or sodium diatrizoate [5] gradients. The GABA concentrations obtained in this work from synaptosomes of sucrose density gradient centrifugations [3] were higher, and those obtained by centrifugation into sodium diatrizoate gradients [5] were lower than those found in previous experiments [12]. These differences are most probably due to interference by sucrose and sodium diatrizoate with protein determinations by the method of Hartree [7]. In the present work these compounds have probably been removed more carefully from the protein precipitates.

The reason for the apparently high values of aspartate in the sodium diatrizoate fractions is not known. Interference of an unknown impurity with aspartate determinations cannot be ruled out. The somewhat higher yield of protein in the synaptosomal fraction from sodium diatrizoate density gradient centrifugations is in agreement with previous observations [13]. This may not reflect higher yields of synaptosomes, but rather a larger proportion of impurities (myelin fragments etc.) as was suggested by electron microscopy of the fractions [13].

DISCUSSION

Fluorescamine as a spray reagent for the detection and quantitative analysis of primary amines, amino acids, peptides, aliphatic or aromatic amino groups

TABLE II

BRAIN GABA CONCENTRATIONS OF YOUNG RATS TREATED WITH 4-AMINOHEX-5-YNOIC ACID, AN IRREVERSIBLE GABA-T INHIBITOR

Comparison of two methods, thin-layer electrophoresis and ion-exchange column chromatography. The GABA values are the Means of duplicate determinations.

Age of rats (days)	Drug dose (mg/kg)	Drug administration prior to sacrifice (h)	Brain GABA concentration		
			Ion-exchange column chromatographic determination (nmole/mg)	Thin-layer electrophoretic determination (nmole/mg)	(Percent of value obtained by ion-exchange column chromatography)
3	—	—	1.03	1.09	106
			1.19	1.02	86
			1.30	1.10	85
			1.36	1.57	115
3	200	4	1.27	1.04	82
			1.39	1.09	78
	500	4	1.49	1.53	103
	750	4	1.34	1.35	101
3	200	16	5.39	5.46	101
	500	16	6.07	6.53	108
	750	16	7.30	6.74	92
			5.68	6.21	109
	1500	16	6.29	6.44	102
			6.86	6.92	101
10	—	—	1.32	1.47	111
			1.21	1.42	117
			1.44	1.66	115
10	200	4	4.35	4.87	112
			2.95	2.48	84
	500	4	6.33	5.64	89
			4.40	4.08	93
			6.22	5.56	89
	750	4	4.79	4.79	100
			6.64	6.08	92
			6.55	7.84	120
	1500	4	6.30	6.52	103
			6.32	6.93	110
10	500	16	14.41	11.33	79
	750	16	15.29	13.09	86
			18.52	14.08	76
			18.74	14.13	75
	1500	16	12.98	14.97	115
				Mean ± S.D.	98 ± 13

containing drugs or drug derivatives on thin-layer plates has been used for several years [1, 2]. A survey of the literature shows that several modifications of spraying techniques have been suggested from time to time [14—19] but the procedure of Feliz and Jimenez [14] is still the most widely used, mainly for qualitative mapping of peptides and related purposes. In a recent paper [20] this method was compared with ninhydrin and *o*-phthalaldehyde staining. It was stated, that the sensitivity obtained for amino acid determinations with fluorescamine was no better than that with ninhydrin. In our hands this spraying technique was not satisfactory either: high background fluorescence and low yields in fluorescent derivatives were obtained. Substitution of triethylamine by triethanolamine as base decreased background fluorescence considerably. Therefore, we used the method routinely for some time, although the fluorescent reaction products developed only slowly. It was necessary to store the plates overnight, before their evaluation by in situ fluorescence scanning was possible. The dipping method described in this work considerably improves staining of thin-layer chromatograms with fluorescamine. Its applicability should be wide and its use contraindicated only for primary amino group containing compounds of low polarity with considerable solubility in the toluene—*n*-butanol mixture.

Some practical considerations: the alkaline solution should be freshly prepared every day and not used for more than 40 plates. The fluorescamine solution must be prepared daily. About 15 ml of this solution are needed per 20 × 20 cm plate. Even though the staining procedure is highly reproducible, the differences between two plates can be significant. It is therefore advisable to run standards on each plate and to observe the precautions found to be important for quantitative evaluation of fluorescent spots by direct scanning [21]. Two or three standards are normally sufficient, so that seven or eight tissue samples can be separated on each chromatogram.

The combination of thin-layer electrophoresis and staining with fluorescamine has proved, in our hands, a practical method of reasonable sensitivity. The precision and the sensitivity of the method is influenced considerably by instrumental characteristics and was in our case obviously limited by the scanning device. Both reproducibility and sensitivity could therefore be improved with better instrumentation.

Since an equivalent of only 5—10-μl aliquots of brain extracts are normally applied for GABA determinations, sample application by manual methods is feasible. In the case where samples with low concentrations of amino acids are to be determined, the advantages of automated sample application are enormous. The cumbersome concentration of the samples to a few microliters is thereby eliminated. About 800 μl of an ethanol—water mixture (3:1) can be applied as a 12-mm streak within 30 min; 10 samples of this volume are applied simultaneously. Automated sample application therefore makes thin-layer chromatographic and thin-layer electrophoretic methods attractive for routine assay procedures in biochemistry, as was exemplified by its application to amino acid analyses in fractions from density gradient centrifugation.

An important aspect of the method described here is its rapidity. Using the automated sample applicator and two thin-layer electrophoretic chambers, it was possible to run at least 14 plates during a normal 8 h working day. Since

TABLE III

DISTRIBUTION OF GABA, GLUTAMIC ACID AND ASPARTIC ACID IN MOUSE BRAIN CORTEX PARTICULATE FRACTIONS

Brain cortex homogenates in 0.32 *M* sucrose were fractionated by differential centrifugation according to Gray and Whittaker [3]. The crude synaptosomal fraction was purified further by centrifugation into a three step sucrose density gradient [3]. The values are means of three independent experiments ± S.D.

Fraction	GABA		Glutamic acid		Aspartic acid	
	μmole per g brain cortex	Percent of total amount of brain cortex	μmole per g brain cortex	Percent of total amount of brain cortex	μmole per g brain cortex	Percent of total amount of brain cortex
Homogenate	2.3 ±0.26	100	10.2 ± 1.0	100	3.64 ±0.6	100
Supernatant (S_2)	1.9 ±0.4	82.6	8.06 ± 0.23	79.0	3.26 ±0.21	89.6
Crude synaptosomal fraction (P_2)	0.70 ±0.1	30.4	2.3 ± 0.2	22.5	1.56 ±0.07	42.9
Purified synaptosomal fraction (B)	0.48 ±0.08	20.9	1.40 ±0.16	13.7	0.68 ±0.21	18.7

TABLE IV

AMINO ACID CONCENTRATIONS IN SYNAPTOSOMAL FRACTIONS OF MOUSE BRAIN CORTEX. COMPARISON OF THREE METHODS.

The values are means ± S.D. of three independent experiments.

Synaptosomal preparation	GABA		Glutamic acid		Aspartic acid		Protein
	nmole per mg protein	μmole per g brain cortex	nmole per mg protein	μmole per g brain cortex	nmole per mg protein	μmole per g brain cortex	mg per g brain cortex
Three-step sucrose density gradient according to Gray and Whittaker [3]	29.1 ±1.3	0.44 ±0.02	67.8 ± 6.6	1.03 ±0.1	27.7 ± 5.6	0.42 ±0.09	15.2 ± 0.4
Sucrose—Ficoll density gradient according to Cotman and Matthews [4]	25.8 ±4.2	0.18 ±0.03	77.5 ± 6.3	0.55 ±0.04	25.4 ± 7.1	0.18 ±0.05	7.1 ± 0.5
Continuous sodium diatrizoate gradient according to Tamir et al. [5]	27.7 ±7.2	0.54 ±0.14	78.3 ±13	1.53 ±0.26	78.3 ±15	1.53 ±0.3	19.5 ±0.2

each plate separates 10 samples, a total of 140 samples were separated during this time. Normally separations were performed on two consecutive days, and staining and quantitative evaluations were done on the third day. In other words, about 280 samples can be measured within three working days. In the case of brain extracts or particulate fractions of brain homogenates, quantitative data for three amino acids were obtained.

The fully automated amino-acid analyzer needed 60 min for one GABA determination. Its maximum sample output within 72 h was therefore 72, i.e. only 25% of that achieved with the electrophoretic method. If glutamic and aspartate were included, the appropriate separation had to be prolonged further to 90 min per sample.

Flat bed chromatographic methods allow the preparation of autoradiographs, if radioactively-labelled compounds have been separated. Moreover, radioactivity can be scanned sensitively with appropriate equipment. We are using the method described here not only for quantitative amino acid measurements, but also for the determination of their specific radioactivities in metabolic studies. In this respect the method is unsurpassed by other procedures, as far as rapidity is concerned.

ACKNOWLEDGEMENT

We would like to express our gratitude to Winthrop Laboratories, Fawdon, Newcastle-upon-Tyne, Great Britain, for the generous supply of sodium diatrizoate.

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Note

Profile bei chronischen Erkrankungen

II. Steroidprofile von Patienten mit Psoriasis vulgaris

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(Eingegangen am 1. August 1978; geänderte Fassung eingegangen am 20. Oktober 1978)

Wie wir kürzlich zeigen konnten [1], unterscheiden sich die Steroidprofile von Patienten mit chronischer Niereninsuffizienz von denen gesunder Kontrollpersonen durch eine extrem starke Verschiebung des Verhältnisses von Dehydroepiandrosteron (DHEA) zu Androstendiol. Während das Verhältnis DHEA zu Androstendiol im Blut von Gesunden etwa 10:1 beträgt, ist die Relation bei Urämikern auf etwa 1:1 verschoben.

Ein verminderter DHEA-Gehalt im Plasma und Gewebe und verminderte Ausscheidung im Urin wurde auch bei anderen Krankheitsbildern beobachtet: Nach Untersuchungen von Sonka et al. [2] soll ein Mangel an DHEA über eine verminderte Hemmung der Glukose-6-Phosphat-Dehydrogenase als pathogenetischer Faktor eine wichtige Rolle bei verschiedenen Erkrankungen spielen. In der Folge konnte durch Holzmann et al. [3] gezeigt werden, dass Psoriatiker gegenüber Gesunden einen um durchschnittlich 40—70% erniedrigten DHEA-Spiegel im Serum aufweisen. Weitere Untersuchungen über den Steroidstoffwechsel von Psoriatikern erbrachten den Nachweis, dass Erythrozyten aus Psoriatikerblut nicht nur eine verminderte Menge DHEA gegenüber Erythrozyten gesunder Kontrollpersonen enthalten, sondern auch an Androsteron, Androstendion und Etiocholanolon verarmt sind. Gleichzeitig wurde ein erhöhter Gehalt von Androstendiol in den Erythrozyten von Psoriatikern gefunden

[4]. Von den gleichen Autoren wurde eine verminderte Ausscheidung von C_{19}-Steroiden im Urin von Kranken mit Psoriasis vulgaris gefunden [5].

Die Ergebnisse von Holzmann et al. über verminderte DHEA-Spiegel und erhöhten Gehalt an Androstendiol im Blutplasma von Psoriatikern weisen eine überraschende Parallele zu unseren Befunden über Plasmasteroidprofile von Urämikern auf, wenn auch bei letzteren wesentlich stärkere Unterschiede im Gehalt dieser beiden Steroide gefunden wurden.

Holzmann und Mitarbeiter hatten die Steroidmengen durch Einzelbestimmungen ermittelt. Da Profile einen direkten Vergleich von Steroidgehalten erlauben, wollten wir mit dieser Methode, die bisher weder bestätigten noch widerlegten Ergebnisse der Untersuchungen von Holzmann überprüfen.

EXPERIMENTELLES

Untersuchungsgut und Methodik

Da zur Aufnahme von Steroidprofilen mit der Gaschromatographie–Massenspektrometrie (GC—MS) Plasmamengen zwischen 50 und 60 ml benötigt werden, wurden die Plasmaprofile in der hier vorgelegten Untersuchung von drei Plasmapools zu jeweils 75 ml, die von Psoriatikern stammten, aufgenommen. Zur Verfügung standen zwei gepoolte Plasmaproben von Frauen und eine gepoolte Plasmaprobe von Männern. Gleichzeitig wurden 16 Einzelurinproben (jeweils 40 ml) von sechs weiblichen und zehn männlichen Psoriatikern in verschiedenen Krankheitsstadien und Altersgruppen untersucht. Zehn Plasmaproben (200 ml) von gesunden Blutspendern und zehn Urinproben (40 ml) von gesunden Probanden dienten als Kontrollen.

Die Aufarbeitung von Urin und Plasma bis zur GC-MS-Analyse erfolgte wie kürzlich beschrieben [1, 6].

Plasma wurde mit physiologischer Kochsalzlösung verdünnt über XAD-4 gegeben und der organische Rückstand an Sephadex LH-20 fraktioniert. Die Steroidkonjugate wurden enzymatisch verseift und die freigesetzten Steroide verschiedenen säulenchromatographischen Reinigungsschritten unterworfen.

Die Urinsteroide wurden nach der Methode von Pfaffenberger und Horning verseift und extrahiert [7].

Derivatisierung

Die Steroide wurden mit N-Methyl-N-trimethylsilyltrifluoracetamid (MSTFA) als Reaktant und Lösungsmittel bei Raumtemperatur in 24 h zu den Trimethylsilylether umgesetzt. Die Reaktion wurde im Mikromassstab in einem verschlossenen Schmelzpunktröhrchen durchgeführt. Die Probe wurde sofort für GC- oder GC—MS-Messungen eingesetzt, da nach etwa drei Tagen mit überschüssigem MSTFA eine Reaktion von Keto- und sterisch gehinderten Hydroxylgruppen stattfindet.

Chemikalien

Für die Chemikalien gelten die gleichen Bezugsquellen wie in [1] angegeben.

Verwendete Geräte

Gaschromatograph. Mit Glaskapillarsäule ausgerüstetes Carlo Erba-2300-Gerät, Glaskapillarsäule (24 m) nach statischer Methode mit SE-30-Film belegt [8, 9] . Injektortemperatur: 275° ; Detektor: Flammenionisationsdetektor. Temperaturprogramm: 150—300°, 2°/min; Split: 1/30; Durchflussgeschwindigkeit (Wasserstoff): 2 ml/min.

Gaschromatograph—Massenspektrometer-Kombinationen. CH-7 Varian-MAT-Gerät mit gepackter Säule, belegt mit 3% SE-30 auf Supelcoport 100—210 mesh. Injektortemperatur: 270°; Temperaturprogram: 200—300°, 4°/min; Durchflussgeschwindigkeit (Helium): 20 ml/min. Separator: Biemann—Watson (zweistufig); Ionisierungsenergie: 70 eV. Die Kombination wurde mit einem Computer (Spectrosystem 100, Varian-Computer 620/L) gekoppelt.

LKB-2091-Gerät mit Glaskapillarsäule, 25 m (nach statischer Methode mit SE-30 belegt). Injektortemperatur: 275° ; Temperaturprogramm: 150—300°, 2°/min; Durchflussgeschwindigkeit (Helium): 5 ml/min; Ionisierungsenergie: 70 eV. Registrierung des Totalionenstroms bei 20 eV; LKB-2130-Datensystem mit PDP-11-05-Computer der Firma Digital Equipment.

ERGEBNISSE UND DISKUSSION

Fig. 1 zeigt das Glaskapillargaschomatogramm der Steroidsulfatfraktion aus einem Plasmapool weiblicher Psoriatiker, dem in Fig. 2 ein Steroidprofil aus dem Plasma einer gesunden Frau gegenübergestellt ist. Hauptsteroid ist in beiden Plasmaproben das DHEA, jedoch ist in dem in Fig. 1 dargestellten Profil aus Psoriatikerplasma Androstendiol in stark erhöhter Menge vorhanden.

Auf die Zuordnung der übrigen GC-Peaks wurde in dieser Arbeit verzichtet, da das hier diskutierte Ergebnis hiervon nicht berührt wird. Entsprechende Arbeiten wurden bereits früher durchgeführt [1, 6].

Bei der Trimethylsilylierung ergab jedes Steroid bei exaktem Einhalten der Reaktionsbedingungen nur ein GC-Signal, von wenigen Ausnahmen abgesehen. In den Gaschromatogrammen einer Testmischung von 15 Steroiden zeigte sich, dass die Silylierung der 17-Ketogruppe der Androstanone in der angegebenen Reaktionszeit ca. 10% beträgt. Bei verlängerter Reaktionszeit wurden Androsteron und Etiocholanolon wesentlich langsamer als DHEA zum 17-Enolether umgesetzt, noch langsamer reagierte 11-Ketoandrosteron. Eine Enoletherbildung der 11-Ketogruppe hingegen wurde nie beobachtet. Von den Pregnanen des Testgemisches wurden α-Tetrahydrocortisol und Tetrahydrocortisol mit MSTFA in der angegebenen Zeit etwa nur zur Hälfte umgesetzt, alle anderen reagierten offenbar vollständig.

Die Ergebnisse dieser Derivatisierungsmethode sind gut reproduzierbar und für solche Untersuchungen, wie hier beschrieben, gut geeignet. Beispielsweise lieferten auch Wiederholungsmessungen mit jeweils frisch angesetzten Steroidtrimethylsilylethern aus Urinextrakten nahezu identische GC-Profile. Auf die Bestimmung von Standardabweichungen wurde verzichtet, da in dieser Arbeit nur qualitative und halbquantitative Auswertungen durchgeführt wurden.

In Übereinstimmung mit den Befunden von Oertel et al. [10] ist ein Men-

genverhältnis von Dehydroepiandrosteronsulfat zu Androstendiolsulfat im Blutplasma von Psoriatikern von 5:3 vorhanden. Dieses Verhältnis wurde bei unseren Messungen in etwa gleichem Ausmass beobachtet und ist im Gaschromatogramm aus den Peakhöhen der beiden Steroide ersichtlich.

Die Steroidprofile im Urin von Psoriasiskranken wiesen kein einheitliches Muster auf, das sie charakteristisch von denen aus Urin von Kontrollpersonen unterscheiden liess.

Die hier vorliegenden Ergebnisse bestätigen die von Holzmann et al. beschriebene Veränderung des Verhältnisses DHEA zu Androstendiol im Serum von Patienten mit Psoriasis vulgaris. Nicht nachvollziehen können wir hingegen die Beobachtungen einer unterschiedlichen Ausscheidungsrate an Steroiden im Harn. Der Urinsteroidgehalt ist stark abhängig vom Alter, so dass

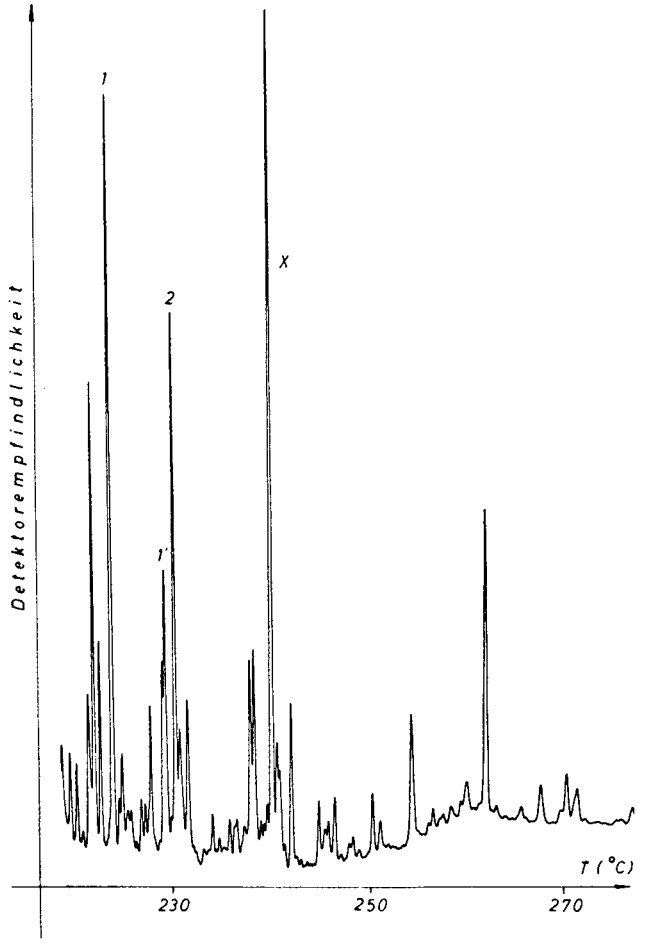

Fig. 1. Glaskapillargaschromatogramm der Steroidtrimethylsilylether, Sulfatfraktion aus Plasma weiblicher Psoriatiker. 24-m-Glaskapillarsäule, SE-30; Temperaturprogramm: 150—300°, 2°/min. 1 = DHEA (3β-Hydroxy-5-androsten-17-on); 1' = Enol von DHEA; 2 = Androstendiol (3β,17β-Dihydroxy-5-androsten); x = Arzneimittelmetabolit.

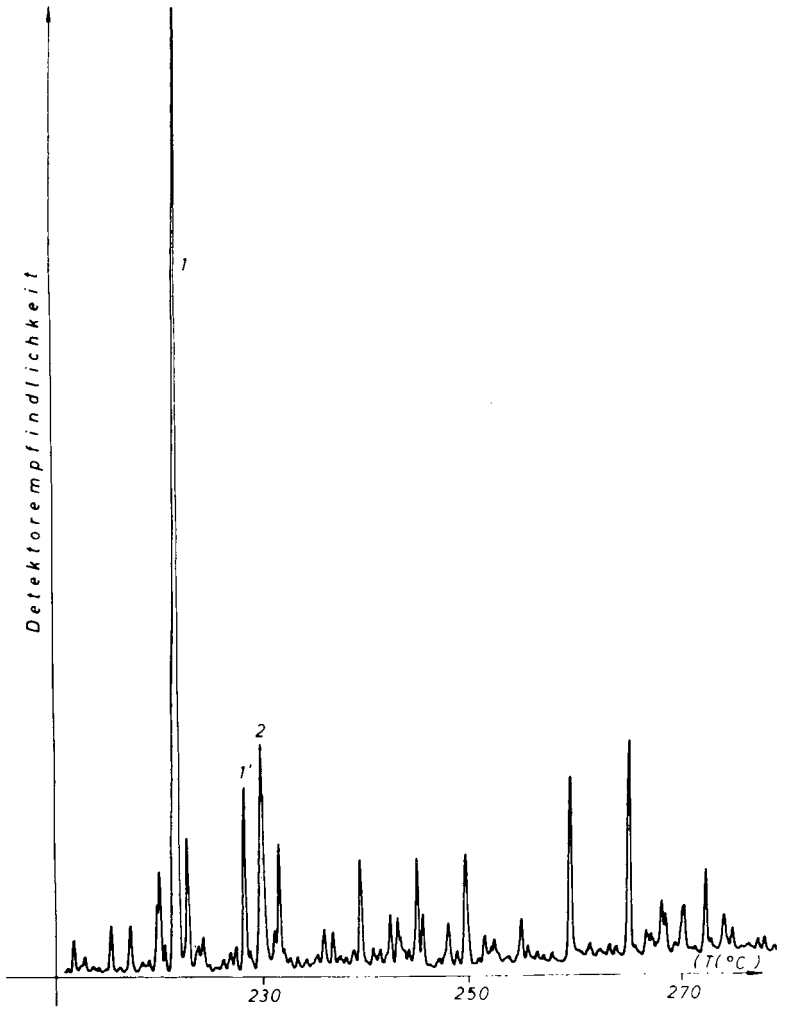

Fig. 2. Glaskapillargaschromatogramm der Steroidtrimethylsilylether, Sulfatfraktion aus Plasma einer gesunden Frau. Übrige Angaben wie in Fig. 1.

relative Schwankungen in der Grössenordnung von 50—100% durchaus noch als normal angesehen werden können [7].

Bei Radioimmunoassaymessungen, wie Holzmann et al. sie durchgeführt hatten, können immer nur einzelne Steroide erfasst und quantitativ gemessen werden. Mit der Massenfragmentographie, wie sie von der Arbeitsgruppe Sjövall [11, 12] eingesetzt wird, kann man weitere Informationen über die Zusammensetzung der Steroide in biologischen Proben erhalten. Neben bekannten Steroiden, die quantitativ bestimmt werden können, lassen sich auch unbekannte erfassen, von denen allerdings kein vollständiges Massenspektrum, sondern nur der Massenfragmentlauf ("fragment ion current") eines oder mehrerer Bruchstücke aufgezeichnet wird. Steroide, die diese Bruchstücke nicht enthalten, entziehen sich dem Nachweis. Mit der GC—MS-Kombination lassen

sich dagegen über den Totalionenstrom nun auch unbekannte Steroide auffinden und anhand ihrer Massenspektren interpretieren. Da die hier angewandte Methode zusätzlich einen direkten GC-Vergleich der Steroidmenge erlaubt, kommen die starken Änderungen in den Steroidprofilen (siehe Fig. 1 und 2) in Plasma von Gesunden und Psoriatikern viel stärker zum Ausdruck als bei Einzelmessungen. Nachteil dieser Methode ist eine relativ hohe Reinheitsanforderung an die isolierte Steroidfraktion. Liegt diese Fraktion nur als Spur in nicht abgetrennten Begleitstoffen vor, sind die Steroidmassenspektren oft mit Beimengungen anderer Verbindungen vermischt, was leicht zu Fehlinterpretationen führen kann. Bei Radioimmunoassaymessungen kommt man hingegen mit einer Extraktion der freien Steroide und bei der Massenfragmentographie mit nur einigen Trennstufen zur Anreicherung der Steroidfraktion aus, wodurch eine quantitative Messung erleichtert wird.

Die Bestätigung der von Holzmann et al. mittels anderer Methoden erhobenen Befunde durch unsere Messungen mit der Kombination GC—MS lassen weitere Untersuchungen über die Ursachen der Psoriasis vulgaris sinnvoll erscheinen, zumal dies die einzigen gesicherten Stoffwechselveränderungen bei dieser Erkrankung sind, die bis heute nur symptomatisch-empirisch therapiert werden kann.

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Journal of Chromatography, 162 (1979) 579—584
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Note

Mass fragmentographic quantification of urinary N,N-dimethyltryptamine and bufotenine

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(Received August 18th, 1978)

The N,N-dimethylated metabolites of tryptamine and serotonin, dimethyltryptamine (DMT) and bufotenine, have been identified in human plasma and urine using a variety of analytical techniques including gas chromatography—mass spectrometry (GC—MS) [1—4]. An enzyme activity capable of forming the dimethylated compounds from the endogeneous non-methylated substrates has been demonstrated in several mammalian tissues [5—7].

The physiological significance of the N-methylating pathway of indoleamine metabolism and of the methylated end products remains unknown at present. Because of the known psychotropic properties of the dimethylated amines, their possible involvement in the chemical pathogenesis of mental disorders has received wide interest [8—11]. Comparative studies have been conducted on the activity of the N-methylating enzyme and on both the blood levels and the urinary excretion of the dimethylated amines in normal subjects and mentally disturbed patients. Somewhat contradictory findings have been reported. The dimethylated amines have been detected more frequently in the urine of psychotic patients than in the urine of normal controls [2, 12, 13]. Also, an elevated activity of the N-methylating enzyme has been claimed as present in the plasma of schizophrenic patients [14]. In other studies, however, no significant differences in either the urinary excretion or the blood levels of the dimethylated metabolites, or in the N-methylating enzyme activity have been detected [4, 8, 9, 15].

Several of these studies are based on the qualitative identification or a semiquantitative estimation of the dimethylated metabolites in the extracts of urine and plasma [1, 2, 9, 13]. A mass fragmentographic (MF) isotope dilution assay for DMT has been presented and applied to clinical studies [4, 15—17]. Also gas chromatography with nitrogen detection has been used for the quantifica-

tion of DMT [18]. Although mass spectrometry (MS) has been used for the identification of bufotenine in the urine [2], no quantitative MF technique has been so far presented.

In this paper we describe a quantitative isotope dilution assay for DMT and bufotenine based on MF. Both compounds can be assayed from the same sample as trimethylsilyl (TMS) derivatives. The deuterated analogues of the two compounds have been synthesized for use as carrier substances and internal standards. The isolation procedure for the dimethylated amines has been modified by introducing the non-ionic XAD adsorbent resin for extraction of the amines from aqueous solution. This facilitates the handling of large urine samples and applied together with a thin-layer chromatographic step reduces the contamination of the GC—MS instrument.

EXPERIMENTAL

DMT and bufotenine were obtained from Sigma (St. Louis, Mo., U.S.A.). The XAD resin (type 2, particle size 0.3—1.0 mm) was purchased from Serva, Heidelberg, G.F.R. Kieselgel G Type 60 was obtained from E. Merck, Darmstadt, G.F.R. The thin-layer chromatographic (TLC) plates (20 × 20 cm, 0.25 mm in thickness) were prepared with a Desaga TLC Spreader and prewashed with methanol—diethyl ether (4:1, v/v). The plates were activated at 110° for 60 min prior to use. The silylation reagents were the products of Pierce, Rockford, Ill., U.S.A. The deuterated analogues were prepared by an acid catalyzed exchange reaction [19]. Morning urine samples were obtained from 26 healthy medical students (10 females, 16 males). The samples, to which no preservatives were added, were frozen and kept at - 18° until analyzed.

Isolation of the dimethylated amines

The internal standards (1 nmole of deuterated DMT and bufotenine) were added to 150 ml of the sample, which was then adjusted to pH 11 with 20% NaOH. If a precipitate was formed, it was removed by centrifugation. The adsorbent resin (5 g per 100 ml of sample) was added and the suspension was stirred mechanically for 20 min. The suspension was then poured into a column plugged with glass wool (1 cm I.D.). After the urine passed through, the retained resin was washed with 15 ml of water at pH 11. The amines were eluted from the resin with 20 ml of ethyl acetate at a flow-rate of 2 ml/min. The ethyl acetate was evaporated under nitrogen to 20—30 μl in a conical centrifuge tube and was then applied to silica gel G plates, which were developed in toluene—acetic acid—ethyl acetate—water (16:8:4:1, v/v). An area of 1 cm^2 was scraped around the site of application and the amines were eluted from the silica gel with two 1-ml portions of methanol, which was evaporated to dryness under nitrogen.

Derivatization and GC—MS

For GC—MS the amines were converted into TMS derivatives. The evaporation residue was reacted in a mixture of bis-trimethylsilyltrifluoroacetamide (BSTFA), pyridine, trimethylsilyldiethylamine (TMSDEA) and trimethylchlorosilane (TMCS) (100:100:30:1, v/v) at 85° for 30 min [20]. The volume

of the reagent was reduced under nitrogen to 20—25 μl and samples of 2—4 μl were injected into the GC—MS instrument.

A Varian Aerograph Model 1700 gas chromatograph coupled to a Varian MAT CH-7 mass spectrometer was used for GC—MS (electron impact ionization, 70 eV). The column was 1% OV-101 on 80—100 mesh Gas-Chrom Q operated at 190° for DMT and at 210° for bufotenine. Multiple ion detection (MID) was carried out with an Altema AL-5 detector. For standardization three samples of known composition as well as a water blank were carried through the entire analytical procedure with each set of analyses.

Creatinine was determined from the samples using the Jaffè reaction (normal values 0.9—1.7 g per 24 h).

RESULTS

Since relatively large volumes of urine had to be processed (at least 150 ml) to achieve reliable quantification of DMT, we have used the non-ionic XAD adsorbent resin to remove the dimethylated amines from aqueous solution. The amines could be eluted from the resin with a small volume of ethyl acetate (20 ml per 7.5 g of resin).

The bulk of the contaminating material moved readily with the solvent in TLC, whereas DMT and bufotenine possessed very low R_F values in the solvent selected and thus could be easily located at the site of application after the run.

The TMS derivatives of DMT and bufotenine were eluted as single, well defined GC peaks. The mass spectra of the two dimethylated amines as TMS derivatives are presented in Fig. 1. In the case of DMT the TMS group is attached to the indole nitrogen. Its spectrum has a molecular ion at *m/e* 260 and an intense fragment at *m/e* 202 resulting from side-chain cleavage between the α and β carbons. In the case of bufotenine the TMS groups are attached to the indole nitrogen and to the phenolic hydroxyl group. In its spectrum the molecular ion is found at *m/e* 348 and the fragment produced by the side chain cleavage at *m/e* 290.

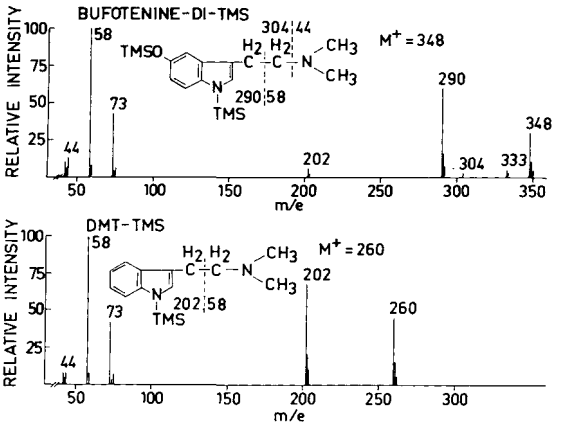

Fig. 1. Mass spectra and fragmentation pattern of DMT and bufotenine as TMS derivatives.

When the molecular ions of both compounds were used for MID, the runs were free from contaminating background peaks. The use of the fragments at *m/e* 202 and 290 resulted in occasional background interference. The detection limit of the assay varied slightly from day to day depending on the condition of the MS instrument. When the molecular ions were used for MID, DMT could be detected at a concentration of 0.1—0.15 ng/ml urine and bufotenine at 0.25—0.30 ng/ml urine. At least 150 ml of sample were required to achieve the detection limits reported above. The recovery after the entire analytical procedure was 40—45% for DMT and 25—30% for bufotenine. Eight analyses of a sample containing 0.5 ng/ml of DMT and bufotenine gave a coeffiecient of variation of 4.4% for DMT and 5.7% for bufotenine.

The results of quantitative analyses of urinary DMT and bufotenine of 26 healthy students are presented in Fig. 2. DMT was excreted at an average rate of 96 ng/g creatinine, the value being 105 ng for males and 81 ng for females. In case of bufotenine the excretion rate averaged 950 ng/g creatinine, 990 ng for males and 875 ng for females. If one exceptionally high value of DMT excretion is excluded, a mean excretion rate of 88 ng/g creatinine is obtained

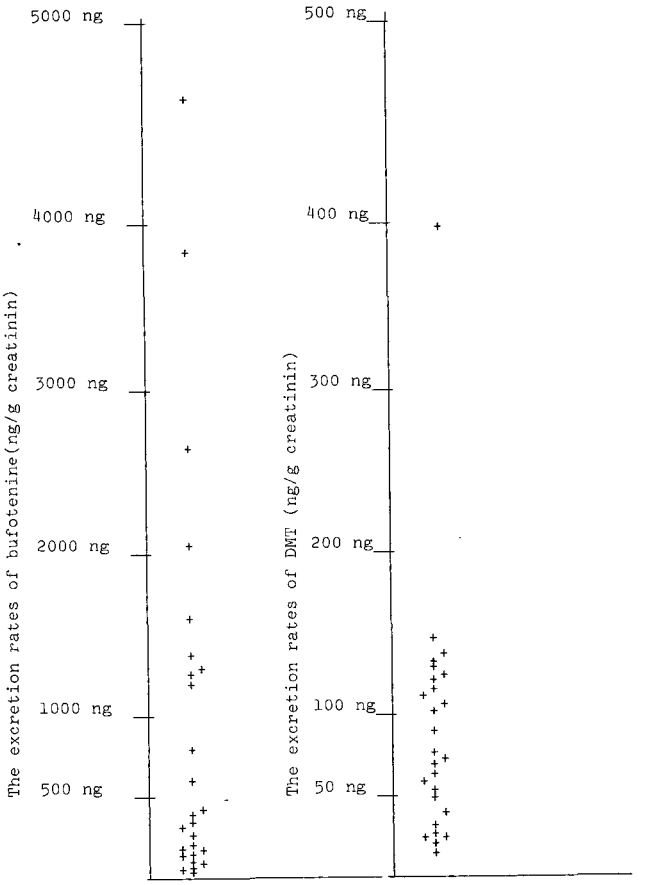

Fig. 2. The excretion rates of DMT and bufotenine calculated per g of urinary creatinine.

for males. After this exclusion the difference in the excretion rates of either DMT or bufotenine between the sexes becomes insignificant.

DISCUSSION

The most commonly employed isolation procedures for DMT and bufotenine include an initial solvent extraction step. Further purification is achieved either by back extraction into acidic aqueous solution and re-extraction into the organic solvent or by means of TLC. The initial solvent extraction step either requires large volumes of the organic solvent or reduction of the sample volume by vacuum evaporation or by lyophilization. The latter procedures, besides being laborious, lead to low yields due to decomposition and adsorption of the amines on to the laboratory ware. These problems could be overcome by employing the XAD adsorbent resin for the initial extraction of the amines from aqueous solution.

The urinary excretion of bufotenine determined with the present method far exceeds that of DMT. In contrast to the DMT values, which form a fairly homogeneous group, the bufotenine values have a very wide inter-individual variation with a majority at the lower end of the scale. Of the 26 subjects studied, 16 excreted less than 600 ng/g creatinine with a smaller cluster between 1000 and 2000 ng. A few higher values are observed between 2000 and 5000 ng. Whether the greater excretion of bufotenine as compared to DMT is due to differences in the synthesis of these compounds or differences in their elimination from human body is not known. However, experiments with parenterally administered DMT and bufotenine have been carried out [8, 21]. Only 0.07% of a dose of DMT was recovered in the urine, in the case of bufotenine the comparable figure was found to vary between 1 and 6% implying that a greater proportion of endogeneously synthesized bufotenine may be excreted unchanged in urine compared to DMT.

The DMT excretion rates reported earlier using GC—MS and GC with nitrogen detection [16, 18] are in reasonable agreement with those obtained with the present method. In the case of bufotenine, although estimates based on TLC techniques have been presented [1, 9, 13], a reliable quantitative method has been lacking so far. In the studies published on the comparison of bufotenine excretion in normal subjects and mentally disturbed patients, about half of the subjects investigated fell below the detection limits of the assays employed [1, 9, 13] and the possible differences between the low excretors of the two groups were consequently not detectable.

Even though the same enzymatic activity has been suggested as being responsible for the production of both DMT and bufotenine [5, 6], no correlation was observed between the excretion rates of these compounds. The availability of the non-methylated substrates, in addition to differences in the degradative metabolism, may explain this disparity.

Because of the low urinary recovery of the parenterally administered DMT and bufotenine, doubts have been expressed as to whether determination of the free amines as such can give any valid information about the metabolic significance of these compounds. However, since the metabolites of the degradative pathway (3-indole acetate, 5-hydroxyindole acetate) are not specific for the

N-methylated indoleamines, the determinations of these amines, in addition to metabolic studies using labelled tracers, appear to be the only way of obtaining information about their quantitative metabolism in the human body. Whether determinations of blood levels rather than the urinary excretion of DMT and bufotenine would be more useful, is doubtful, since the mode of release of these compounds into the circulation is not yet known. If they are released into the circulation sporadically, as may well be the case, the blood levels would vary greatly as a consequence and their determinations from single blood samples would be of limited significance. Frequent or continuous sampling is prevented by the large volumes of plasma needed for quantitative determinations. The urinary excretion, even though it represents only a fraction of the total amount of the compound metabolized, gives a summation over a longer period and can serve as a more reliable indicator of the overall quantitative metabolism of DMT and bufotenine.

ACKNOWLEDGEMENTS

This work has been supported by the National Research Council for Medical Sciences, Finland. Skilful technical assistance has been provided by Ms. Hilkka Rönkkö.

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Note

Determination of inosine and adenosine in human plasma using high-performance liquid chromatography and a boronate affinity gel

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(Received September 14th, 1978)

The determination of nucleosides from biological fluids by the reversed-phase partition [1—6] or ion-exchange [7—12] mode of high-performance liquid chromatography (HPLC) has been the subject of much recent research. The direct analysis of plasma nucleosides has in our experience been especially difficult because of the extremely low, variable, endogenous levels in the normal human population, and the tendency for the compound of interest to be obscured by other components found in plasma.

Uziel et al. [3] offered a solution to the selectivity aspects of nucleoside analysis through the pre-treatment of urine with an affinity gel which contains a boronate group (now offered commercially as Affi-Gel® 601). We have found this material to be extremely useful for the pre-fractionation and concentration of plasma nucleosides prior to injection in HPLC, thus greatly increasing sensitivity as well as selectivity. The normal human plasma levels of at least two nucleosides, inosine and adenosine, were reliably measured in all subjects examined using this boronate affinity gel. The identity and absolute quantitative value of these two compounds has been verified by the enzymatic peak-shift method.

METHODS

Instrumentation

A Varian Aerograph 4100 high-pressure liquid chromatography system with a Rheodyne Model 7120 loop injector for sample introduction and a UV absorbance detector which monitors at 254 nm was used throughout the study. A 250 × 4.6 mm stainless-steel column was packed with Spherisorb 5 μm ODS reversed-phase material (Spectra Physics, Santa Clara, Calif., U.S.A.). Packing was done from a butanol slurry using a DST-100 constant-pressure pump (Haskell Engineering, Burbank, Calif., U.S.A.) operating at a delivery pressure of

6000 p.s.i. An LS-8000 liquid scintillation counter (Beckman Instruments, Fullerton, Calif., U.S.A.) was used for ^{14}C counting.

Boronate affinity gel column

Affi-Gel® 601 (Bio-Rad Labs., Richmond, Calif., U.S.A.), a boronate affinity gel, was used for the batch separation. The manufacturer's specifications are 100—200 mesh, 100 mequiv. boronate per g dry wt., 6 ml settled bed volume per g packing material. The Affi-Gel 601 (0.25 g) was weighed out and allowed to swell overnight in 0.25 *M* ammonium acetate (pH 8.8). This was poured into a 0.7 × 4 cm column. The column was stored at 4° when not in use. After 4 months of intermittent use, the columns have shown no deterioration under the conditions of this assay, although they have occasionally been repacked when air spaces developed.

Sample preparation

A 5-ml volume of blood was drawn via venipuncture, and immediately plunged into an ice-bath. The plasma was separated at 500 *g* at 4°, and then ultrafiltered through a 2100 CF 50 membrane cone (Amicon, Lexington, Mass., U.S.A.) at 4°. Recoveries of inosine and adenosine through the ultrafiltration were quantitative.

Plasma samples were obtained as described from five male and one female normal subjects between the ages of 29 and 45 years. The blood samples were obtained in the morning with no restriction on breakfast eating habits.

Chromatography

To 1 ml of ultrafiltrate, 0.1 ml of 2.5 *M* ammonium acetate (pH 8.8) was added. This mixture was then applied to the top of a boronate affinity gel column, and the sample was washed on with 1 ml of 0.25 *M* ammonium acetate (pH 8.8). The column was then washed further with 7 ml of the same buffer. Elution of the nucleosides was carried out with 6 ml of 0.1 *M* formic acid as used by Davis et al. [4] for urinary nucleoside separation using a synthesized boronate affinity gel.

The 6-ml solution of 0.1 *M* formic acid eluate was lyophilized to dryness, and the residue dissolved in 200 µl of water, resulting in a nucleoside concentration equal to five times that in the original plasma. Fifty to one hundred microlitres of this solution were then injected onto the reversed-phase HPLC system described above, using a solvent system of 0.05 *M* H_3PO_4 in water, adjusted to pH 3.05 with sodium hydroxide.

A chromatogram of normal human plasma is shown in Fig. 1.

Reagents

Ammonium acetate (A.C.S.; J.T. Baker, Phillipsburg, N.J., U.S.A.), and formic acid (reagent, A.C.S.; M.C. & B., Norwood, Ohio, U.S.A.) were used in the preparation of solutions for analysis. [8-^{14}C]Inosine was purchased from Schwarz/Mann (Orangeburg, N.Y., U.S.A.) and had an activity of 35 mCi/mmole. Radiopurity was assayed by thin-layer chromatography (silica gel; *n*-butanol—2 *N* NH_4OH, 10:2) and found to be greater than 98%. [8-^{14}C]-Adenosine was purchased from New England Nuclear (Boston, Mass., U.S.A.) and had an activity of 54.7 mCi/mmole and a manufacturer's specification for radiopurity greater than 97%.

Inosine (Ajinomoto Co., Japan) and adenosine (Grade A; Calbiochem, La Jolla, Calif., U.S.A.) were dissolved in water at varying concentrations and used in the preparation of standard curves.

Purine nucleoside phosphorylase (EC 2.4.2.1), a product of Boehringer (Mannheim, G.F.R.), had an activity of 25 units/mg in a suspension of 1 mg/ml and was used for the enzymatic peak-shift measurements.

RESULTS

Fig. 1 illustrates a UV absorbance pattern of boronate-treated plasma on the HPLC reversed-phase column. Peaks marked INO and ADO are identical in retention time to inosine and adenosine, respectively. To verify the identity of these peaks, duplicate injections of the sample were made and the pooled fractions corresponding to the peaks were collected and adjusted to pH 7—7.4. Three microliters of the purine nucleoside phosphorylase solution were added and allowed to digest for 1 h. The fractions were lyophilized, reconstituted to 0.2 ml, and reinjected. Quantitative conversion to the corresponding base (hypoxanthine or adenine) and complete elimination of the original peak was found in all samples tested. Although other small peaks can be found that correspond to various nucleosides, the concentrations represented are too low to allow the use of this quantitative peak-shift technique.

Six subjects were examined for normal circulating levels of inosine and adenosine, and the results, in μg/ml plasma, are shown in Table I.

Precision of the assay

Sources of error in the assay may be divided into two categories. The first is variation associated with application of the sample onto the boronate column, and collection of the acid fraction, lyophilization and concentration. The

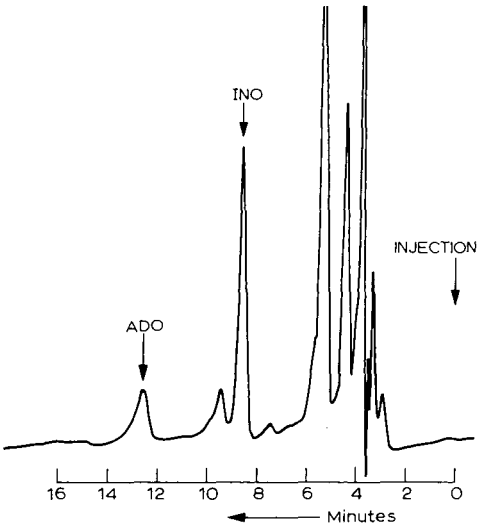

Fig. 1. Chromatogram of boronate-treated normal human plasma (subject J.C.); conditions as indicated in text. Injection volume was 75 μl, attenuation 0.04 a.u.f.s.

second source of error is the chromatographic analysis and quantitation by HPLC.

The Affi-Gel 601 boronate column efficiency was evaluated for both adenosine and inosine in plasma. Pooled, normal human plasma ultrafiltrate was spiked with [^{14}C]inosine to a concentration of 0.77 μg/ml or [^{14}C]adenosine at 0.49 μg/ml. The ultrafiltrate was treated as described under Chromatography, applied to the boronate column and the acid fraction counted for ^{14}C determination. Inosine and adenosine determinations were carried out in quadruplicate with recoveries in excess of 90%, as shown in Table II.

TABLE I

NORMAL CIRCULATING LEVELS OF ADENOSINE AND INOSINE

Subject	Adenosine (μg/ml)	Inosine (μg/ml)
S.K.G.	0.052	0.094
J.M.	0.034	0.13
R.N.	0.030	0.18
LV	0.087	0.31
T.G.	0.018	0.15
J.C.	0.149	0.60

TABLE II

RECOVERIES OF ADENOSINE AND INOSINE FROM THE BORONATE COLUMN

Compound	Mean recovery (%)	Standard deviation (%)
Adenosine	94.4	2.5
Inosine	92.1	2.5

TABLE III

PRECISION OF HPLC ASSAY

Subject	Inosine			Adenosine*		
	μg/ml	Mean	S.D. (%)	μg/ml	Mean	S.D. (%)
S.G.	0.093	0.0936	2.2	0.052	0.052	2.2
	0.092			0.052		
	0.096			0.050		
J.M.	0.134	0.131	6.1	0.033	0.034	5.1
	0.122			0.033		
	0.137			0.036		
R.N.	0.186	0.183	2.8	0.031	0.030	12
	0.186			0.033		
	0.177			0.026		

*Concentrations are calculated to original concentration in plasma.

The precision of the HPLC assay was estimated by three different repetitive injections of the boronate column concentrate from three subjects. The results of these determinations are given in Table III and demonstrate a mean standard deviation of 3.7% for inosine and 6.4% for adenosine.

The overall precision of the assay was estimated by dividing the original plasma of the three subjects into two aliquots (six samples). Each aliquot was concentrated from the boronate column and analyzed in duplicate by HPLC. The average coefficient of variation for the inosine assay was 13% and 7% for the adenosine assay.

DISCUSSION

Plasma levels of nucleosides are of potential interest in a number of disease states. It has already been noted by Hartwick and Brown [1] that an increased level of circulating adenosine is found in the plasma of patients suffering from adenosine deaminase (ADA) deficiency. In that study [1] levels of adenosine in normal patients were not found. This result could be explained by a lack of sufficient sensitivity when a simple direct injection method is used. It is of interest to note that elevated adenine levels had previously been found in the red blood cells of ADA-deficient patients [13].

Nucleoside levels are also of interest in the study of Lesch-Nyhan disease, in which hypoxanthine—guanine phosphoribosyltransferase activity is severely reduced by an inborn error of purine metabolism [14]. Various nucleosides have been considered as possible therapeutic treatments.

Some workers [3—6] have already exploited the boronate affinity gel as a preliminary extraction procedure for modified nucleosides in urine and serum. Excretion patterns of these compounds are being studied as possible cancer markers [3—5] and the patterns may also be useful in evaluating the effects of chemotherapy on cancer [5, 6].

The method reported here will offer an expanded range of applications directly related to its increased sensitivity and selectivity over direct-injection, reversed-phase HPLC column methods. Use of this procedure will enable drug metabolism studies to be carried out since endogenous circulating levels of nucleosides will now be detectable.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Bruce Black of Bio-Rad Labs. for his gift of Affi-Gel® 601 before it became commercially available.

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Journal of Chromatography, 162 (1979) 591—595
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Note

Assay of etomidate in plasma by capillary gas chromatography with nitrogen-selective detection

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(Received October 11th, 1978)

Two procedures have been described in the literature for the determination of the intravenous anaesthetic etomidate in plasma: (1) a gas-chromatographic (GC) method which requires a laborious extraction procedure and is not sensitive enough to determine very low plasma concentrations, unless plasma samples larger than 1 ml plasma are used [1]; (2) a mass-fragmentographic method which requires a gas chromatograph—mass spectrometer and may therefore not be very suitable for routine analysis [2].

In the present communication a method for the assay of etomidate in plasma is described, which is more sensitive and less laborious than the previously published GC method [1] and which can be applied to pharmacokinetic studies in man. The use of a support-coated open tubular (SCOT) column together with a nitrogen-selective detector has proved to be very suitable in this respect. In addition to etomidate it was found that propoxate could be analysed equally well with this method.

EXPERIMENTAL

Materials and methods

Etomidate [(R)-(+)-ethyl-1-(1-phenylethyl)-1H-imidazole-5-carboxyl] and propoxate [(+)-propyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate] were kindly supplied by Janssen Pharmaceutica (Beerse, Belgium). Pentane was from Baker (Phillipsburg, N.J., U.S.A.), redistilled; ethanol, p.a. was from Merck (Darmstadt, G.F.R.). The analysis was performed on a dual-column 5710 (Hewlett-Packard) gas chromatograph with a N—P detector (Model 18789A). Column: SCOT, 10 m × 0.4 mm I.D., Duran 50 glass. Support layer: Tulla-

nox, silanized fumed silica, particle size <10 μm (Cabot Corp., Boston, Mass., U.S.A.). Stationary phase: Carbowax 20M. Temperatures: column 170°, injection port 200°, detector 300°. Gas flow-rates: carrier (helium) 10 ml/min, hydrogen 3 ml/min, air 100 ml/min, auxiliary gas (helium) 15 ml/min.

Extraction procedure

To 1.0 ml plasma in a centrifuge tube were added 25 μl ethanol containing 125.0 ng propoxate as internal standard. After homogenization the mixture was extracted once with 5 ml pentane on a Cenco whirlmixer for 15 sec. After centrifuging for 5 min at 2500 *g*, the upper organic layer was removed with a pasteur pipette and transferred to a conical evaporation tube. The solvent was evaporated to dryness at 40° in a stream of dry nitrogen on a water-bath. The residue was dissolved in 100 μl ethanol and 1—2 μl of this solution was brought on to the needle of the solid GC injection system, which was previously used for the determination of underivatized nitrazepam in plasma [3]. After evaporation of the solvent the residue was injected into the gas chromatograph.

Preparation of calibration curves

The concentration of etomidate was calculated with the aid of calibration curves prepared by adding known amounts of etomidate to 1.0 ml blank plasma. These standard samples were analysed by the same procedure as described previously and the ratios of the peak heights of etomidate to internal standard were plotted against the known concentrations of etomidate. The same procedure was followed for estimating the extraction yield of etomidate from plasma at various concentrations, except that propoxate (125 ng) was used as an external standard. The ratios found were compared to the ratios of standard amounts of the drugs. Before analysis of a sample series, calibration was always carried out using at least two plasma samples containing a known concentration of etomidate.

RESULTS AND DISCUSSION

Fig. 1 shows gas chromatograms of extracts from plasma samples taken at 8 and 240 min after the intravenous injection of 17 mg etomidate to a surgical patient, as well as the chromatogram of a blank extract. There is no interference from endogenous plasma substances or metabolites and retention times are short. Propoxate — which is structurally very closely related to etomidate — was chosen as an internal standard in the assay of etomidate and the two peaks are well separated, both at low and high concentrations.

Identification of the compounds eluting from the gas chromatograph was carried out by means of a combined gas chromatograph—mass spectrometer (LKB-2091 with PDP-11 computer system). The mass spectra of etomidate and propoxate show a parent peak at 244 *m/e* and a peak with a relative intensity of 100% at 105 *m/e*, representing in both cases the removed ethylbenzene moiety. On applying mass chromatography for the analysis of a plasma extract containing etomidate and propoxate, peaks with *m/e* values of 105 and 244 coincide with the GC retention time of etomidate, and peaks with *m/e* values of 105 and 258 have the retention time of propoxate (Fig. 2).

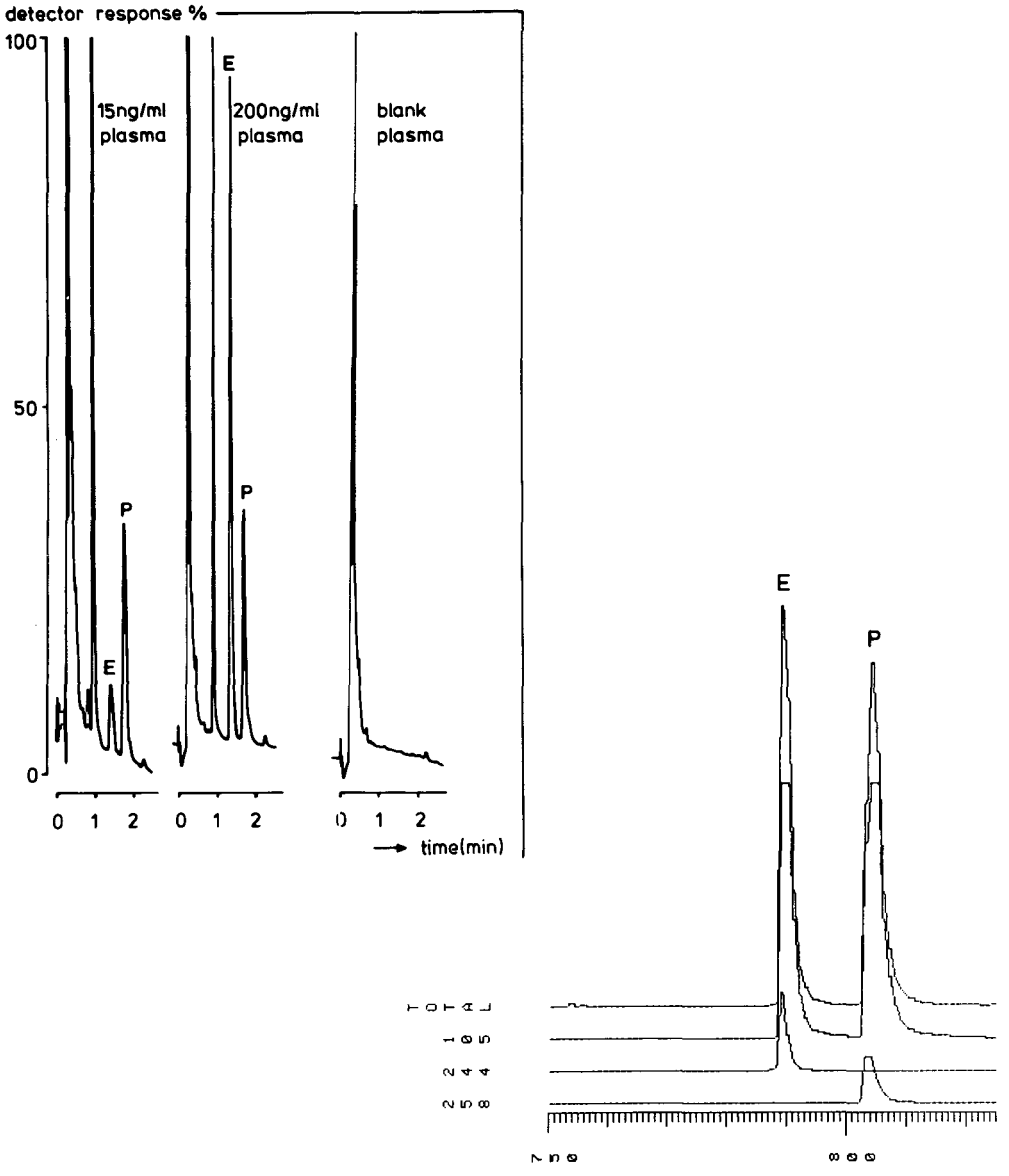

Fig. 1. Gas chromatograms of a plasma extract obtained from a male surgical patient immediately before (right), and 8 min (centre; 200 ng etomidate per ml plasma) and 240 min (left; 15 ng etomidate per ml plasma) after receiving 17 mg etomidate intravenously. E = etomidate, P = propoxate (internal standard; 125 ng/ml plasma).

Fig. 2. Chromatogram of a plasma extract containing etomidate and propoxate. The number on the X-axis refers to the numbers of the mass spectra taken during the particular GC—MS run. E = etomidate, P = propoxate.

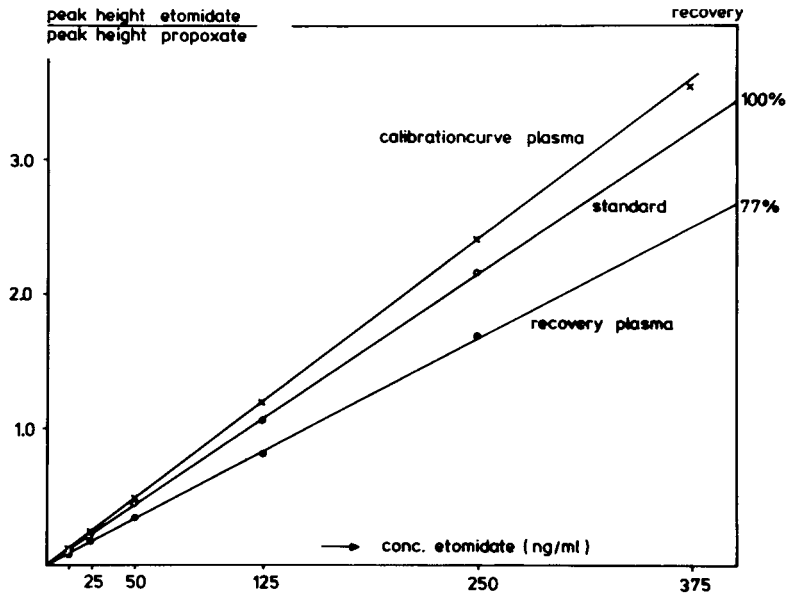

Fig. 3. Peak height ratio of etomidate to propoxate (125 ng) as a function of known etomidate concentrations. The standard curve was obtained with stock solutions of the two compounds. The recovery line was obtained by extraction of etomidate from plasma, using propoxate as external standard. The calibration graph was obtained by extraction of etomidate from plasma, using propoxate as internal standard.

It can be concluded that etomidate and propoxate both leave the GC column unchanged, so that they are being determined in intact form. According to the standard curve (Fig. 3) there is a linearity between the detector response ratio (peak height etomidate:peak height propoxate) and the concentration of etomidate between 12.5 and 375 ng/ml plasma. In Fig. 3 the extraction yields for etomidate in the concentration range 12.5—250 ng/ml plasma are also given. In spite of the short extraction time (15 sec) the recovery of etomidate from plasma is satisfactory (77%) and constant over the whole concentration range. The reproducibility of the whole procedure is reflected in the mean calibration curve of Fig. 3 (n = 5; S.D. $\leqslant$4.9%). It appears that the present method permits the accurate and specific determination of underivatized etomidate and propoxate in plasma in relatively low concentrations. The detection limit is about 5 ng etomidate per ml plasma. The use of the capillary Carbowax 20M SCOT column appears to be a definite improvement in the analysis of etomidate and many samples can be analysed in a short time. A solid injection system is required in order to prevent deterioration of the column support, caused by organic solvents.

Preliminary results have been obtained with surgical patients [4].

ACKNOWLEDGEMENTS

The authors wish to thank Drs. N.P.E. Vermeulen for taking the mass spectra, Mr. L. Bontje and co-workers for drawing the capillary columns, and Mr. L.W. Gerrése and co-workers for constructing the solid injection system.

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Journal of Chromatography, 162 (1979) 596—600
Biomedical Applications

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Note

Direct urinary assay method for N^1-methylnicotinamide by soap chromatography

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(Received August 15th, 1978)

Various analytical methods have been reported for the measurement of urinary excretion of N^1-methylnicotinamide (1-MNA). One of the earlier methods used the formation of a fluorescent condensation product between 1-MNA and acetone in strong alkali and the removal of interfering fluorescent impurities by charcoal [1]. This method has been improved by various workers, with the removal of the impurities by ion-exchange [2, 3] or descending-paper chromatography [4].

Recently, an improved method for the determination of 1-MNA in urine, based on the removal of interfering substances with a single strong cation-exchange chromatographic step, followed by quantitative high-performance liquid chromatographic (HPLC) assay of the isolated 1-MNA was reported [5]. While this method was shorter than former methods, it also required cleanup before the HPLC assay.

We report here a rapid quantitative method for 1-MNA in rat and human urine by direct injection onto a reversed-phase HPLC column operating in the soap chromatography mode.

This system employs a reversed-phase packing in combination with a hydrophilic eluent containing methanol as an organic modifier and a small concentration of a detergent which forms an ion-pair with an ionized form of a solute. To keep the solutes in the preferred ionic forms, the pH of the solution is controlled. This is a rather simple, rapid, and specific method for the direct assay of 1-MNA in urine, requires no pretreatment of the sample, and has a high degree of precision and accuracy.

EXPERIMENTAL

Materials

N^1-Methylnicotinamide chloride was obtained from Sigma (St. Louis,

Mo., U.S.A.). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Glass-distilled methanol (Burdick & Jackson, Muskegon, Mich, U.S.A.) and deionized water were used.

Preparation of urine samples

Sprague-Dawley rats were placed in metabolism cages (two rats per cage) and urine was collected for 24 h. To remove debris, collected urine was centrifuged on a GLC-1 (Sorvall, Norwalk, Conn., U.S.A.) at 3000 rpm for 15 min, decanted, and the supernatant stored at -70° if not used immediately. An aliquot of the above sample (or of fresh normal human urine) was passed through a 0.45-μm Nalge membrane filter (Fisher Scientific Co., Silver Spring, Md., U.S.A.) before direct HPLC analysis.

Recovery of N^1-methylnicotinamide

A stock solution of 1-MNA chloride (10 μg/μl) was prepared in deionized water. Aliquots of urine (1 ml) were spiked with 60, 150, and 300 μg of 1-MNA, and 1-MNA recoveries were determined by direct injection of a few microliters of spiked urine onto an HPLC column.

Apparatus

All analyses were performed at ambient temperature on a Waters Assoc. (Milford, Mass., U.S.A.) Model ALC 202 liquid chromatograph with U6K septumless injector and a Schoeffel (Westwood, N.J., U.S.A.) Model SF-770 variable wavelength (200—700 nm) ultraviolet (UV) detector. Chromatograms were recorded on a 10 mV full-scale Omniscribe recorder (Houston Instruments, Austin, Tex., U.S.A.) operated at 0.1 in./min chart speed.

A 25 cm × 4.6 mm I.D., Partisil-10 ODS analytical column (Whatman, Clifton, N.J., U.S.A.) was used. A 4 cm × 2.1 mm I.D. precolumn, packed with Waters Assoc. pellicular Bondapak C_{18}/Corasil (37—50 μm), was used to ensure the stability of the analytical column. The column was eluted with a mixture of 0.005 *M* SDS, 47.5% methanol, and 0.01% H_2SO_4, at a flow-rate of 1.5 ml/min. The eluent was de-gassed by ultrasonification for 10 min before use. Peak areas and retention times were determined with a Hewlett-Packard (Avondale, Pa., U.S.A.) 3352-A laboratory data system linked through a Hewlett-Packard 1865 A/D converter to the UV detector output of the liquid chromatograph. The output from the data system was registered on a Texas Instruments' (Houston, Tex., U.S.A.) silent 700 thermal line printer.

RESULTS AND DISCUSSION

The recent introduction of soap chromatography [6, 7] has enlarged the scope of HPLC application to biochemical analysis. The method has a particular advantage when applied to urine analysis, since the bulk of the excreted compounds are polar, show UV absorption and will not be retained. We have developed a rapid HPLC method to measure 1-MNA in urine without prior cleanup.

The result of a direct injection of 3 μl of rat urine onto the column is shown in Fig. 1. N^1-Methylnicotinamide is separated from the interfering compounds

under the conditions shown. Several methanol concentrations in the mobile phase were investigated; namely, 40, 45, 47.5 and 50%. All provided adequate resolution of 1-MNA from interfering compounds in urine, and the retention time of 1-MNA was increased by lowering the methanol concentration. Lower SDS (0.002 *M*) concentrations also gave longer retention of 1-MNA. The average retention time for ten identical injections (1—3 μl) was 16.4 ± 0.15% (± S.D.) under the conditions in Fig. 1.

A standard calibration curve covering the range 0.1—0.9 μg of the 1-MNA standard showed excellent linearity (correlation coefficient 0.999). Linearity of response of 1-MNA in urine for urine injection volumes covering the range 1—6 μl was also excellent (correlation coefficient 0.999). The method was used to determine the 1-MNA content of normal human urine, which was found to be 56 μg/ml. Recovery experiments were carried out on 1 ml of control urine samples spiked with 60, 150 or 300 μg of 1-MNA. The overall recovery and its coefficient of variation (C.V.), as shown in Table I, was reasonable, indicating that the method is precise and accurate. The concentration of 1-MNA in rat urine by this method was found to be 117 μg/ml, whereas the method reported in ref. 5 gave 112 μg/ml. This indicates good agreement between the two methods. However, it should be noted that the present method utilizes

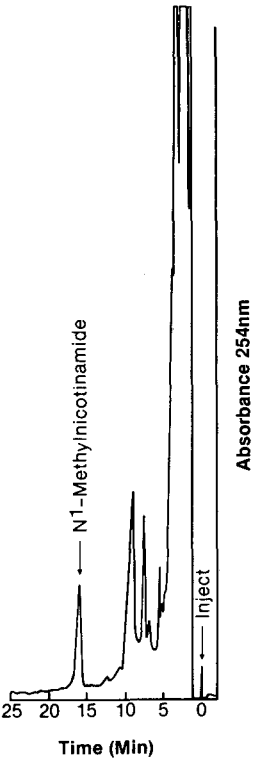

Fig. 1. Soap chromatography of N^1-methylnicotinamide in urine. Conditions: column, Partisil-10 ODS; detection, 254 nm, 0.04 a.u.f.s.; eluent, water—methanol—SDS (52.2:47.4:0.01, v/v/w) with 0.01% added sulfuric acid.

TABLE I

RECOVERY OF 1-MNA ADDED TO URINE SAMPLES

Amount of 1-MNA added (μg)	Recovery (%)
60	91.9
	105.8
	104.1
150	95.5
	100.5
	102.7
312	99.0
	97.7
	103.4
Mean	100.1
C.V.*(%)	4.5

*C.V. (%) = (S.D./mean) × 100.

direct injection of urine onto the HPLC column and the analysis is effected in less than 20 min. In contrast, the method in ref. 5 required pre-cleanup before HPLC analysis, resulting in several hours for the overall assay.

The identity and purity of the 1-MNA peak was established by the following techniques. (A) Alteration of the SDS concentration (0.002 *M vs.* 0.005 *M*) and methanol content (40% *vs.* 47.5%) of the mobile phase gave identical quantitative results for urinary 1-MNA content. (B) A UV spectrum run on collected urinary 1-MNA peaks was identical to that of authentic 1-MNA. The peak effluents were combined and taken to dryness on a rotary evaporator and reconstituted with 3 ml of deionized water for UV spectral analyses. The reference cell and standard compound solutions were prepared from the mobile phase (0.005 *M* SDS, 47.5% methanol, 0.01% H_2SO_4) and treated as above. Both the standard and collected urinary 1-MNA peaks showed a similar UV spectrum with a maximum at 265 nm. (C) The HPLC peak height ratios of urinary and standard 1-MNA were determined and compared at two wavelengths, 254 nm (a general wavelength) and 265 nm (absorption maximum for 1-MNA). The HPLC peak height ratio for 254:265 of urinary 1-MNA (0.65) was similar to that of standard 1-MNA (0.66), and was also similar to the 254:265 absorption ratio from the UV spectrum (0.66).

These techniques, especially the matching of ratios of both standard and sample compound, indicates that the component of interest (1-MNA) in urine is completely resolved from contaminants, thus the peak height ratio method in combination with the other two could be used to obtain an accurate quantitative analysis. The soap chromatographic method described here is hence suitable for assaying 1-MNA in urine, since this compound is well retained and readily separated from other UV-absorbing components. Furthermore, the need for tedious processes for cleanup previously required is avoided by direct injection of a few microliters of urine samples onto the HPLC column. Repeated

injections of urine cause no notable deterioration in column performance. The method can be used for a reliable quantitative assay of 1-MNA for studies in human nutrition, and NAD tryptophan metabolism associated with certain diseases including cancer.

ACKNOWLEDGEMENTS

This research was sponsored by the National Cancer Institute under Contract No. N01-CO-75380 with Litton Bionetics, Inc. We would like to thank Ms. Elaine Yateem for manuscript preparation.

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Journal of Chromatography, 162 (1979) 601—604
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Note

Determination of methyldopa and metabolites in human serum by high-performance liquid chromatography with electrochemical detection*

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(Received September 19th, 1978)

Methyldopa [(S)-3-(3,4-dihyroxyphenyl)-2-methylalanine] is an effective antihypertensive agent which has been used extensively for the treatment of hypertension. Previous studies [1—5] have shown that the absorption, biotransformation and incidence of side effects produced by methyldopa vary in individual patients and may be significantly altered in patients with impaired renal function [6]. Thus, correlating serum levels of methyldopa and its metabolites with therapeutic response may provide a more effective means of using this valuable agent.

The quantitative analysis of methyldopa in biological specimens has most commonly been carried out by fluorometric procedures which are protracted and rather non-specific [1—5]. Other methods utilizing [^{14}C]methyldopa in conjunction with paper and thin-layer radiochromatography [7] or gas chromatography requiring derivatization procedures prior to analysis, have also been reported [8, 9]. Recently, a technique employing high-performance liquid chromatography (HPLC) with ultraviolet detection has been described for assay of methyldopa in serum and urine [10, 11].

This report describes a highly sensitive and selective method for the determination of methyldopa and its principal biotransformation products in serum, utilizing HPLC with electrochemical detection. This recently developed detection system [12] has proved to be extremely sensitive and specific for catecholamines, and compounds of similar chemical structure present in biological

*A preliminary report of these investigations was presented to the Annual Meeting of the Society for Pediatric Research, New York, April, 1978.
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material [13—16]. The technique presented requires a minimum sample work-up time, making this method attractive for routine analyses.

EXPERIMENTAL

All chemicals employed were of reagent grade. Water used in the preparation of buffers was glass-distilled over ethylene diamine tetraacetic acid (EDTA).

High-performance liquid chromatography

The apparatus consisted of a pumping system (Waters Assoc., Model 6000) and an electrochemical detector (Bioanalytical Systems, Model LC-2A). A glass column, 500 mm × 2 mm I.D. (Altex Scientific) was dry-packed with Vydac SCX cation-exchange resin, particle size 30—44 μm (Altex Scientific). The eluent was 20 m*M* ammonium dihydrogen phosphate (pH 2.55) containing 0.1 m*M* EDTA pumped at a flow-rate of 0.4 ml/min with a column pressure of 300—350 p.s.i. The inclusion of EDTA in elution buffers of pH values less than 4.0 was shown to be necessary to suppress erratic fluctuations in the background current, presumably due to electroactive materials present on the resin or in the pump itself. The working electrode potential was set at +0.54 V against an Ag/AgCl reference electrode, and the detector operated at a sensitivity of 2—20 nA f.s.d. depending on the concentration of methyldopa in the biological specimen.

Procedure

To 1.0 ml serum, 50 μl of concentrated (70%) perchloric acid were added. The sample was immediately agitated on a Vortex mixer for 30 sec and kept on ice for 15 min. After centrifugation for 15 min at 1000 *g* (4°), an aliquot of the acid-extracted supernatant was filtered through a Centriflo membrane cone (Amicon) by re-centrifugation at 1000 *g*. An appropriate volume (usually 10 μl) of the ultrafiltrate was injected onto the chromatographic column and the value obtained represented the amount of unconjugated methyldopa present. In order to determine total drug levels, the remainder of the sample was kept in a boiling water bath for 20 min. The hydrolyzed sample was then processed in the same manner as the non-hydrolyzed one. Working standards of methyldopa or its various metabolites containing 0.1—10.0 μg/ml were prepared from outdated normal human serum and treated identically as above.

RESULTS AND DISCUSSION

A representative chromatogram demonstrating the resolution of methyldopa, its metabolite methyldopamine and the endogenous catecholamine neurotransmitters, norepinephrine and dopamine, is shown in Fig. 1A. In addition, 3,4-dihydroxybenzylamine, an exogenous catechol used routinely as an internal standard for tissue catecholamine determinations, has also been included. Under these conditions, serum concentrations of methyldopa in the range of 50—100 ng/ml ($2—5 \cdot 10^{-7}$ *M*) were routinely detected. A chromatogram of a patient's serum prior to and following hydrolysis at 90° is shown in Fig. 1B. Since only the unconjugated species of methyldopa can be detected directly,

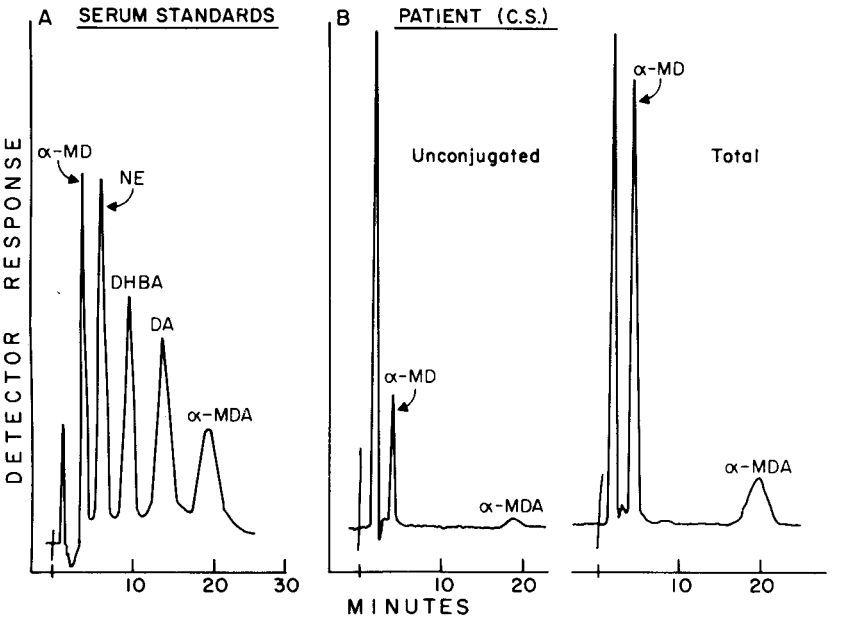

Fig. 1. (A) High-performance liquid chromatogram of reference compounds in human serum. Each peak represents 10 ng of standard compound: α-MD = methyldopa, NE = norepinephrine, DHBA = dihydroxybenzylamine, DA = dopamine, α-MDA = methyldopamine. (B) Elution profile of patient serum before (left) and after (right) hydrolysis at 90°. Unconjugated α-MD, 0.8 μg/ml; total α-MD, 3.4 μg/ml; unconjugated α-MDA, 0.2 μg/ml; total α-MDA, 1.0 μg/ml. Conditions: 500 × 2 mm I.D. Vydac SCX column, with 20 m*M* ammonium dihydrogen phosphate and 0.1 m*M* EDTA as eluent; flow-rate, 0.4 ml/min; electrochemical detection at +0.54 V.

the absolute quantity of sulfated metabolite present in a given specimen is represented by the difference between the total (determined after hydrolysis) and unconjugated (determined prior to hydrolysis) compounds. The decarboxylated metabolite of methyldopa, methyldopamine, can also be quantitatively determined in patient serum by this method, as demonstrated in Fig. 1B. Like the parent compound, methyldopamine also appears to be present both as an unconjugated and sulfate-conjugated species.

Standard curves for methyldopa in human serum were linear over the range 0.05—5.0 μg/ml. Therefore, serial dilutions of sera were routinely performed to insure that measurements fell on the linear portion of the standard curve. The average recovery of methyldopa added to serum when concentrations between 0.05 and 10.0 μg/ml were studied was 89.2 ± 4.1% (mean ± S.D., $n = 35$).

In a preliminary series of investigations, this method has been applied to the quantitation of methyldopa in sera obtained from hypertensive pediatric patients with normal renal function (serum creatinine <1.3 mg/dl). The mean serum concentration of unconjugated methyldopa 8 h after a morning dosage (7.5 mg/kg) was 0.63 ± 0.23 μg/ml, which represents 58% of the total methyldopa present in serum. This value is essentially identical to those recently reported by Myrhe et al. [3]; however, the absolute values reported in this

study were slightly lower. This discrepancy most likely results from the longer interval between dosage and sampling time (8 vs. 4 h), or possibly because the fluorescence method used by Myrhe and coworkers [3, 4] cannot distinguish between methyldopa and methyldopamine, resulting thereby in falsely elevated values.

Serum volumes of 1 ml were used in our investigation, but when sample size is limited, as in pediatric applications, the whole procedure can be successfully performed with serum volumes as small as 100 μl [6]. In addition, this method for the determination of methyldopa and its metabolites in human serum has several important advantages over those previously described. It is more rapid, sensitive and specific than fluorometric techniques and the ultrafiltration purification eliminates the alumina adsorption step [11]. Furthermore, sample derivatization is not needed as for gas chromatographic analysis [8, 9]. The use of electrochemical detection also provides a greatly enhanced sensitivity compared to ultraviolet absorption [10, 11]; this factor also enables the analysis of much smaller volumes. In the procedure of Walson et al. [10] the lower limit of detection was 1 μg/ml, using 1-ml serum samples. Mell and Gustafson [11] processed 5—30-ml aliquots of urine for each analysis, and, moreover, the entire procedure was more time consuming since an alumina adsorption step was used. Both these systems analyzed only parent drug; the current procedure enables analysis of metabolites as well. This technique should facilitate the quantitation of methyldopa and its metabolites in biological specimens acquired from patients on antihypertensive drug regimens.

ACKNOWLEDGEMENT

This investigation was supported by a USPHS grant (HD-08580).

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Journal of Chromatography, 162 (1979) 605—614
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Note

Simultaneous determination of diazepam and its metabolites N-desmethyldiazepam, oxydiazepam and oxazepam in plasma and urine of man and dog by means of high-performance liquid chromatography

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(Received September 14th, 1978)

Benzodiazepine derivatives are widely used all over the world for a variety of clinical indications [1]. Recently, it has become obvious that several drug-associated events, such as drowsiness, hangover and other CNS depressions, are related to intrinsic pharmacological properties. Due to different pharmacokinetics and metabolism, a variety of active metabolites may be formed and these may be responsible for some of the adverse effects. Knowledge of the pharmacokinetic behaviour of diazepam, the most widely used benzodiazepine derivative, is limited by the analytical assay procedure. Due to extensive biotransformation and/or tissue distribution, parent drug and metabolites are present in only trace amounts in body fluids. Several attempts have been made to determine parent compound and metabolites by means of gas—liquid chromatography with electron capture detection [2—4], gas chromatography—mass spectrometry [5, 6], thin-layer chromatography [7, 8] and high-performance liquid chromatography (HPLC) [9—12]. Until now, none of these methods (except that of Kabra et al. [12]) has allowed the simultaneous determination of all the main metabolites of diazepam. Kabra's method [12] however, requires a large sample volume (2 ml).

The method described here affords the simultaneous determination of diazepam, N-desmethyldiazepam, oxydiazepam and oxazepam in 0.2 ml of sample. The method has been applied to pharmacokinetic studies in dog and man and also for routine monitoring in man, for which some results are presented.

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, Emmen, The Netherlands). A stainless-steel column, 10 cm × 4.6 mm I.D., commercially packed with LiChrosorb RP8, particle size 5 μm, (Chrompack, Middelburg, The Netherlands) was used. The injection loop was 100 μl size. Detection of the benzodiazepines and metabolites was effected at 230 nm; the detection limit is 30 ng/ml.

Solvent

The solvent was a mixture of water—methanol—acetonitrile (500:450:50, v/v) and the flow-rate was 1.6 ml/min, at a pressure of 250 atm.

Drugs

Diazepam, N-desmethyldiazepam, oxydiazepam (3-hydroxydiazepam), oxazepam, and flunitrazepam were obtained from Hoffmann-La Roche (Mijdrecht, The Netherlands).

Animals

Beagle dogs from the Central Animal Laboratory of the University of Nijmegen were used in this study. The benzodiazepines were administered at a dose of about 2 mg/kg. The dogs were kept under light anaesthesia (nitrous oxide, oxygen, halothane). Induction was achieved with pentobarbital (Nembutal®). A constant urine flow was achieved by continuous infusion of dextrose solution (5%, w/v).

Subjects

Ten subjects, all employees of the Department of Clinical Pharmacy, Nijmegen, participated in this study. Oxydiazepam and oxazepam were administered orally in doses ranging between 2 and 30 mg. Two volunteers took a mixture of oxazepam and oxydiazepam. The drugs were taken orally in the morning, 1.5 h after a standard breakfast [13]. Blood samples of 0.2 ml were collected at scheduled intervals by fingertip puncture (Microlance No. 433, Becton & Dickinson). Spontaneously voided urine was collected for 60 h.

Sample preparation

Plasma and urine. Ten microlitres of acetonitrile, containing 400 ng of flunitrazepam as internal standard, were added to 0.1 ml of plasma or urine and 2 ml of diethyl ether, and was mixed for 1 min. The mixture was then centrifuged for 5 min at 4000 rpm (2600 *g*) in a Heraus Christ centrifuge. The ether layer was transferred and evaporated to dryness. A 0.2-ml aliquot or the eluent was added to the residue and 0.1 ml was injected onto the column.

Deglucuronidation of the samples. Plasma or urine (0.2 ml) was incubated with 25 μl beta-glucuronidase (100,000 units/ml; Sigma, St. Louis, Mo. U.S.A.), 0.4 ml of KH_2PO_4 buffer (0.067 *M*) and one drop of 0.2 *M* acetic acid for 24 h.

Ten microlitres of acetronitrile containing the internal standard (400 ng flunitrazepam for plasma and 1000 ng for urine) were added together with 0.8 ml Na_2HPO_4 (0.067 *M*) buffer. The mixture was extracted with ether, centrifuged, evaporated to dryness and treated for injection onto the column as described above.

Recovery

The recovery of the extraction for diazepam is 96 ± 7%, for N-desmethyldiazepam 91 ± 7%, oxydiazepam 94 ± 7% and oxazepam 89 ± 6%. The calibration curves were linear for the concentration range 10 ng to 10 μg (r = 0.999). The sensitivity limit for all the derivatives was 30 ng/ml.

RESULTS

Chromatography

Fig. 1a shows a high-performance liquid chromatogram for a reference sample of the calibration curve extracted from human ACD plasma. Flunitrazepam (F) is used as internal standard. There is an excellent separation between diazepam (D) and its main metabolites, N-desmethyldiazepam (DD), oxydiazepam (OD) and oxazepam (OX). Fig. 1b gives an example of an extract of a plasma sample of a dog, 30 min after an intravenous bolus injection of 2.18

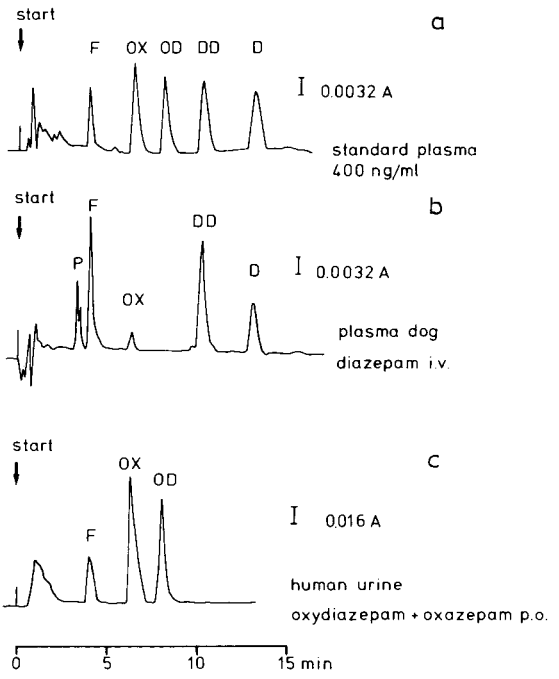

Fig. 1. HPLC chromatograms of diazepam (D) and its metabolites, N-desmethyldiazepam (DD), oxydiazepam (OD) and oxazepam (OX), obtained from: (a) reference sample; (b) dog plasma; (c) human urine. F is the internal standard flunitrazepam.

TABLE I

RELATIVE RETENTION TIMES OF DIAZEPAM AND ITS METABOLITES

Compound	Relative retention time (k')
Flunitrazepam	6.50
Oxazepam	10.67
Oxydiazepam	13.30
N-Desmethyldiazepam	17.00
Diazepam	21.80
Chlordiazepoxide	10.07
Pentobarbital	5.58

mg/kg was given. Note the pentobarbital (P) peak just before the flunitrazepam. Pentobarbital (Nembutal®) was used as the anaesthetic. No oxydiazepam could be detected and only small amounts of oxazepam.

Fig. 1c is an example of an extract of human urine containing relatively large amounts of oxazepam and oxydiazepam, after oral intake of both drugs simultaneously. No interfering compounds are seen. The relative retention times of diazepam and its metabolites are given in Table I. Chlordiazepoxide (Librium®) is also well separated from the other benzodiazepine derivatives, which may be important when measuring plasma concentrations following ingestion of a mixture of benzodiazepines [14].

Benzodiazepines in a beagle dog

Fig. 2 shows the structural formulae of diazepam and its main metabolites. Fig. 3 shows the pharmacokinetics of diazepam and its metabolites in a beagle dog after an intravenous dose of 2.18 mg/kg. Note that diazepam is rapidly eliminated ($t_{½}$ = 80 min) and converted mainly to N-desmethyldiazepam. This metabolite is relatively slowly eliminated ($t_{½}$ = 10 h) by hydroxylation at the C_3 position, which results in the formation of oxazepam (Fig. 2).

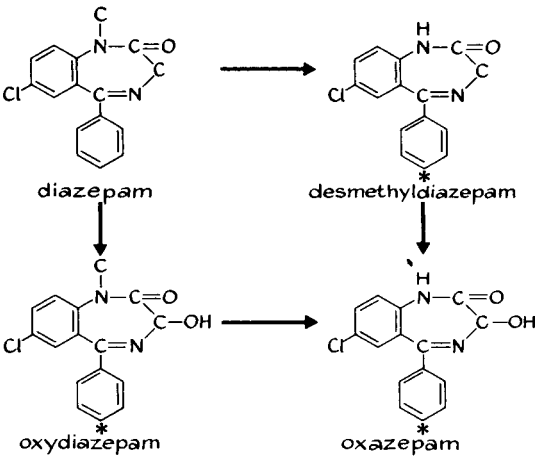

Fig. 2. Structural formulae of diazepam and its main metabolites, N-desmethyldiazepam, oxydiazepam and oxazepam. * = Position vulnerable for hydroxylation.

Diazepam may also be slowly hydroxylated at the C_3 position to give 3-hydroxydiazepam or oxydiazepam, which is present in very minute concentrations as the glucuronide. The N-demethylation of oxydiazepam is fast and results in oxazepam.

After deglucuronidation of the plasma samples only oxydiazepam and oxazepam were present as glucuronides. The formation of oxazepam glucuronide from oxazepam is shown in Fig. 4. Oxazepam, given as an intravenous bolus injection to a beagle dog, was eliminated slowly ($t_{1/2}$ = 3 h) as the free drug, and as the glucuronide with a $t_{1/2}$ of 8 h. The drug is mainly excreted as its glucuronide (24.5% in 7 h) and very little as the free drug (0.07%).

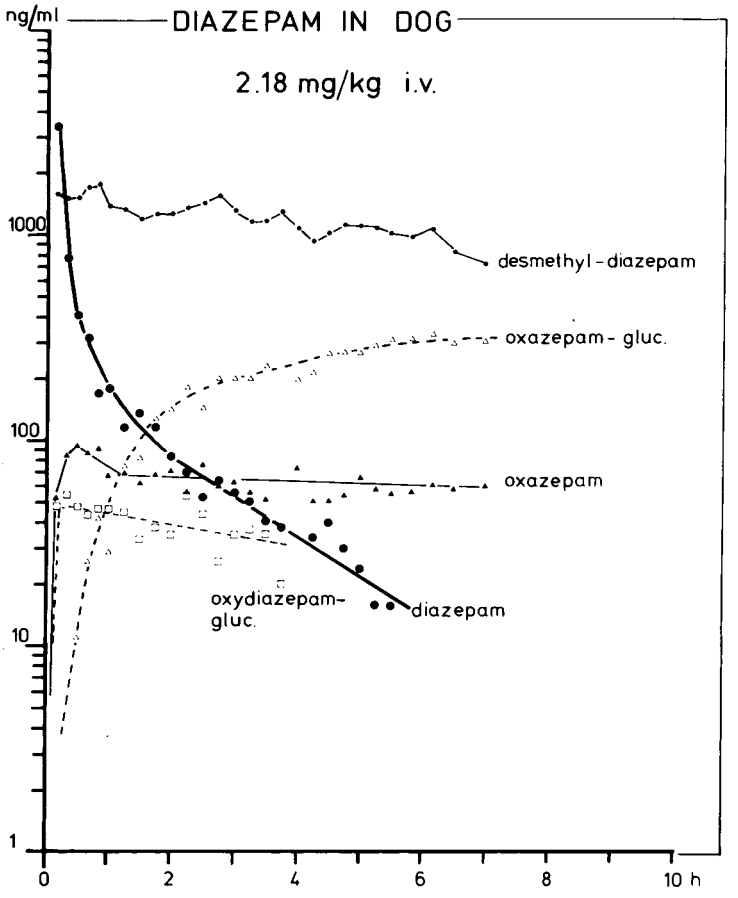

Fig. 3. Pharmacokinetics of diazepam in a beagle dog. Diazepam is rapidly eliminated ($t_{1/2}$ = 80 min) and converted to N-desmethyldiazepam ($t_{1/2}$ = 10 h), oxydiazepam glucuronide, oxazepam and oxazepam glucuronide.

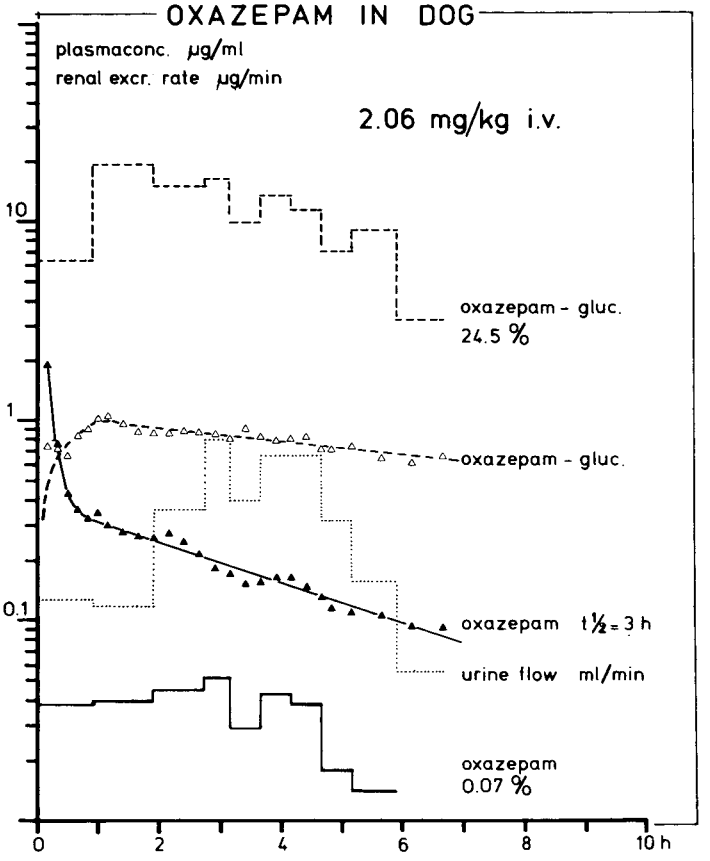

Fig. 4. Pharmacokinetics of oxazepam in a beagle dog. Oxazepam is eliminated by glucuronidation and renal excretion of free drug and glucuronide.

Oxydiazepam and oxazepam in man

The method of extraction and HPLC analysis of benzodiazepines can also be applied to the pharmacokinetic study of diazepam and its metabolites in man. Fig. 5 shows the pharmacokinetics of oxydiazepam and its metabolite oxazepam after an oral dose of 17.8 mg of oxydiazepam (Temazepam®). The renal excretion rate of oxydiazepam glucuronide has a $t_{1/2}$ of 10 h, the metabolite, oxazepam glucuronide, has a $t_{1/2}$ of 12.5 h. Less than 1% of the free drugs is excreted by the kidneys.

The plasma concentrations of oxydiazepam and metabolite show a much shorter $t_{1/2}$ of about 3 h, perhaps indicating an alpha-phase in the plasma elimination curve. We have to remember that in plasma, oxydiazepam is measured

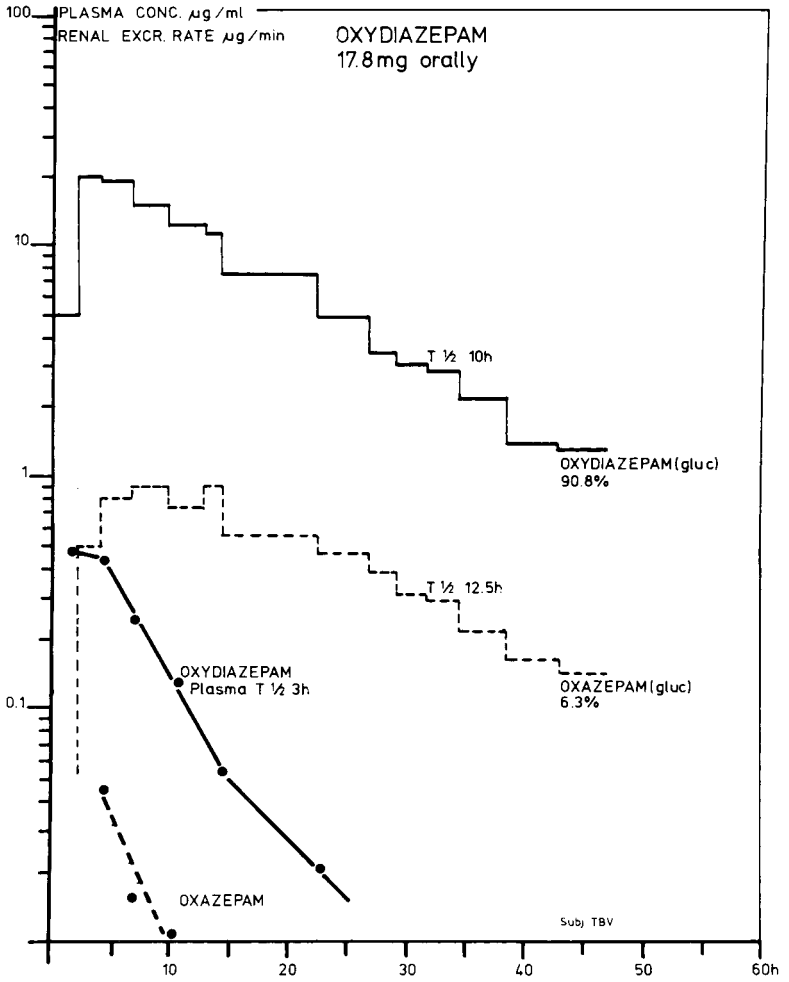

Fig. 5. Pharmacokinetics of oxydiazepam in man after an oral dose of 17.8 mg. The plasma elimination curve, which shows a much shorter $t_{1/2}$ than that of the renal excretion rate of both drugs, shows only the alpha phase of the elimination curve.

as the free compound while in urine the glucuronide is measured. Oxazepam, as parent drug, is excreted almost entirely as glucuronide, with a $t_{1/2}$ of 11.5 h (Fig. 6 and Table II). The renal excretion rates of oxydiazepam glucuronide and oxazepam glucuronide are independent of urinary pH and urine flow. The renal clearance constant varies between 20 and 60 ml/min. Table II summarizes the pharmacokinetic parameters of oxydiazepam and oxazepam in man.

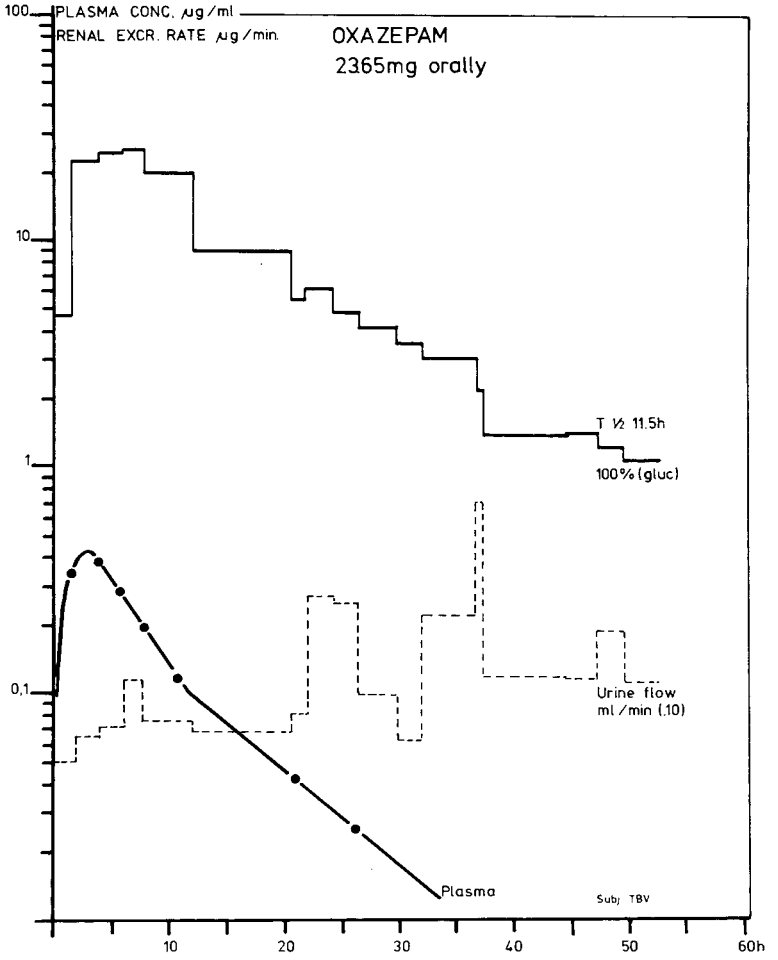

Fig. 6. Pharmacokinetics of oxazepam in man after an oral dose of 23.6 mg. The alpha and beta phases of the plasma elimination curve can be observed. Oxazepam is excreted almost solely as the glucuronide.

TABLE II

PHARMACOKINETIC PARAMETERS OF OXAZEPAM AND OXYDIAZEPAM IN MAN

Compound	*n*	Percentage excreted as glucuronide	Percentage of metabolite excreted as glucuronide	$t_{½}$ (h)	$t_{½}$ of metabolite (h)
Oxydiazepam	5	86.6 ± 9.6	5.8 ± 3.8	7.7 ± 1.7	9.7 ± 2.4
Oxazepam	10	98.3 ± 10.7		7.5 ± 2.1	

DISCUSSION

The method described in this communication permits the study of the pharmacokinetics of diazepam and its metabolites. The small amount of blood (0.2 ml plasma) required, together with the sensitivity limit of 30 ng/ml allows the convenient use of volunteers as subjects in these experiments. Also volunteers are able to collect blood samples of 0.2 ml in a fashion which greatly simplifies and facilitates the experimental design [12]. There are few papers describing the pharmacokinetics of oxydiazepam [15, 16] and oxazepam [15, 17], probably due to difficulties encountered in analysis.

The glucuronidation of oxazepam and subsequent renal excretion of oxazepam glucuronide is the main pathway for elimination of the drug. Similar behaviour is found for lorazepam [18—20], which indicates that glucuronidation of benzodiazepines only takes place at the C_3-hydroxyl group.

Pharmacokinetic processes in man are slower than in the dog. The half-life of diazepam in man is about 40 h [21, 22] and of N-desmethyldiazepam about 60 h [21, 22]. These two drugs have been thoroughly investigated, and that accumulation of the parent drug and metabolite occurs at a consumption rate of diazepam of 5—30 mg/day has been known for about ten years [17]. In analogy to dogs, oxydiazepam and oxazepam may be present in man with a $t_{1/2}$ of 7—10 h from the first dose of diazepam, but, as can be seen from Fig. 3, at a plasma concentration ten times less than that of N-desmethyldiazepam.

This HPLC method is superior to others, as it allows the determination of the main metabolites of diazepam and requires only a small volume of blood. Therefore it can be used in routine patient monitoring in hospitals and as a tool to establish the clinical importance of oxazepam and oxydiazepam.

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Journal of Chromatography, 162 (1979) 615—621
Biomedical Applications

CHROMBIO. 286

Note

Determination of an aromatic retinoid and its main metabolite by high-performance liquid chromatography

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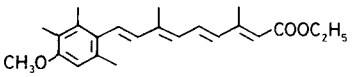
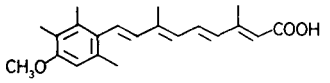
(Received August 16th, 1978)

Many therapeutic successes have been achieved with the retinoic acid analogue, Ro 10-9359 [ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, Fig. 1], in psoriasis patients. The compound



Ro 10-9359



Ro 10-1670

Fig. 1. Retinoid Ro 10-9359 and metabolite Ro 10-1670.

is both therapeutically effective in the mouse and prophylactically effective on epithelial tumors [1—3]. The need to determine the bioavailability of different oral formulations of Ro 10-9359, to monitor clinically-effective plasma levels of patients undergoing oral retinoid therapy and to determine the pharmacokinetic parameters necessitated the development of a sensitive and specific assay for the determination of Ro 10-9359 and its pharmacologically-active main metabolite Ro 10-1670 in plasma (Fig. 1).

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MATERIALS AND METHODS

All work with the retinoids and the internal standard retinoic acid must be carried out in darkened rooms, and all glassware must be amberized to prevent photoisomerisation of these compounds.

Reagents

All reagents must be of analytical reagent grade (>99% purity). The organic solvents were purchased from E. Merck (Darmstadt, G.F.R.).

Columns

The columns were 0.25 m × 3.0 mm I.D. stainless steel, containing 5 μl silica gel (Partisil, Whatman, Clifton, N.J., U.S.A. or LiChrosorb, Merck) and generating 20,000—40,000 plates per m. The temperature of the columns was 20°.

Instrumental parameters

Pump: Milton-Roy 5000. Injector: Altex high-pressure sample injection valve. Detector: Cecil 212 UV detector at 360 nm.

Mobile phases

The isocratic mobile phases were mixtures of: (A) hexane—tetrahydrofurane —glacial acetic acid (98:1.5:0.6, v/v/v) and (B) hexane—methylbenzoate— propionic acid (87.5:12.5:0.35, v/v/v). The mixtures were boiled before use.

Analytical standards

All analytical standards were of pharmaceutical grade purity (> 99%). These included Ro 10-9359 (ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, $C_{23}H_{30}O_3$), Ro 10-1670 (all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $C_{21}H_{26}O_3$) and the internal standard (all-*trans*-retinoic acid, $C_{20}H_{28}O_2$).

Preparation of standard solutions

Place 10.0 mg of the retinoids or the retinoic acid in separate 100-ml volumetric flasks and dissolve in 100.0 ml of tetrahydrofuran to obtain stock solutions containing 100 ng/μl. These stock solutions could be stored without alteration for 3 months at - 20°.

To prepare the standard solutions, aliquots of 10.0 ml of the stock solutions were adjusted to 100.0 ml with hexane. Volumes (10.0 ml)of these solutions containing 10 ng/μl were diluted to 100.0 ml with hexane in volumetric flasks, forming the standard solutions of the retinoids and the internal standard containing 1 ng/μl. These standard solutions could be stored undecomposed for 2 weeks at 5°.

Procedure

To a 20 ml amberized centrifuge tube, add 0.5 ml of plasma, 50 μl of the internal standard solution (= 50 ng retinoic acid), and 4.5 ml of a 0.1 *M* buffer pH 6 solution (citrate—NaOH, Titrisol; Merck). The plasma samples thus

prepared could be stored for 1 week at - 20°. Extract the aqueous phase with 10 ml of hexane by shaking for 10 min on a reciprocating shaker (Heidolph) at 60 rpm. Along with 10 samples, process two samples of 0.5 ml of control plasma, containing 50 µl Ro 10-9359 and Ro 10-1670 standard solutions, respectively (= 50 ng retinoid). Centrifuge the samples at 2000 *g* at room temperature for 4 min. Transfer approx. 9 ml of the upper organic layer into another amberized 20-ml conical centrifuge tube. Evaporate the organic layer to dryness at 40° under a stream of clean, dry nitrogen. Dissolve the residues, cooled to 4°, in two 100-µl aliquots of the mobile phase and filter through a small swab of cotton wool into a microtube. Inject approx. 100 µl. Plasma samples which were not analysed immediately after collection could be stored without alteration for 3 months at - 20°.

Calibration curves

Calibration curves of the peak height ratios (retinoid:internal standard) versus the concentration of the retinoids were prepared, analysing 0.5 ml of the plasma samples, each containing 100 ng/ml of the internal standard and different concentrations of the retinoids (Fig. 2).

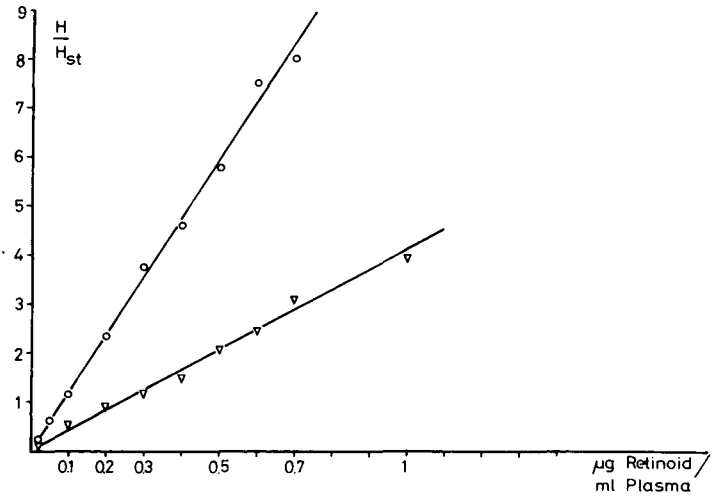

Fig. 2. Calibration curves for Ro 10-9359 (o) and the metabolite Ro 10-1670 (▽). Mobile phase: mixture A.

RESULTS

The assay allows the simultaneous determination of the drug Ro 10-9359 and its main metabolite Ro 10-1670 after extraction from plasma, using their UV absorbance at the absorption maximum of 360 nm. So far, more than one thousand plasma samples from rats, rabbits, dogs and humans have been analysed using this assay.

Using the mobile phase A and a flow-rate of 1.2 ml/min, the retention times of Ro 10-9359, of Ro 10-1670 and of the internal standard were 2.6,

6.0 and 4.1 min, respectively. Under these conditions, a new metabolite of Ro 10-9359, found only in human plasma [4] after multiple dosing of Ro 10-9359, is not separated from the peak of the main metabolite Ro 10-1670 in the chromatogram (Fig. 3).

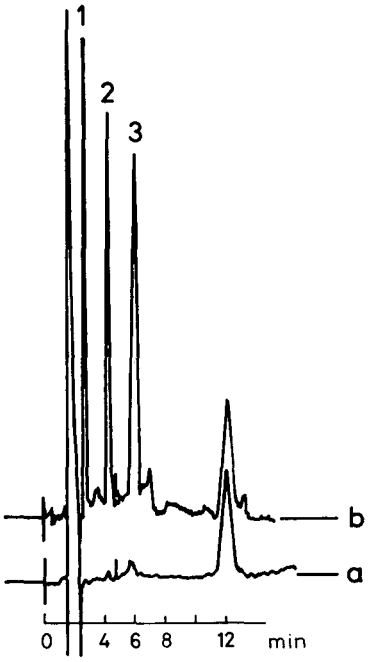

Fig. 3. Chromatograms of the HPLC analysis of: (a) 0.5 ml control plasma and (b) 0.5 ml plasma containing 105 ng/ml Ro 10-9359 (peak 1), 100 ng/ml internal standard (retinoic acid, peak 2) and 210 ng/ml Ro 10-1670 (peak 3). Stationary phase: LiChrosorb SI 60 (Merck); mobile phase: hexane—tetrahydrofurane—glacial acetic acid (98:1.5:0.6, v/v/v) at 360 nm.

Using the mobile phase B and a flow-rate of 1.2 ml/min, the retention times of Ro 10-9359, Ro 10-1670 and the internal standard were 3.2, 8.1 and 4.2 min, respectively. Under these conditions, the peak of the new metabolite, not detectable in Fig. 4, is separated sufficiently from the Ro 10-1670 peak and appears 7 min after injection (Fig. 4).

The calibration curves for Ro 10-9359 and Ro 10-1670 were linear from 10 to 1000 ng/ml of the retinoid, using the mobile phase A, and from 20 to 500 ng/ml of the retinoid, using the mobile phase B.

The relative standard deviation of the whole assay ranges from ± 10 to ± 20% for the unaltered drug and the metabolite Ro 10-1670, within the range of concentration of 50 to 1000 ng/ml and to ± 10 to ± 25% within 10—50 ng/ml of the retinoid. These relatively high values are due to bad extractability specific to these substances and the low concentrations to be determined.

The concentration of each retinoid is determined from the slope of the calibration curves and the peak height ratio, retinoid:internal standard (i.s.) as

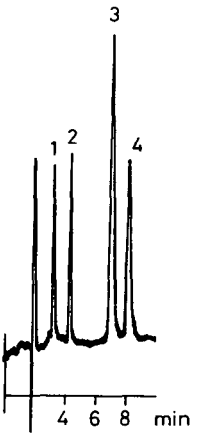

Fig. 4. Chromatogram of the HPLC analysis of 0.5 ml human plasma following multiple dosing of Ro 10-9359, containing 80 ng/ml Ro 10-9359 (peak 1), 100 ng/ml internal standard (retinoic acid, peak 2), 240 ng/ml of the metabolite Ro 10-1670 (peak 4) and an unknown amount of a new metabolite (peak 3). Stationary phase: LiChrosorb SI 60 (Merck); mobile phase: hexane—methyl benzoate—proprionic acid (87.5:12.5:0.35, v/v/v) at 360 nm.

follows:

$$\text{ng/ml Ro 10-9359} = \frac{\text{Peak height Ro 10-9359 (mm)}}{\text{Peak height i.s. (mm)}} \cdot f$$

$$\text{ng/ml Ro 10-1670} = \frac{\text{Peak height Ro 10-1670 (mm)}}{\text{Peak height i.s. (mm)}} \cdot f$$

Average factors (f_A) for Ro 10-9359 and Ro 10-1670, using the mobile phase A, were 84 ng/ml (S.D. = 9 ng/ml) and 235 ng/ml (S.D. = 31 ng/ml), respectively. Using the mobile phase B, they were 90 ng/ml (S.D. = 10 ng/ml) for Ro 10-9359 and 260 ng/ml (S.D. = 34 ng/ml) for Ro 10-1670.

As already mentioned, along with 10 samples, two samples of control plasma containing 50 ng internal standard, 50 ng Ro 10-9359 and 100 ng Ro 10-1670, were analysed in order to control the calibration curves. From these control samples the daily factors (f_D) were calculated and compared with the average factors. If the factors were within the limits given by the standard deviation of the assay, the average factors were used for calculation. If they were within twice the standard deviation, the actual factors were put into the equation, and if there was a large difference between the daily and the average factors, the assays had to be repeated.

The overall recoveries of Ro 10-9359 and Ro 10-1670 determined with the radioactive compounds, were 75 ± 10% and 65 ± 10%, respectively.

The same recoveries were obtained when more polar organic solvents were used, e.g. ethyl acetate, but the chromatograms contained fewer impurities after extraction with hexane.

The limits of detection are in the order of 10—20 ng/ml for Ro 10-9359 and Ro 10-1670, using a 0.5-ml aliquot of plasma.

DISCUSSION

The retinoids Ro 10-9359 and Ro 10-1670 have a high UV absorption at 360 nm, which was utilized for their quantification in the low nanogram range. Both compounds can be analysed either in human or animal plasma (rat, rabbit, dog) using the same procedure and calibration curves. As was shown in an assay with radioactive Ro 10-9359 and Ro 10-1670, the formation of the retinoid—protein complex in plasma is reversible, and the retinoid is extracted by hexane.

Adsorption chromatography was selected in preference to reversed-phase chromatography because of better sensitivity (columns with 20,000—40,000 plates per m could be used) and the fact that the resolution of the *cis*-isomers of the all-*trans* compounds, produced by exposure to light or by biotransformation, was achieved.

The assay presented is based on the comparison of the peak heights of the retinoid with that of an internal standard. For this analytical procedure it is essential to use an adequate internal standard.

Retinoic acid is an ideal internal standard because of the similar chemical and physical behaviour. Irregularities in extracting the compounds from the plasma and the loss of compounds due to absorption on to the glass walls are taken into account. Retinoic acid absorbs enough UV light at 360 nm, the UV absorption maximum of Ro 10-9359 and Ro 10-1670, and is always available in a pure form*.

The disadvantage of this internal standard is its sensitivity to light. Since Ro 10-9359 and Ro 10-1670 are also very sensitive to light the whole analytical procedure has to be done in a darkened room.

Care must be taken in slowly thawing the frozen plasma samples in order to avoid the formation of solid protein particles. After careless thawing, the plasma might not be homogenous and, since the lipophilic retinoids are bonded to a great extent to these proteins, the determination can be inaccurate.

Ro 10-9359 has been shown previously [5] to be highly metabolized and to form at least 21 metabolites. These were found in urine, bile and blood. Most have a shortened tetraen side chain, with a UV adsorption maximum near 260 nm, and do not impair the assay.

The oxygenated metabolites of Ro 10-9359, with an intact tetraen side chain found in rat bile and which show an absorption maximum near 260 nm, are separated sufficiently well with this high-performance liquid chromatographic assay to prevent their interference.

The attempt to perfect a quantitative method of determination on thin-layer plates and a gas chromatographic assay failed because of the extreme photosensitivity and the thermal instability of the retinoids Ro 10-9359 and Ro 10-1670.

Application of the assay to plasma samples containing the retinoids Ro 10-9359 and Ro 10-1670

Typical plasma level curves of the drug and the main metabolite after oral

*Retinoic acid is the active principle of AIROL Roche.

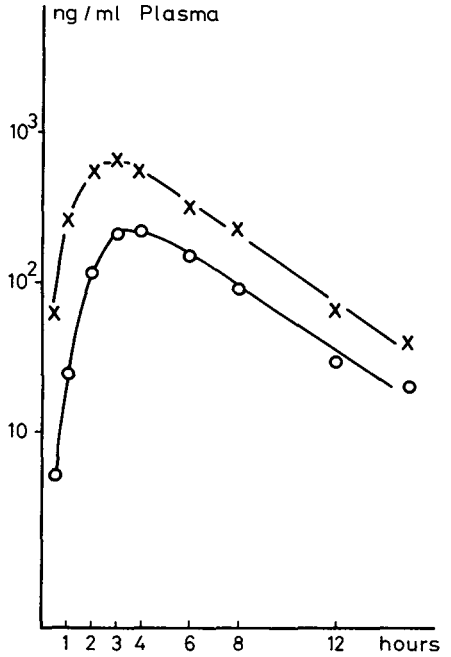

Fig. 5. Plasma levels of Ro 10-9359 (o) and Ro 10-1670 (x) after oral administration of 2 mg/kg of Ro 10-9359 to an adult male.

administration of 100 mg of the drug to an adult male are shown in Fig. 5. The drug reaches its maximum concentration of 230 ng/ml in plasma, 3—4 h following oral administration, showing a relatively slow absorption. After the same period, the metabolite reaches its maximum concentration of 700 ng/ml. Twenty-four hours after administration the concentrations declined to immeasurable amounts.

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Author Index

Errata

J. Chromatogr., 146 (1978) 273—281

Page 278, legend to Fig. 2 should read "Verlauf der Eichkurven für Aldosteron bei Verwendung des Antikörpers gegen (●) Aldosteron-Oxim (PIH; Plasma) und (o) Aldosteron-18,21-Disuccinat (NIH, Lot 088)".

Page 280, ref. 3 should read "B. Kliman und R.E. Peterson, J. Biol. Chem., 235 (1960) 1639".

J. Chromatogr., 162 (1979) 237—243

Page 240, Results, 4th and 5th lines should read "with an acetylation rate of 12 to 47%".

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

APPARATUS

N-1266

ISOELECTRIC FOCUSING AND ELECTROPHORESIS

A new booklet by Pharmacia Fine Chemicals describes the new flat bed system for isoelectric focusing and electrophoresis. In "Focus with Pharmacia" details about the following products of Pharmacia are given:

- Pharmalyte™, a new series of carrier ampholytes,
- the flat bed apparatus FBE 3000,
- the power supply ECPS 2000/300, capable of delivering 300 mA at 2000 V,
- the power supply EPS 500/400, capable of delivering 400 mA at 500 V,
- Sephadex IEF, the support medium for preparative focusing,
- agarose powders for electrophoresis and immunoelectrophoresis.

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-1240

HORIZONTAL ELECTROPHORESIS CELL

Bio-Rad Labs.' Model 1415 horizontal, large capacity, electrophoresis cell is designed with interchangeable accessories for a number of electrophoresis methods. The 125 × 430 mm cooling platform accommodates multiple separations. The entire platform can be used lengthwise to obtain high resolution, or crosswise for preparative separations. The lid is hinged to the unit for easy opening, and includes a condensation coil which prevents dripping on gels or fogging of the lid. The reversible cooling platform has a light colored glass surface for high cooling efficiency and high visibility of colored separations, and a dark plastic surface for visibility of immunoprecipitates.

N-1249

SEMI-MICRO ELECTROPHORESIS SYSTEM

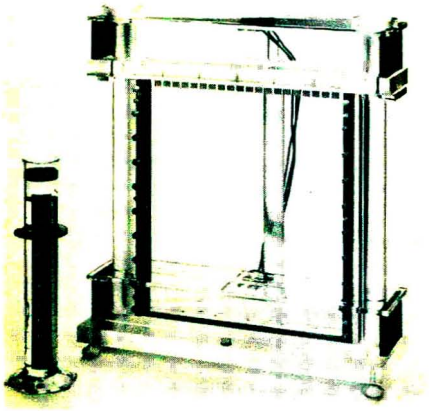
A 4-page bulletin (PB 337R) from Gelman describes the semi-micro electrophoresis system for high-volume electrophoresis. With the system, 1 to 32 tests can be run simultaneously using up to four different procedures. The total electrophoresis time is 15–30 minutes.

The bulletin also describes other Gelman products and chemicals for electrophoresis. Prices and ordering information are included.

N-1241

SLAB ELECTROPHORESIS CELL FOR DNA AND RNA SEQUENCING

100 or more DNA bases can be sequenced electrophoretically in the new 330 × 430 mm Bio-Rad slab cell using the chemical cleavage method of Maxam and Gilbert or the enzymatic method of Sanger and Coulson. The Super Slab is also suitable for RNA sequencing, and for the two-dimensional electrophoresis of proteins using a 290 mm tube gel in the first dimension and the 430 mm slab in the second. The gels are sized for autoradiography on standard sheets of X-ray film. The Bio-Rad Super Slab is an uncooled, single slab cell designed to use plain rectangular glass plates. A precision-molded upper buffer chamber gasket produces a leak-free seal without using matched glass plates. With the Slab a comb which forms 20 wells is supplied.



N-1235

FLUORIMETRIC SUGAR ANALYSER

The new Cenco fluorimetric sugar module (FSM) is a medium-pressure liquid chromatograph for the separation and detection of reducing and non-reducing carbohydrates with detection limits in the lower nanomole range. The module is available as a selfcontained unit with provision for output directly to a mV recorder. Alternatively, the FSM may be coupled to a specially designed integrator–controller terminal where the parameters are automatically monitored and controlled by a microprocessor system, the peaks integrated and the data displayed on a printer. Detection of carbohydrates is done by reaction fluorimetry in the effluent stream. The principle of the method is the chromatographic separation of the borate complexes of the various carbohydrates on an anion-exchange column. The reagent in an unreactive state is part of the eluent and is activated after the column. The fluorescent reaction products of the sugars are passed through a flow-through filter fluorimeter for detection.

CHEMICALS

N-1238

NEW BIOCHEMICAL SEPARATION TECHNIQUE

A prize-winning technique, called "hydrophobic chromatography", forms the basis for exploratory kits and individual polymers marketed by the Miles Laboratories Research Products Division. This technique for isolating and studying proteins and cells, is developed by Professor S. Shaltiel of the Weizmann Institute of Science in Israel and Professor J. Porath of the University of Uppsala in Sweden. Hydrophobic chromatography makes use of a series of water-insoluble polymers, each member of which bears hydrophobic hydrocarbon chains of a given length. Materials can be separated according to their preferential binding to a particular polymer containing the appropriate carbon chain.

N-1263

HPLC COLUMNS FOR CARBOHYDRATE SEPARATION

Bio-Rad Laboratories' Carbohydrate Analysis HPLC Columns are designed for the rapid analysis of high fructose corn syrup, and similar applications. Narrow, well separated peaks are obtained from sugars such as maltose, dextrose, fructose and ribose is less than 10 minutes running time. The columns are packed with Aminex Carbohydrate HPX-87 cation-exchange resin consisting of a tight size range of sulfonic acid resin which has been converted to the calcium salt under controlled conditions to minimize osmotic shock and particle fracturing. The columns are packed according to an optimized procedure.

CALENDAR OF FORTHCOMING MEETINGS

April 18–20, 1979
Carlsbad, Czechoslovakia

2nd Danube Symposium on Progress in Chromatography

Contact:
Dr. Karel Macek, Institute of Physiology CSAV, Budejovicka 1083, Prague 4, Czechoslovakia (Further details published in Vol. 160, No. 1)

April 30–May 3, 1979
Brussels, Belgium

27th Annual Colloquium on Protides of the Biological Fluids

Contact:
Colloquium "Protides of the Biological Fluids", Secretariat, c/o Lipid and Protein Department, Institute for Medical Biology, Alsembergsesteenweg 196, B-1180 Brussels, Belgium

May 7–10, 1979
Boston, Mass., U.S.A.

4th International Symposium on Column Liquid Chromatography

Contact:
Professor Barry L. Karger, Chairman, Organizing Committee, 4th International Meeting on Column Liquid Chromatography, Northeastern University, Institute of Chemical Analysis, Boston, Mass. 02115, U.S.A. (Further details published in Vol. 154, No. 2)

June 11–15, 1979
Budapest, Hungary

4th International Symposium on Analytical and Applied Pyrolysis

Contact:
Dr. L. Leisztner, c/o Research Laboratory for Inorganic Chemistry of the Hungarian Academy of Sciences, Bedaörsi ut 45, H-1112 Budapest, Hungary

June 19–20, 1979
Venice, Italy

10th International Symposium on Chromatography and Electrophoresis

Contact:
Dr. A. Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy

June 21–22. 1979
Venice, Italy

6th International Symposium on Mass Spectrometry in Biochemistry and Medicine

Contact:
Dr. A. Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy

June 17–23, 1979
Frankfurt/Main, G.F.R.

Achema 79

Contact:
Dechema, P.O. Box 970146, D-6000 Frankfurt/Main 97, G.F.R.

June 26–29, 1979
Strasbourg, France

International Symposium on Affinity Chromatography and Molecular Interaction

Contact:
Dr. J.M. Egly, Faculté de Médecine, Institut de Chimie Biologique, 11, rue Humann, 67085 Strasbourg Cedex, France

August 27–31, 1979 Helsinki, Finland	**27th IUPAC Congress** Contact: Dr. J. Larinkari, P.O. Box 244, SF-00131 Helsinki 13, Finland
September 24–28, 1979 Lausanne, Switzerland	**Chromatography '79. 14th International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.
October 21–24, 1979 Philadelphia, Pa., U.S.A.	**18th Annual Meeting of ASTM Committee E-19 on the Practice of Chromatography** Contact: Tim Bradley, Spectra-Physics, 2905 Stender Way, Santa Clara, Calif. 95051, U.S.A.
October 22–25, 1979 Houston, Texas, U.S.A.	**Expochem '79** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.
October 31 – November 2, 1979 Williamsburg, Va., U.S.A.	**The Society of Forensic Toxicologists Annual Meeting** Contact: Robert V. Blanke, Medical College of Virginia, MCV Station, Box 696, Richmond, Va. 23298, U.S.A.
May 27–30, 1980 Balaton Lake, Hungary	**4th Symposium on Ion Exchange** Contact: Professor J. Inczédy, Organizing Committee 4th Symposium on Ion Exchange, P.O. Box 28, Veszprém, H-8201 Hungary
June 30–July 4, 1980 Cannes, France	**13th International Symposium on Chromatography** Contact: Groupement pour l'Avancement des Méthodes Spectroscopiques et Physicochimiques d'Analyse (GAMS), 88 Boulevard Malesherbes, 75008 Paris, France
July 20–26, 1980 Lancaster, Great Britain	**SAC 80** Contact: The Secretary, Analytical Division, The Chemical Society, Burlington House, London W1V 0BN, Great Britain
September 6–12, 1980 Liège, Belgium	**International Solvent Extraction Conference ISEC '80** Contact: Conference Secretary, ISEC '80, Department of Chemistry, University of Liège, Sart Tilman, B-4000 Liège, Belgium

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

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.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

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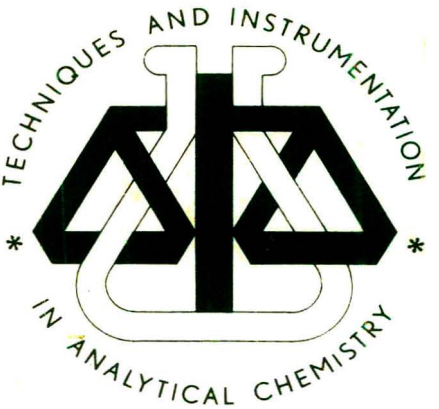
Evaluation and Optimization of Laboratory Methods and Analytical Procedures



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D.L. MASSART, A. DIJKSTRA *and* L. KAUFMAN.

with contributions by S. Wold, B. Vandeginste *and* Y. Michotte

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