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For contents see p. VII

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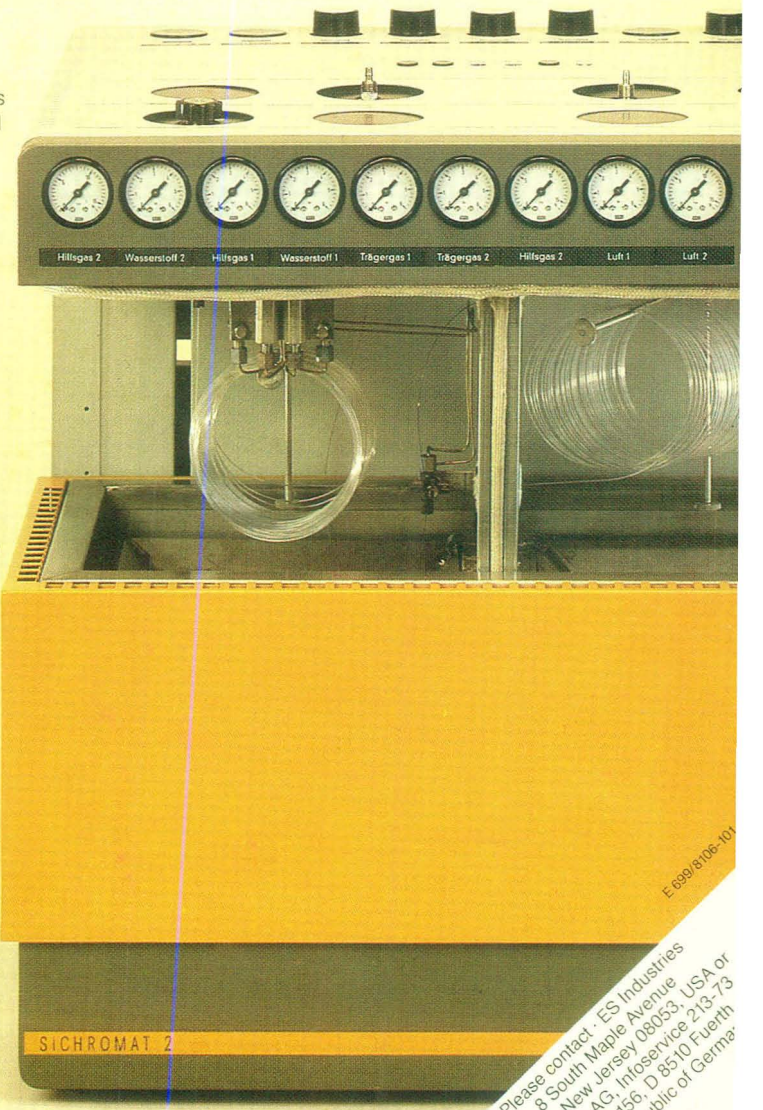

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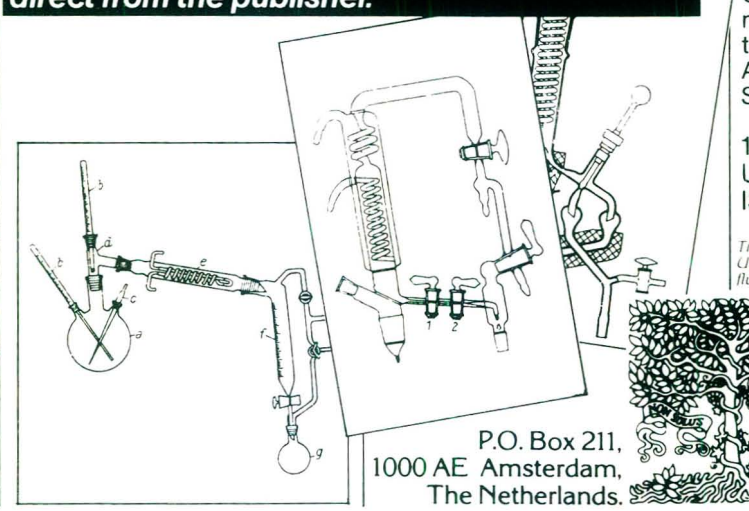

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A Survey of Techniques and Applications

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PART A: TECHNIQUES

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BIOMEDICAL APPLICATIONS

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SPECIAL ISSUE

8TH INTERNATIONAL SYMPOSIUM ON BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY

Hradec Králové and Nové Město nad Metují (Czechoslovakia), September 5–8, 1982

Edited by

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CONTENTS

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CHROMBIO. 1548

PREFACE

The Eighth International Symposium "Biomedical Applications of Chromatography" was held in Hradec Králové and in Nové Město nad Metují castle (Czechoslovakia) from September 5—8, 1982. The symposium was organized by the Chromatography Group of the Czechoslovak Chemical Society and sponsored by the Chromatography Group of the Society for Clinical Chemistry and Laboratory Diagnostics of the German Democratic Republic, the Institute of Physiology of the Czechoslovak Academy of Sciences, and the Charles University Faculty of Pharmacy. The Symposium was attended by 107 active participants from 16 countries.

This Symposium is the eighth in a series of meetings organized by both societies mentioned above. This series started in 1966 in Leipzig and was devoted to the clinicochemical applications of chromatography. In the sixties the leading techniques in the field were thin-layer chromatography and classical column liquid chromatography, and the applications dealt in their vast majority with basic biomedical research. Today the main chromatographic technique is high-performance liquid column chromatography and the main domain besides proteins, enzymes and their constituents is drug monitoring and pharmacokinetic studies. These trends were naturally reflected in this Symposium.

The program of the Symposium was divided into five sections:

A. Plenary lectures (held in the castle of Nové Město nad Metují),
B. General techniques,
C. Amino acids, peptides and proteins,
D. Metabolic and pathological studies,
E. Drugs.

In Section A there were nine plenary lectures (out of which only three are presented in this special issue):

1. E. Jellum (Oslo, Norway): The strategy and perspectives of multicomponent separation techniques,
2. A. Kuksis (Toronto, Canada): The strategy of lipid separation by chromatography and quantitation procedures,
3. J. Janča (Brno, Czechoslovakia): The perspectives of field-flow fractionation in biomedicine,
4. J. Turková (Prague, Czechoslovakia): Perspectives of high-performance bioaffinity chromatography,
5. A.F. Fell (Edinburgh, Great Britain): New perspectives in multichannel detection for chromatography,

6. J. Čoupek (Prague, Czechoslovakia): New developments in the field of sorbents for HPLC,
7. H. Gleispach (Graz, Austria): The application of stable isotopes to metabolic studies using GC—MS—data system,
8. M. Novotný (Bloomington, IN, U.S.A.): Metabolic profiling in the animal models of human diseases,
9. J.A.F. de Silva (Nutley, NJ, U.S.A.): Analytical strategies for therapeutic drug monitoring.

There were 79 papers reported during this meeting out of which there are 24 papers published in this issue in extenso.

KAREL MACEK

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CHROMBIO. 1523

APPLICATIONS OF RAPID-SCANNING MULTICHANNEL DETECTORS IN CHROMATOGRAPHY

PLENARY LECTURE

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Department of Pharmacy, Heriot-Watt University, Edinburgh, EH1 2HJ (Great Britain)

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SUMMARY

A review of progress in the field of multichannel detection in column and thin-layer chromatography is presented, together with some novel applications of a computer-based, linear photodiode array UV—visible spectrophotometer for detection in high-performance liquid chromatography (HPLC). Computer-aided methods for simultaneous monitoring of the elution profile at three wavelengths with automatic peak detection and capture of UV spectra are described. The continuous calculation of absorbance ratios during elution is discussed as an index of peak homogeneity. A novel technique for the enhancement of qualitative identification in HPLC, based on transformation of captured spectra to the second derivative or to the decadic logarithm, is proposed. These developments are exemplified by a model system of diacetylmorphine and its principal metabolites and degradation products, morphine and 6-acetylmorphine. The potential utility of three-dimensional projections of (A,λ,t) data is discussed in the context of pharmaceutical, bioanalytical and forensic applications.

INTRODUCTION

Of the numerous physical methods advocated as detectors in column liquid chromatography (LC) and in thin-layer chromatography (TLC), those based on the measurement of transmittance, absorbance or, in the case of TLC, reflectance of radiation in the UV—visible range have found most application

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[1]. In the absence of a truly universal detector, the UV—visible spectrometer, based on the combination of a monochromator and photomultiplier tube (PMT), has proved to have a wide dynamic range, high signal-to-noise ratio (SNR) and long-term reliability for the detection of those compounds of pharmaceutical and bioanalytical interest with appreciable absorption in the UV—visible spectrum. However, many analytes display only moderate absorptivity and a relative lack of selectivity, which leads to problems of sensitivity and specificity, especially in bioanalytical studies. These problems may sometimes be overcome by exploiting natural or selectively-induced fluorescence for fluorimetric detection, which in favourable cases may be two or more orders of magnitude more sensitive as a chromatographic detector [2].

A major limitation of conventional UV—visible and fluorimetric detectors in chromatography is that only one wavelength resolution element is registered at a time. Although this may have the advantage of simplicity, the disadvantage implied by lack of detection flexibility and the consequent use of non-optimal wavelengths for the detection of multiple components in a chromatogram, imposes a significant limitation on the information-gathering power of the chromatographic method. To a certain degree this disadvantage can be overcome by the use of a scanning spectrophotometer in the stop—flow mode for LC [3] or at specified positions on the chromatoplate in TLC [4].

More convenient, however, are the rapid-scanning spectrophotometric detectors based on electronic, electromechanical or multichannel devices. One of the earliest approaches to rapid-scanning UV detection in column LC was the device of Bylina et al. [5], based on the ingenious combination of a conventional monochromator and PMT with a cathode-ray tube. The high-intensity quartz phosphor screen functioned as a radiation source. Electronic scanning of the screen in the axis of the monochromator slit caused the point source to generate a spectrum in the range 230—750 nm, permitting the transmission spectrum of the eluate to be recorded in 10 msec and the absorbance ratio at any wavelength pair to be calculated. An electromechanical analogue of this device is the oscillating mirror detector developed by Denton et al. [6] for high-performance liquid chromatography (HPLC). This was successfully used to demonstrate three-dimensional presentation of spectral data (A,λ,t) acquired as "time-slices" in the elution profile of uracil, cytosine and adenine. The normal elution chromatogram could be presented at stationary wavelength, optimised for each component. However, the low source intensity of the Bylina device and electromechanical problems of wavelength registration in Denton's detector have led to their being superceded by optical multichannel methods of detection in chromatography.

The advent of optical multichannel detectors based on the linear photodiode array (LDA), the silicon intensified target (SIT) vidicon or the charge-coupled device (CCD) has transformed the technology of detection systems in spectroscopy [7—10] and consequently in column LC [11—20], TLC [21—23] and in gel electrophoresis [23, 24]. These devices are, of course, limited to the detection of analytes with appreciable absorption or fluorescence in the UV—visible spectrum. They do, however, offer particular advantages for detection in chromatography. The rapid acquisition of spectral data should lead to a time-domain multiplex advantage, since simultaneous detection of N spectral

resolution elements reduces the observation time by a factor of N to yield an improvement in SNR by $\sqrt{N}$. An intrinsic advantage of the optical multichannel detector is that spectral data in digital form can be readily stored, manipulated and presented in any desired format. The wide variety of digital algorithms available for transformation of spectral data has led to the proposal of several new strategies for detection in LC [20].

The SIT vidicon, first proposed as a detector for HPLC by Rogers [25], was evaluated by McDowell and Pardue [14], who used a grating polychromator and reversed optics to disperse the eluate transmission spectrum across the front surface of the detector. In the plane of dispersion, each of the several hundred photosensitive diodes acts as a spatially separated resolution element, corresponding to a specific nominal wavelength and monitored by a scanning electron beam [7, 8]. The entire spectrum is registered in computer memory and can be manipulated by a number of digital techniques: ensemble averaging (to improve SNR); presentation of chromatograms at a number of observation wavelengths; and spectral deconvolution of overlapping chromatographic peaks. These concepts were extended by Klatt [15], who used the SIT vidicon to present spectral data during elution, followed by post-run processing by laboratory computer to generate three-dimensional (A,λ,t) surface projections of oil sample eluates, to characterise different degrees of hydrogenation. Moreover, the dispersed spectrum can be optically segmented across the vidicon tube surface to give sub-nanometer resolution if required [16].

The SIT vidicon has also shown promise as a fluorescence detector in HPLC. This approach has been developed by Christian and co-workers [9, 10, 17] to generate the three-dimensional emission–excitation matrix (I_f, λ_f, λ_{ex}), using a computer-aided videofluorimeter based on the SIT vidicon and twin polychromators. The emission–excitation matrix, captured at any time t during the elution of petroleum hydrocarbon fractions, has been shown to yield highly characteristic qualitative data of (I_f, λ_f, λ_{ex}, t) in four-dimensional space [17]. More recently, Warner and co-workers [18, 19] have applied similar techniques for detection in HPLC and have made an extensive analysis of the data reduction algorithms required to apply the videofluorimetric detector efficiently to petrochemical analysis.

Christian and co-workers [21] have further examined the potential of the SIT vidicon for detection in two spatial dimensions in TLC. By illuminating the HPTLC chromatoplate with uniform excitation radiation, the fluorescence spectrum can be produced by dispersion at sequential points along the elution axis d_1 of a single track. This method yields a three-dimensional record (either as a contour plot or as an isometric projection) of (I_f, λ_f, d_1) without mechanical scanning, and has been successfully applied to resolve the overlapping spots of two porphine derivatives [21]. By sacrificing the emission wavelength variable and using a fixed-emission wavelength filter, the system can be modified to record the fluorescence intensity over the entire surface of the HPTLC chromatoplate to give a three-dimensional record of ($I_f^{\lambda(f)}$, d_1, d_2), where the spatial dimension d_2 is normal to the elution track d_1 [21]. Thus three-dimensional isometric plots can be presented for multiple elution tracks on a single chromatoplate, or for separations achieved by the technique of two-dimensional TLC (reserving the term "dimension" for its correct mathematical

connotation [26]). The SIT vidicon has also been employed for rapid non-mechanical scanning of the densitometric profile of separations by TLC [22, 23] or by gel electrophoresis [23, 24].

Early work using the LDA detector either in single-beam [11] or in dual-beam configuration [12] demonstrated its potential usefulness as an LC detector much less expensive than the SIT vidicon. The 256-element Reticon LDA required Peltier cooling (—30°C) to sufficiently reduce the background noise level. Digital methods for ensemble averaging (or integrating) spectra at the rate of 20 or more per second were used to improve the SNR. Milano et al. [11] demonstrated the practicality of presenting chromatograms at any wavelength in the 256-nm range captured. They were the earliest to use the first derivative spectrum to eliminate a component from a chromatogram. In principle, the observation wavelength is selected to coincide with the λ_{max} of the component to be eliminated, where $dA/d\lambda = 0$ [11, 13]. Unfortunately, the method yields bipolar chromatograms and suffers from lack of sensitivity, except for sharp peaks whose spectral inflection points are close to the observation wavelength.

A more general method recently proposed [20] for the improved resolution of overlapping bands in HPLC, involves transformation of the elution profile to its second or higher even derivative in the time domain (d^2A/dt^2, d^4A/dt^4). As discussed later in this paper, the even derivative of a Gaussian band is sharper than the original band. This effect can therefore be exploited to enhance the resolution of peaks in HPLC [20, 27] and in TLC [28], albeit at the expense of a deterioration in the SNR. Another technique for the analysis of overlapping chromatographic peaks is the method of spectral deconvolution [13, 15], which requires among other things that the spectra to be deconvoluted are well defined and sufficiently dissimilar [20].

Several digital techniques for extending detection capability in LC have been given renewed impetus by the recent commercial development of microcomputer-controlled LDA spectrophotometers. These techniques include:

calculation of absorbance ratios at a pair of wavelengths specified for each peak as an index of chromatographic homogeneity [5];

generation of second and higher even derivative spectra in the wavelength domain for enhanced qualitative discrimination [20];

transformation of the elution profile to its corresponding second and higher even derivative in the time domain for the improved detection and resolution of overlapping peaks [20, 27, 28];

deconvolution in the wavelength domain of peaks which overlap in the elution profile, using zero order, first or higher derivative transformation of the absorption spectra [13, 15, 20];

conversion of spectral data to logarithmic format for direct comparison in computer-aided library searches [29, 30].

Moreover, the availability of data in digital form, stored and manipulated by an on-board microcomputer, permits the facile execution of conventional operations:

scaling to improve detection of both minor and major components in one chromatogram;

ensemble averaging for improved SNR throughout the spectrum;

"clustering" of adjacent diodes for improved SNR at a given nominal wavelength;

subtraction of spectra for compensation;

or division of spectra for qualitative comparison purposes.

The addition of a bench-top microcomputer to the LDA spectrophotometric detector enables multiwavelength plotting of chromatograms and the presentation of pseudoisometric or isometric three-dimensional displays of (A,λ,t) data [31].

The present paper reports the development of computer-aided algorithms, based on a commercial LDA spectrophotometer, and their application in HPLC for the detection and characterisation of a model system of diacetylmorphine (heroin) and its potential metabolites and degradation products. This drug system was selected as being illustrative of problems encountered in pharmaceutical manufacture, in bioanalytical investigations and in forensic toxicology.

EXPERIMENTAL

Reagents and materials

HPLC grade acetonitrile (Rathburn Chemicals, Walkerburn, Great Britain) was used as received. Tetrabutylammonium phosphate (Pic A reagent; Waters Assoc., Milford, MA, U.S.A.) was diluted to 0.005 *M* with glass-distilled water and then filtered through a Millipore® 0.45-μm MA filter using an all-glass apparatus. Eluent was degassed under reduced pressure in an ultrasonic bath for 10 min. Diacetylmorphine (DAM), 6-acetylmorphine (6AM) and morphine (MOR) were kindly provided by the Home Office Central Research Establishment, Aldermaston, Great Britain and were shown to be chromatographically homogeneous. The internal standard *p*-hydroxybenzoic acid (PHBA; Koch-Light, Colnbrook, Great Britain) and sulphuric acid (BDH, Poole, Great Britain) were of reagent grade. Standard solutions of DAM, 6AM and MOR were prepared in eluent as used for HPLC (vide infra) or in acetonitrile—0.02 *M* sulphuric acid (4:1). The internal standard PHBA was incorporated at 50 μg/ml (for pharmaceutical dosage forms) or at 0.20—0.40 μg/ml for dilute solutions at biological concentrations, to give peak height comparable with the anticipated peak heights of interest.

Equipment

The modular liquid chromatograph assembled in the laboratory comprised a constant-flow LC pump with integral pulse damping (Gilson Model 302; Villiers-Le-Bel, France), a Rheodyne injection valve (Model 7125; Berkeley, CA, U.S.A.), furnished with either 20-μl or 50-μl loops. The 100 x 5 mm I.D. stainless-steel column (Shandon Southern Instruments, Runcorn, Great Britain) was slurry-packed with a microparticulate aminopropyl bonded silica material (5-μm APS-Hypersil; Shandon Southern Instruments) by the upward displacement technique recommended by the manufacturers.

The optical multichannel detector system employed was the Hewlett-Packard (Palo Alto, CA, U.S.A.) Model HP 8450A UV—visible spectrophotometer, equipped with an 8-μl quartz flow-cell (Model 178-32 QS; Hellma, Mühlheim-Baden, G.F.R.). Spectral resolution was 1 nm per diode in the UV (200—400 nm) and 2 nm per diode in the visible range (400—800 nm). The

spectrophotometer was configured via the RS232C port (9600 Baud) with a Model HP 85 laboratory microcomputer, equipped with 32 kbyte total RAM storage, input-output ROM, print-plot ROM, matrix ROM, and RS232C and HP-IB IEEE-488 interface ports. Two configurations were employed for the present work. In configuration A (Fig. 1A), the HP 85 microcomputer was connected via the HP-IB port to a graphics plotter (Model HP 7225B) and a dual 8-inch floppy-disk mass storage unit (HP 9895A). The alternative configuration (Fig. 1B) employed the on-board tape unit of the microcomputer as a program source. The mass storage unit was connected via HP-IB directly to the spectrophotometer for rapid transfer and storage of "time-slice" spectral data during elution, the fastest sampling rate being 2.3 sec per UV spectrum. Configuration B has the merit of being identical both for data acquisition and data processing, and permits the storage of 1200 UV spectra. However, other configurations proposed by the manufacturers [31] require total instrument shut-down and the disconnection of equipment, to allow the mass storage unit to be reconfigured with the microcomputer for subsequent data processing. In both configuration A and B, the HP 85 microcomputer has been designated as controller, over-riding the central control of the spectrophotometer itself, an arrangement which has proved convenient in practice [32].

A software package ("KEYBOARD") establishes direct control of the LDA spectrophotometer by the HP 85 microcomputer. This allows instructions for

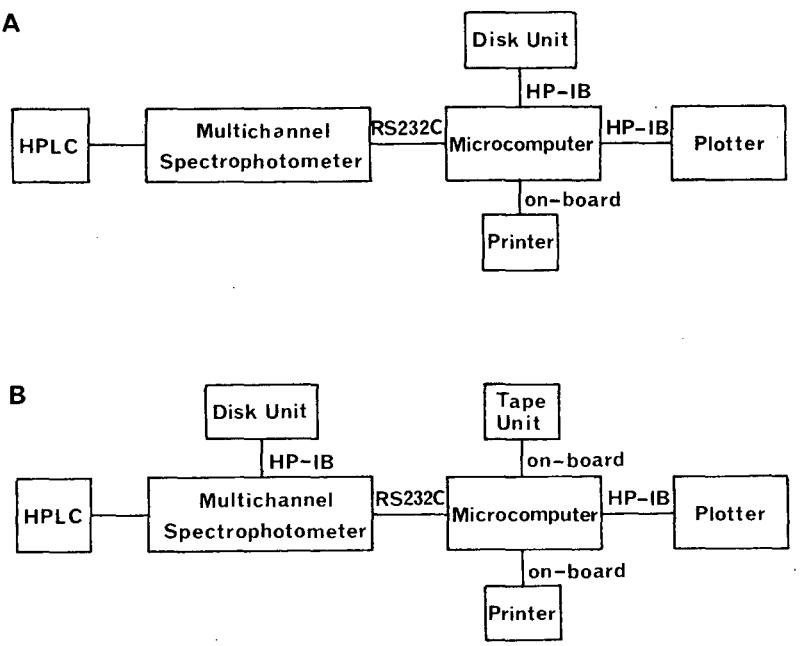

Fig. 1. Configurations of HP 8450A linear photodiode array spectrophotometer and peripherals used for multichannel detection in HPLC. Inter-connection (HP-IB, RS232C) is as indicated, the printer and tape drive units being integral with the HP 85 microcomputer. (A) System for multiwavelength monitoring under HP 85 control, captured spectra being stored by the spectrophotometer. (B) System for continuous spectral capture at 2.5-sec intervals under HP 85 control, captured spectra being stored by the twin disk-drive unit, followed by post-run processing to generate three-dimensional (A,λ,t) chromatograms.

any valid operation by the spectrophotometer to be entered via the alphanumeric keyboard of the HP 85 and translated into a local system code. A suite of programs has been developed to enable the spectrophotometer to function as a detector for HPLC [32]. Using configuration A, absorbance values at up to three wavelengths can be sent to the HP 85 every second and plotted directly on its monitor screen. A peak detection algorithm, based on the first derivative of the elution profile above a selected minimum threshold level, can be used to instruct the spectrophotometer to measure and store a UV spectrum automatically. The spectrophotometer can store up to 92 UV spectra, which can also be captured by manual interrupt. At the end of the chromatographic run, the spectra can be transferred from the spectrophotometer to disk for permanent storage and post-run processing. Firmware routines available on the LDA spectrophotometer include smoothing, transformation to the first or second derivative, spectral deconvolution, logarithmic spectra and spectral subtraction. Peak heights in the chromatogram can be measured automatically at each of the observation wavelengths and corrected for baseline drift. The alternative configuration (B), which allows "time-slice" spectra to be acquired for storage as the (A,λ,t) matrix, permits digital averaging of spectra within the sampling interval. This technique enables the SNR of spectra to be improved, a feature which is of importance for measurement at low absorbance.

Graphics routines have been developed for each of the instrument configurations. The chromatographic data acquired by system A can be presented at each of the selected observation wavelengths, with the absorbance ratio at two wavelengths superimposed. Captured spectra can be plotted above the chromatogram and compared with plots of normalised standard spectra retrieved from data archive. The captured spectra and their derivative or logarithmic transformations can also be compared with the corresponding standard spectra. "Time-slice" spectra acquired using configuration B can be presented as a three-dimensional plot of (A,λ,t). This routine requires typically 30—45 min to generate a pseudo-isometric projection of ca. 240 files at a selected angle (25° in the present report). A hidden-line removal algorithm eliminates any spectral data which would normally be obscured by a peak in the foreground at the observation angle selected. Simple manipulation of the spectral data files enables the projection to be reversed, so that bands hidden by foreground peaks can be more easily observed. Moreover, any particular region in the (A,λ,t) chromatogram can be isolated and presented separately for closer examination if required.

HPLC method and procedure

The published method of Baker and Gough [33] was adapted, using a shorter (100-mm) column packed with 5-μm aminopropyl bonded silica from an alternative source (APS-Hypersil) and an optimised eluent flow-rate of 1.2 ml/min to give more rapid separation. The eluent composition was acetonitrile—0.005 *M* tetrabutylammonium phosphate (85:15, v/v). A test mixture of DAM (retention time 4.0 min) was used to routinely check the column performance, the average number of theoretical plates being 35,000—40,000 plates/m. The retention times for the internal standard (PHBA) and

for morphine were 2.9 and 9.2 min, respectively. The HPLC flow-cell was referenced against air, the detector baseline being balanced for 10 sec prior to chromatography.

After introducing a sample via the loop valve injector, the spectral data were acquired every 1 or 2 sec using configuration A, and at 2.5-sec intervals using configuration B. The observation wavelengths were 254 nm for PHBA and 280 nm for DAM, 6AM and MOR. Data at 300 nm were used to check the baseline. UV spectra were acquired on-line for peaks detected by the gradient-sensitive algorithm described above and stored by the spectrophotometer. At the end of the chromatogram the corrected absorbance of each peak above a pre-set threshold was automatically measured and listed on the HP 85 printer.

Analytical curves of peak height (and of peak height ratio with respect to the internal standard) against analyte concentration were rectilinear and passed through or close to the origin at both low (0—1 mg/ml for DAM) and high sensitivities (0—4 μg/ml for DAM, 6AM and MOR). For replicate 50-μl injections (n = 8) of each component the relative standard deviations (R.S.D.) of corrected peak heights at 280 nm (and of the corresponding peak height ratios to internal standard at 254 nm, in brackets) were: DAM 200 ng, 2.7% (1.3%): 6AM 80 ng, 7.3% (6.6%); MOR 160 ng, 10.5% (10.1%); and for PHBA (10 ng) at 254 nm, 2.3%. At higher concentrations, replicate 10-μl injections (n = 8) of each component gave the following R.S.D. data for peak heights at 280 nm (with peak height ratio to internal standard at 254 nm, in brackets): DAM 5 μg, 2.7% (1.2%); 6AM 2 μg, 2.3% (1.2%); MOR 4 μg, 2.9% (3.5%); and for PHBA (0.5 μg) at 254 nm, 1.9%. The R.S.D. of peak retention times varied from 0.96—1.05% (n = 8). After the chromatographic run, the zero order spectrum captured for each peak was plotted and coded in elution sequence directly above the chromatogram. Standard spectra were superimposed for direct comparison. Spectral data were then further manipulated by smoothing or derivative routines, the results of which could be plotted separately and compared directly with standard spectra similarly treated. When configuration B was employed, the UV spectra (200—400 nm) acquired on disk at 2.5-sec intervals during chromatography were processed by a graphics routine to plot three-dimensional (A,λ,t) chromatograms at 25° in forward or reverse projection, as described above. Superfluous baseline scans between the peaks were suppressed for greater clarity of presentation.

RESULTS AND DISCUSSION

The detection of DAM at high sensitivity, well-resolved from its principal metabolites, is illustrated in Fig. 2. UV spectra could be captured for all components at these low concentrations, although the relatively high noise level necessitated the application of 7-point Savitzky—Golay smoothing for satisfactory comparison of spectra with standards. However, the residual noise level led to unsatisfactory first and second derivatives of the captured spectra. The on-column detection limits at 280 nm, defined in terms of twice peak-to-peak noise level, were: DAM, 15 ng; 6AM, 25 ng; MOR, 65 ng. These limits can be reduced by an order of magnitude by measurement between 210 and 220 nm, where the absorptivities of DAM and its metabolites are greater. The

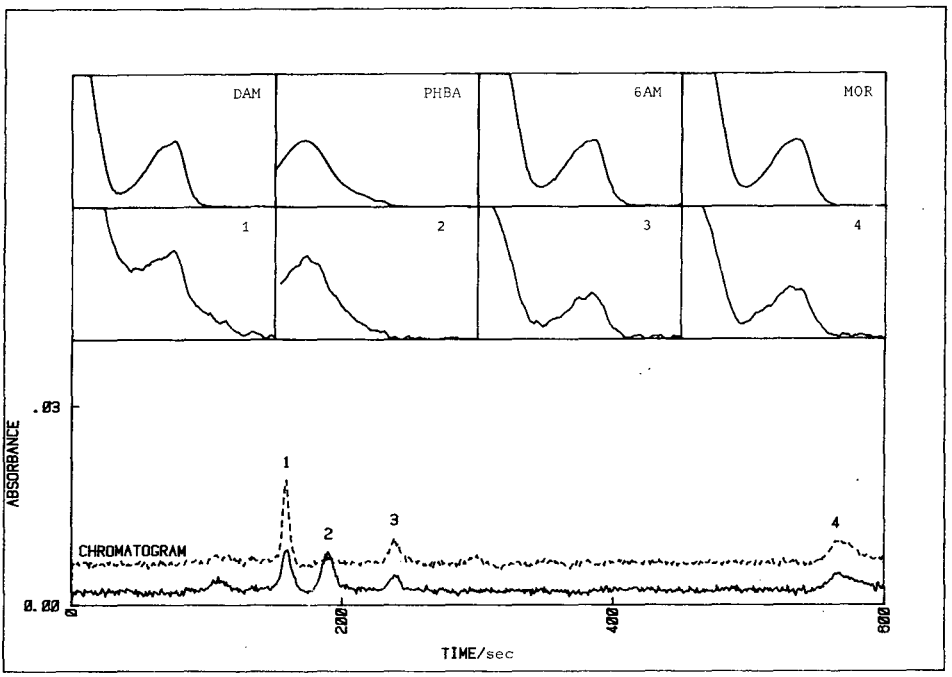

Fig. 2. Chromatograms at 254 nm (——) and 280 nm (— — —) of: (1) diacetylmorphine (DAM) (100 ng); (2) *p*-hydroxybenzoic acid (PHBA) (internal standard, 10 ng); (3) 6-acetylmorphine (6AM) (103 ng); and (4) morphine (MOR) (232 ng). Chromatographic conditions, see text; injection volume, 50 µl. Smoothed UV spectra (230—330 nm) captured during elution (lower row), compared with normalised reference spectra (upper row), correspond to each peak as coded.

quantitative performance at 280 nm is improved somewhat by use of an internal reference standard, PHBA, optimally detected at 254 nm. This flexibility in wavelength selection exemplifies a very useful feature of multichannel detection in HPLC.

At concentrations of DAM and its degradation products, comparable with those found in pharmaceutical dosage forms or forensic samples, low-noise UV spectra can be readily acquired during elution for confirmation of identity (Fig. 3). The absorbance ratio at two pre-determined wavelengths (A_{254}:A_{280}), calculated continuously and superimposed above the chromatogram, yields a sensitive measure of peak homogeneity during elution [34]. In principle, the absorbance ratio profile for a pure component peak should resemble a square-wave function, as is seen for DAM in Fig. 3. Any co-eluting impurity changes the absorbance ratio to higher or lower values, depending on the absorptivities at the pair of wavelengths selected, the concentration and the exact retention position of impurity peak relative to the analyte peak of interest. The numerical value of the absorbance ratio is a qualitative index of identity and gives an estimate of peak purity [34]. A constant absorbance ratio throughout the peak profile gives a good indication of peak homogeneity. The absorbance ratio concept has been usefully extended by Carter et al. [35], who recently used the multichannel detector to eliminate a major component mathematically to reveal various underlying impurities, as a technique for method validation

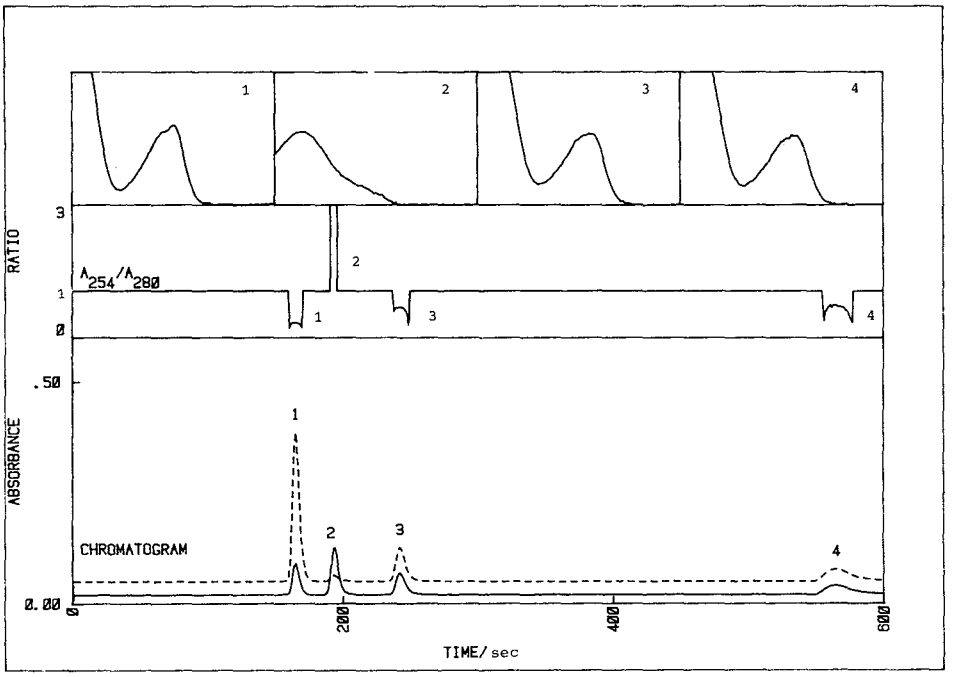

Fig. 3. Chromatograms at 254 nm (——) and 280 nm (— — —) of: (1) diacetylmorphine (4.8 μg); (2) *p*-hydroxybenzoic acid (internal standard, 0.5 μg); (3) 6-acetylmorphine (2 μg); and (4) morphine (2 μg). Chromatographic conditions, see text; injection volume, 10 μl. The continuous absorbance ratio (A_{254}:A_{280}) superimposed above the chromatogram and the normalised UV spectra (230—330 nm) captured during elution (upper row) correspond to each peak as shown.

in pharmaceutical analysis.

A number of functions have been explored for enhanced qualitative evaluation of spectral data captured during elution. The second or higher even derivative spectrum has recently been proposed for identification purposes in pharmaceutical [36], biochemical [37, 38], environmental [39] and forensic analysis [40]. This proposal is based on the fact that, in general, the fine structure and inflection points observed in a zero order UV spectrum are transformed to more finely structured, bipolar peaks in the first or higher derivative ($dA/d\lambda$, $d^2A/d\lambda^2$, . . .). The second derivative is seen as a sharpened, inverted profile, with a minimum corresponding to the maximum in the original peak. Insofar as second and higher even derivative spectra bear a similarity to the zero order spectrum, they are more easily interpreted than the odd derivative functions and have found wider popularity.

In Fig. 4 the zero order spectra of 6AM and MOR, captured during elution (Fig. 3), are compared with the unsmoothed second derivative spectra. The zero order, first derivative, second derivative, and $\log_{10}$ (A) spectra of DAM are compared in Fig. 5. It can be readily seen that, whereas the zero order spectral profiles are relatively smooth and featureless, the second derivative functions present a number of sharpened features for comparison. The second derivative spectra of 6AM and MOR are very similar, an observation

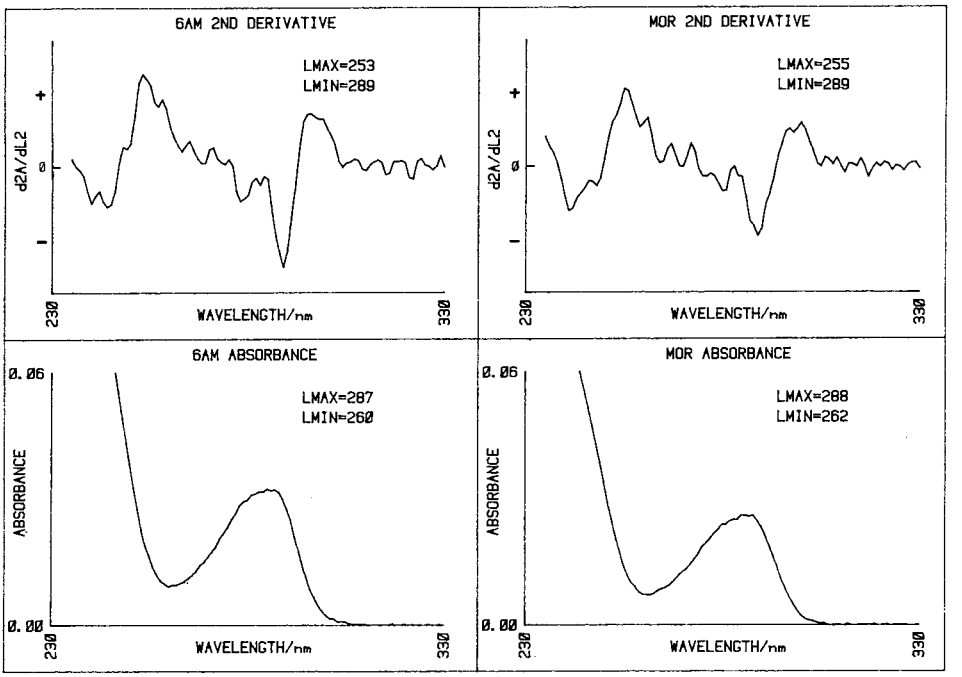

Fig. 4. Zero order and second derivative UV spectra of 6-acetylmorphine (6AM) and morphine (MOR) peaks (cf. Fig. 3).

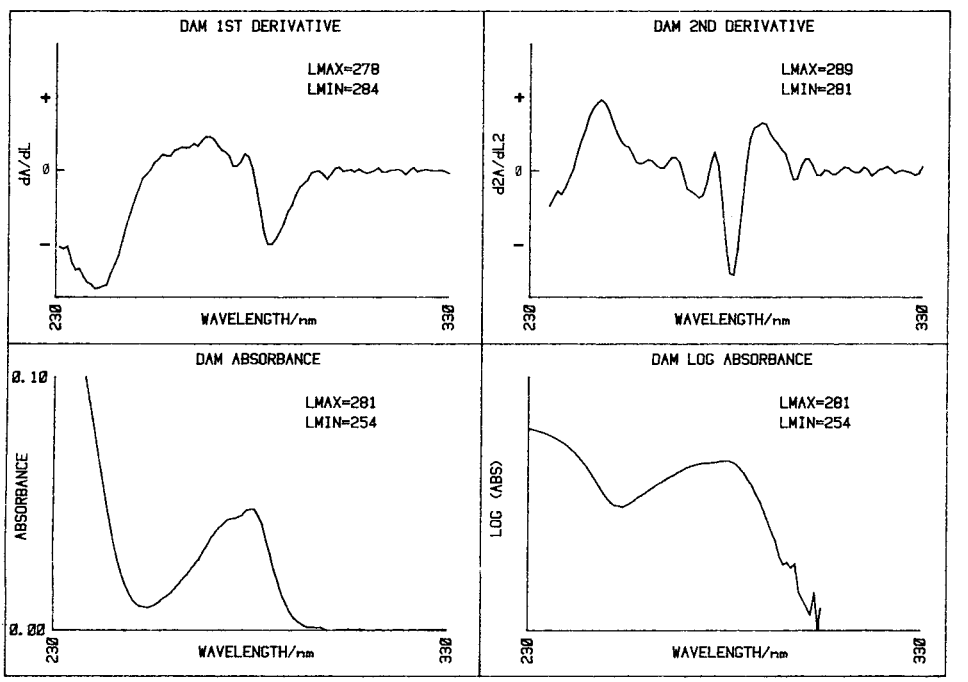

Fig. 5. Zero order, first derivative, second derivative and $\log_{10}$ (A) UV spectra of diacetylmorphine (DAM) peak (cf. Fig. 3).

attributable to the shared feature of a 3-hydroxyl group in the aromatic ring (Fig. 4). However, both 6AM and MOR are readily distinguished from DAM in the second derivative spectrum (Fig. 5). Here the inflection near 275 nm in the zero order spectrum of DAM is seen as a sharpened feature in the second derivative, which although inverted, can be readily related to the original spectrum. The first derivative spectrum of DAM is, however, a relatively featureless disperse function and is more difficult to relate to the zero order profile. The $\log_{10}$ (A) presentation of DAM is relatively uninformative. However, it does offer an alternative method for normalisation, since the concentration term in the Beer—Lambert expression is thereby reduced to a constant displacement of the spectrum on the $\log_{10}$ ordinate, the shape of the profile remaining constant. The noisy baseline at high wavelength reflects the logarithm of values close to zero. Theoretical considerations suggest that the second derivative of $\log_{10}$ (A) spectra should offer a characteristic, concentration-independent profile for spectral comparison purposes [29]. The combination of retention data with zero order and second derivative UV spectra offers a highly selective means for characterising components separated by HPLC.

The presentation of a three-dimensional chromatogram as a pseudo-isometric projection of (A,λ,t) data, using configuration B, is illustrated in Fig. 6, where DAM can be clearly distinguished from 6AM and MOR although the angle of projection partially obscures the internal standard PHBA. By digital manipulation of the data files the viewpoint can be reversed, so that the internal standard and 6AM peaks are more readily visible (Fig. 7). Alternatively, the data files for 6AM could be suppressed for better observation of hidden peaks, as discussed above. Similar three-dimensional projections of the sample used in

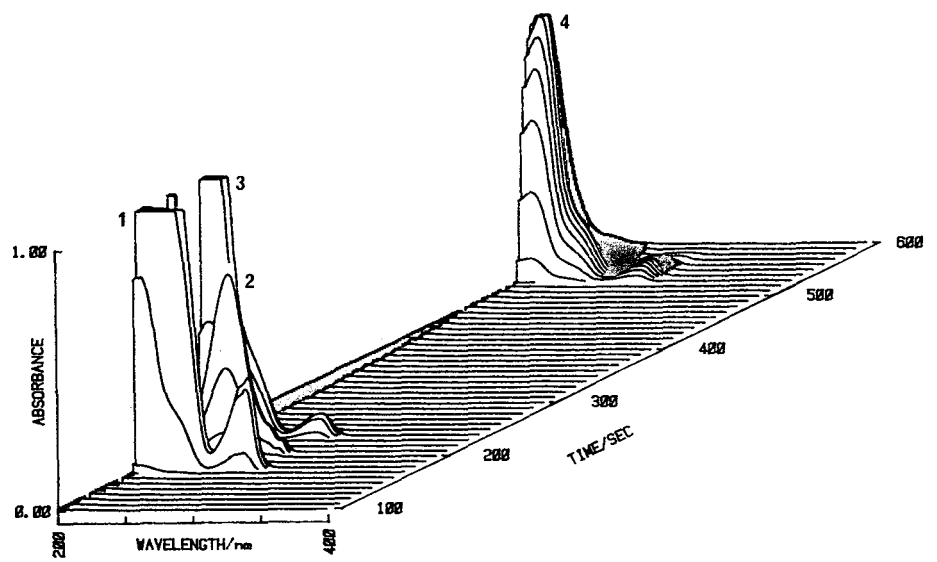

Fig. 6. Pseudo-isometric (A,λ,t) presentation of sequential spectra captured at 2.5-sec intervals during elution. Hidden lines and superfluous baseline spectra have been omitted for clarity. Chromatographic conditions, see text; injection volume, 10 μl. (1) Diacetylmorphine (5.0 μg); (2) p-hydroxybenzoic acid (internal standard, 0.50 μg); (3) 6-acetylmorphine (2.0 μg); and (4) morphine (4.0 μg).

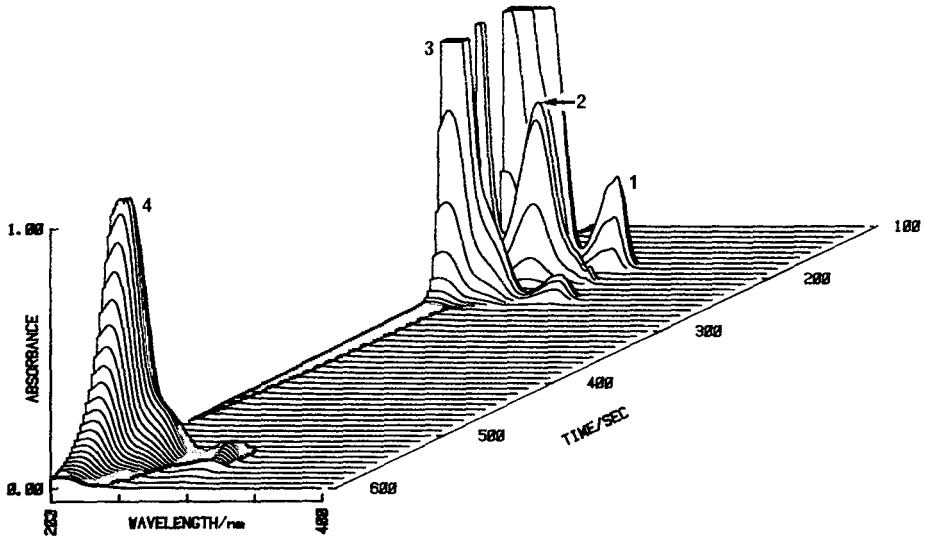

Fig. 7. Reversed presentation of pseudo-isometric (A,λ,t) data in Fig. 6, in order to facilitate observation of hidden features.

Figs. 6 and 7 have been successfully obtained after 100-fold dilution, using the technique of digital integration during the 2.5-sec sampling period [32]. The present configuration for generating (A,λ,t) chromatograms (Fig. 1B) enables immediate post-run data processing to be performed by the HP 85 microcomputer and has been found to be fast, reliable and flexible in use.

It should perhaps be emphasised that the presentation of a three-dimensional projection is only one of many options open to the chromatographer for processing multichannel data. It can be argued that the availability of the (A,λ,t) data as a three-dimensional matrix in digital memory is in itself of greater potential usefulness than the pseudo-isometric projection. The data matrix can be sectioned at any time to give the absorption spectrum for qualitative characterisation or at any wavelength to generate the conventional elution profile. If required, the wavelength of the section can be varied as a function of time to present each peak at its optimum wavelength in the chromatogram. Moreover, the absorbance ratio can be calculated at any pair of wavelengths for each peak. The three-dimensional chromatogram is, however, an attractive and effective means of presenting complex qualitative relationships in a chromatogram. As such it will find use in the biomedical sciences for the characterisation of drugs and their metabolites. This application of three-dimensional chromatography has recently been elegantly confirmed in the screening of metabolites in body fluids [41, 42].

Spectral deconvolution of peaks overlapping in the time domain offers an additional technique for the characterisation of complex chromatograms, provided that the components are defined and available in spectral archive. Work continuing in our laboratory indicates that there is considerable potential in pharmaceutical analysis for deconvolution using zero order and higher derivative spectra [43]. Further benefits may be anticipated from the use of principal component analysis and pattern recognition techniques applied to

three-dimensional data generated in HPLC and TLC [44]. The combination of these computer-aided concepts with rapid-scanning multichannel detectors presages several new experimental strategies for chromatographic detection in the life sciences.

CONCLUSIONS

The optical multichannel detector offers a rapid and versatile approach to detection in HPLC and TLC. By combining retention time data with rapidly-scanned spectra captured during elution, drugs, their degradation products and metabolites can be characterised with greater specificity. Peak homogeneity can be examined by use of absorbance ratios and qualitative features can be enhanced by generating the second derivative of the captured UV spectrum. By monitoring the chromatogram at wavelengths selected according to known spectral characteristics, the quantitative performance of the detector can be optimised.

The acquisition of rapidly scanned spectral data during elution permits several new strategies to be developed for more flexible detection in HPLC. These include the presentation of three-dimensional projections of (A,λ,t) chromatograms for qualitatitive purposes and the optimised selection of detection wavelength after chromatographic separation.

Further developments in optical multichannel detectors based on 16-bit and 32-bit dedicated microcomputers will give faster data handling and greater sensitivity for bioanalytical applications. Such devices will enable digital data-bases of spectral and chromatographic data to be established and rapidly accessed for the application of pattern recognition algorithms and related techniques in the biomedical sciences.

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ANALYTICAL STRATEGIES FOR THERAPEUTIC MONITORING OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

PLENARY LECTURE

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SUMMARY

Therapeutic drug monitoring can involve quantitation in either microgram, nanogram or picogram concentrations present in a complex biological matrix (whole blood, urine or tissue).

The chemical structure of a compound influences not only the analytical method best suited to its quantitation, but also its acid/base character (pK_a) and its extractability. The dose administered, the bioavailability of the dosage form, and the pharmacokinetic profile of the drug govern the circulating concentrations of either the parent drug and/or its metabolites present in vivo, and dictate the ultimate sensitivity and specificity required of the analytical method.

The degree of sample preparation required is dependent on the analytical method used (gas—liquid chromatography, thin-layer chromatography, high-performance liquid chromatography) and on the tolerance of the specific type of detection system to contamination. Factors leading to compound losses during sample preparation (adsorption, stability) are critical at low concentrations and can adversely affect the reliability of an assay, therefore maximizing the overall recovery of the assay is essential not only for high sensitivity but also for good precision and accuracy. Therefore, the criteria to be used in sample preparation should aim to optimize all of the above factors in the overall development of a reliable and validated method for the compound suitable for use in clinical therapeutic monitoring.

INTRODUCTION

Therapeutic drug monitoring can involve quantitation in the microgram (10^{-6} g or ppm), nanogram (10^{-9} g or ppb) or picogram (10^{-12} g or ppt) concentration range. These concentrations are present in a complex biological matrix (whole blood/urine/tissue) from which it must be selectively extracted and cleaned up prior to quantitation [1].

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The chemical structure of a compound influences the analytical method best suited to its quantitation, while the ionizable groups in the molecule determine its acid/base character (pK_a) and its extractability [2]. The dose administered (mg/kg), the bioavailability of the dosage form, and the pharmacokinetic profile of the drug govern the absolute concentrations of the parent drug and/or metabolites to be quantitated [3, 4]. These criteria influence the ultimate sensitivity and specificity required of the analytical method [5].

The degree of clean-up required is dependent on the analytical method used [gas—liquid chromatography (GLC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC)] and on the tolerance of the specific type of detection system to contamination. The options available for processing a biological specimen must be tailored not only to the method itself, but also to the sensitivity and specificity required of it. Factors responsible for compound losses during sample preparation (adsorption, stability) are critical at low concentrations and may adversely affect the reliability of an assay. Consequently, maximizing the overall recovery of the assay is essential not only for sensitivity but also for good precision and accuracy.

The choice of the method selected is governed either by the intrinsic analytical properties of the molecule or its amenability to chemical derivatization to render it compatible to quantitation by a specific method [6, 7]. The type of assay selected will also govern the amount of sample preparation and clean-up required, and the biological specimen best suited for analysis, i.e., whole blood, plasma, or urine [8].

The armentarium of sensitive and specific methods currently available to the analyst is quite diverse and covers a wide linear dynamic range for quantitation (Fig. 1). These include chromatographic techniques with a variety of selective detectors to ensure specificity [e.g., GLC with ionization detectors such as electron-capture (ECD), nitrogen—phosphorus specific detector (NPD), mass spectrometric—chemical ionization detectors (CI—MS), HPLC with UV, fluorescence and electrochemical detectors (oxidative and reductive-polarographic),

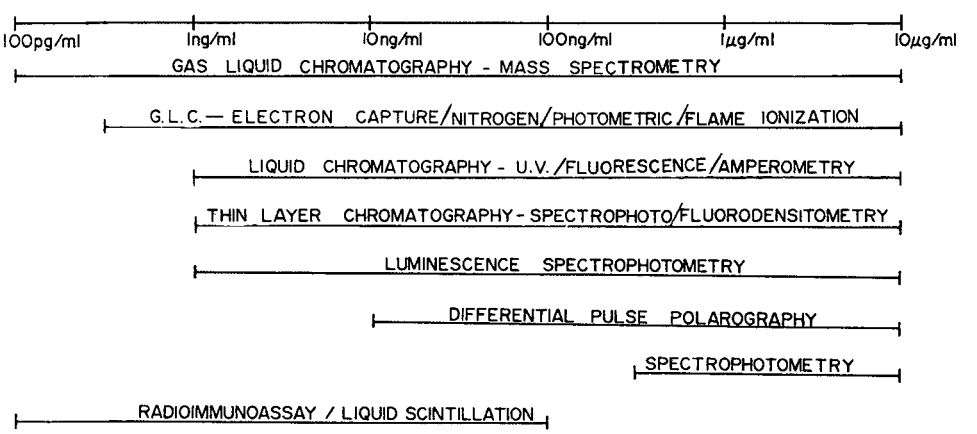


Fig. 1. Practical range of usefulness of analytical techniques.

high-performance thin-layer chromatography (HPTLC) with in situ spectrophotometry/fluorodensitometry or non-chromatographic techniques such as spectrophotometry (UV—Vis), luminescence methods (fluorescence and phosphorescence), differential pulse polarography (DPP) and radioimmunoassay (RIA)], all of which are capable of quantitation over a wide linear dynamic concentration range.

CHEMICAL STRUCTURE AND ANALYTICAL UTILITY

Chemical manipulation of a drug via derivatization is useful in enhancing both the sensitivity and the specificity of the determinate step [6, 7], and has certain inherent advantages even if the intrinsic sensitivity is adequate and/or blood concentration is not a limiting factor. The sample volume extracted can be reduced from milliliter to microliter amounts, and/or the aliquot of the final residue analyzed can be reduced by sample dilution, significantly improving chromatographic analysis by minimizing endogenous interferences, resulting in more accurate, reproducible and reliable quantitation. All of the above factors should be optimized in the overall development of a reliable and validated method for eventual clinical evaluation.

The development of a sequential series of assays is advantageous in developing methods with a wide range of useful sensitivity. Examples of two chemical derivatives used in the development of such a series of assays are shown in Fig. 2 for two nitroimidazoles. Either drug is first separated from metabolites by extraction followed by TLC and elution of intact drug from the silica gel with methanol. An aliquot of this eluate may be hydrolyzed in base to liberate nitrite which is used to diazotize sulfanilamide. This diazonium salt is further reacted to yield an azo-dye chromophore suitable for absorptiometric analysis with a sensitivity limit of about 1 μg/ml. The colored product is designated the Bratton—Marshall chromophore because this same diazotization reaction is the basis for the analysis of sulfanilamide and other sulfa drugs.

If greater sensitivity is required, another aliquot can be reacted to yield the trimethylsilyl (TMS) derivative which is sufficiently volatile to be analyzed by electron-capture GLC. This allows for an increase in sensitivity of three orders of magnitude.

Although not a derivatization procedure, DPP (Fig. 2), with about three times more sensitivity than that of the absorptiometric assay, is also available for these compounds.

METABOLIC PROFILE

Metabolic studies on the in vitro biotransformation of the compound using microsomal (9000 *g*) enzyme preparations and/or in vivo studies using the radioisotopically labelled (^{14}C, ^{3}H) compound should be underway in parallel with method development, so that assay development can also incorporate the quantitation of the major metabolites present in blood or urine using more sensitive and specific chemical methods.

Determination of the total radioactivity in plasma or urine vs. solvent-extractable radioactivity will indicate the extent to which polar non-extractable

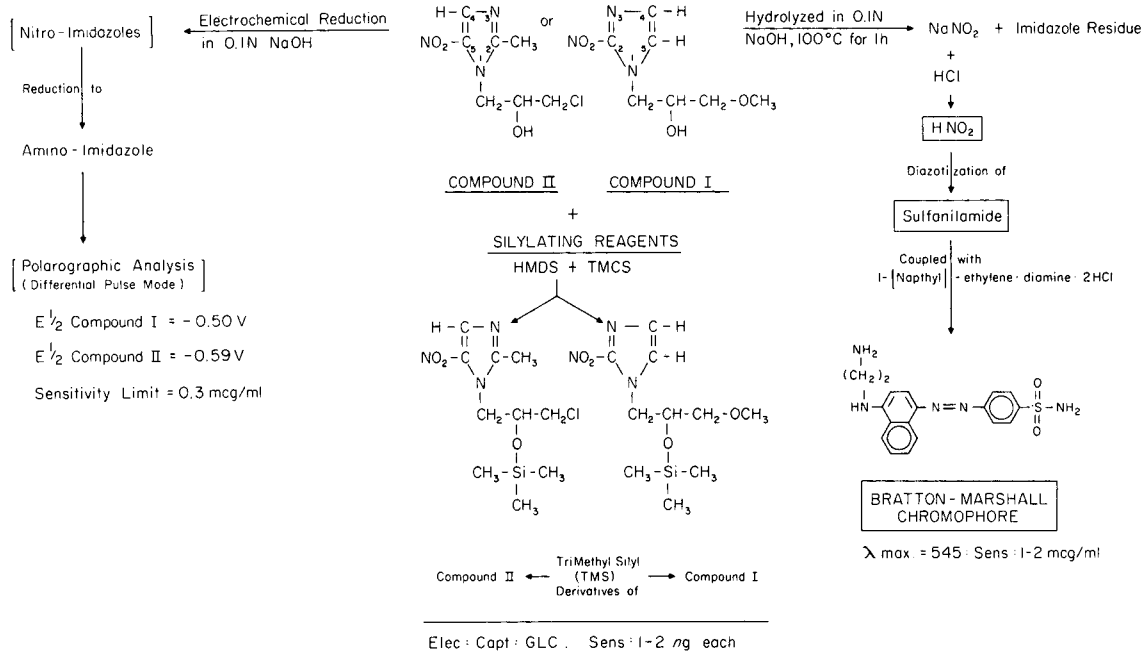


Fig. 2. Chemical reactions of a 2-nitro[I]- and a 5-nitro[II]imidazole used in multiple analytical methods.

metabolites are present. Chromatographic analysis (TLC or HPLC) of the solvent-extractable fraction using either a radiochromatogram scanner or a radiometric detector with HPLC analysis will indicate the relative amounts of the parent drug and any metabolites present; a reliable index of the specificity of the extraction procedure (pH and solvent used).

The plasma profile of ^{14}C-labelled clonazepam (anticonvulsant) in man (Fig. 3) indicated the presence of polar non-extractable metabolites when the total plasma ^{14}C-profile (upper curve) was compared to the diethyl ether extractable [^{14}C]-clonazepam profile (middle curve) in plasma. The excellent correlation between the clonazepam concentration in whole blood (lower dashed curve) determined by TLC—radiometry vs. EC—GLC verified the accuracy and specificity of the latter technique.

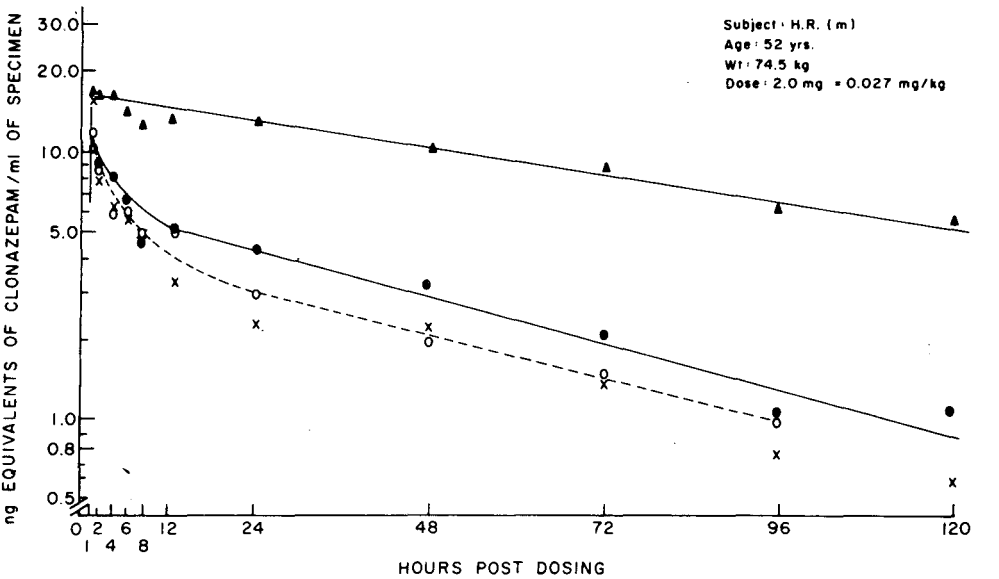


Fig. 3. Radiolabelled drug and its utility in the verification of assay sensitivity and specificity. Plasma levels of radioactivity and of intact clonazepam as a function of time following a 2-mg oral dose of micronized [^{14}C]clonazepam. Plasma total ^{14}C in ng equivalents of clonazepam per ml (▲), plasma [^{14}C]clonazepam in ng/ml (●), blood [^{14}C]clonazepam (×) by radiometry, and blood clonazepam determined by EC—GLC analysis (○) in ng/ml.

PHARMACOKINETIC FACTORS WHICH INFLUENCE SAMPLE PREPARATION

Biotransformation

Chromatographic analysis is necessary to ensure the specificity of analysis for the parent drug and/or any major metabolites present. Any "first pass" biotransformation will be reflected in the ratio of parent drug to major metabolites; indeed the metabolite may be the only measurable component present and dictate the choice of the biological sample to be used, e.g., rapid hydrolysis of an ester to an acid. Radioisotopic data would also indicate the feasibility of developing a chemical assay for the compound in terms of the ultimate sensitivity and/or specificity required of it.

Elimination

The rate and extent of elimination of a drug and/or its metabolites in urine would dictate the utility of analyzing this medium. Drugs that are extensively metabolized by Phase I reactions are eliminated in urine following Phase II reactions as the glucuronide/sulfate/hippurate conjugates [9]. Their concentrations are usually sufficiently high to warrant their analysis in urine as in bioavailability/bioequivalence studies for dosage form evaluation.

SAMPLE PROCESSING VERSUS ANALYTICAL DETERMINATE STEP

Analysis in blood/plasma/tissue

The degree of sample preparation and clean-up required is usually a function of the analytical method to be used and the tolerance of the specific detection system to contamination. The options available for processing whole blood/plasma/tissue homogenate or proteolytic digest are outlined in Fig. 4. The biological specimen undergoes a protein precipitation step, followed by pH adjustment and selective extraction into a suitable solvent, which can then be processed in one of several ways depending on the analytical method to be used [8].

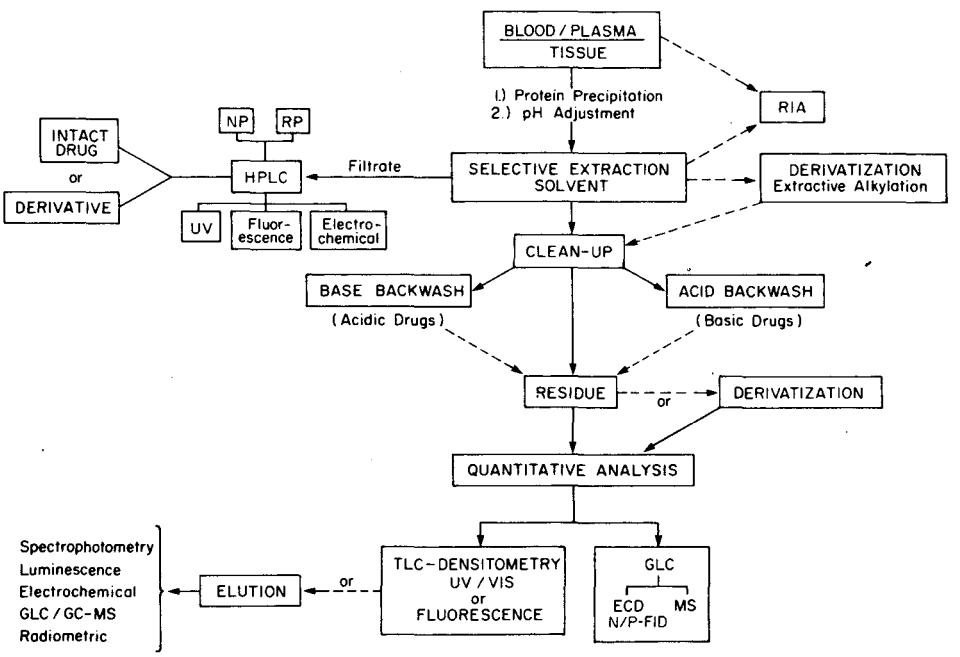


Fig. 4. Flow diagram of the analytical options available for sample processing.

Radioimmunoassay. RIA can be performed either directly in the biological sample (plasma or serum) or in the residue of the solvent extract which can also be used for HPLC analysis.

HPLC. Analysis by HPLC has several advantages that can be collectively optimized for sensitive and specific analysis. Two modes of operation, normal

phase (adsorption) and reversed phase (partition) are the most widely used for drug analysis, although cation/anion-exchange chromatography is also used for highly polar zwitterionic drug molecules such as the β-lactam antibiotics and quaternary ammonium compounds. The sample residue is reconstituted in 50–100 μl of the mobile phase to be used and passed through a 40-μm Millipore filter to remove colloidal lipids/lipoproteins which could precipitate out if a reversed-phase system is used. Drug molecules can be analyzed either as the intact moiety or as a suitable derivative using either UV, fluorescence or electrochemical detection.

GLC. Analysis by GLC may require more extensive sample preparation depending on the drug to be analyzed. The drug in the solvent extract can be derivatized directly by extractive alkylation (N-1-desalkyl-1,4-benzodiazepin-2-ones) followed by a clean-up step, or the intact moiety can be further purified from endogenous impurities by utilizing a backwash step into a base (for acidic drugs) or an acid (for basic drugs), and re-extracted from the aqueous phase by appropriate pH adjustment into a solvent, the residue of which may be derivatized prior to GLC analysis (silylation, esterification, alkylation) using highly selective detectors such as ECD, NPD or CI–MS.

TLC. Analysis by TLC may be performed directly on the residue of the initial solvent extract, unless prechromatographic clean-up is required due to a derivatization step. TLC separation can be used, per se, as an effective clean-up step since the extract applied to the chromatoplate can be processed by multiple development, first in a nonpolar lipophilic solvent system to move the endogenous contaminants from the origin to the solvent front followed by a second development, in a more polar solvent, to resolve the compounds of interest from each other. The compounds can either be quantitated in situ by spectrophotometry (UV–Vis) or fluorodensitometry or be eluted from the silica gel and determined separately by an appropriate physico-chemical method (spectrophotometry, luminescence, electrochemical, radiometric).

Analysis in urine and feces

A flow diagram for the extraction of urine and feces is outlined in Fig. 5. The analysis of these two media requires an aliquot of a representative sample, i.e., an aliquot of urine from a total voidance volume collected over a known excretion period (e.g., 24, 48 h), and for feces an aliquot of a homogenate of a total voidance collected similarly. The sample is filtered to remove particulates and analyzed for the "free" (directly extractable) and "bound" (conjugated) fractions of drug and metabolites present.

Basic extracts of urine or feces are not as heavily contaminated with endogenous compounds as are acidic extracts which have extensive amounts of phenolic and indolic acids, and require additional clean-up, e.g., column chromatography. The conjugated or "bound" fraction has to be hydrolyzed either with acid to cleave hippurates and other amino acid conjugates or incubated with β-glucuronidase/sulfatase at 37°C for 2–12 h in a Dubnoff Incubation shaker to cleave glucuronide/sulfate conjugates. The aglycone(s) are extracted after appropriate pH adjustment, followed by clean-up of the extract either by chromatography (column or TLC) or liquid–liquid partition (acid/base).

Depending on the determinate step, the residue of the final extract may

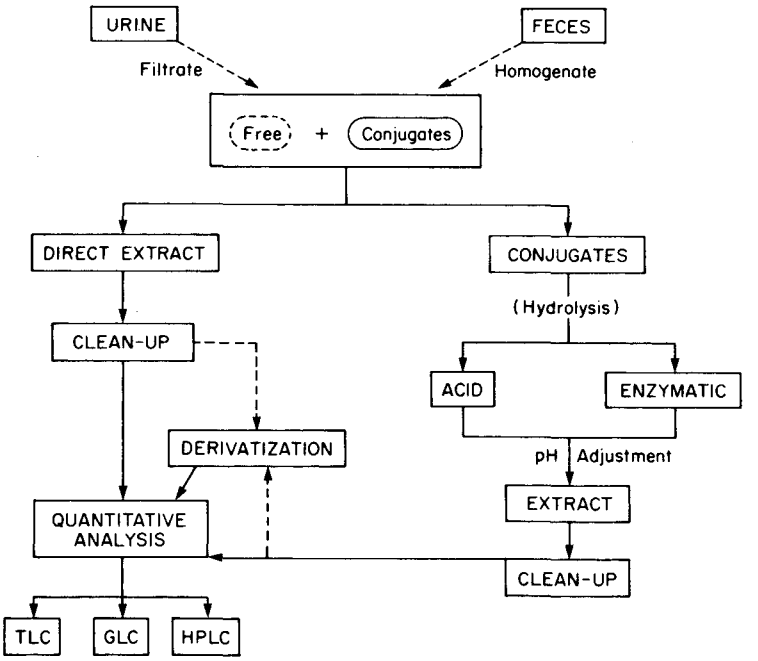


Fig. 5. Sample preparation of urine and feces for the extraction of drugs.

have to be derivatized (silylation of hydroxyl groups, esterification of carboxylic acids) for GLC analyses, or analyzed per se by either TLC densitometry or HPLC (usually reversed-phase) using UV, fluorescence or electrochemical detection. HPLC is generally the method of choice since the components can usually be analyzed without derivatization and concentration is not limiting. Resolution of endogenous impurities not removed by previous clean-up may be a limiting factor.

CHROMATOGRAPHIC TECHNIQUES

Analysis by GLC

Although analysis of the intact molecular moiety (underivatized) is preferred to ensure specificity, derivatization is often necessary for valid analytical reasons. Simple derivatization reactions, such as extractive alkylation and/or silylation, can be used where needed, to yield very sensitive, specific and readily automatable methods. The idiosyncrasies of specific detectors such as the ECD and the NPD have to be considered during sample preparation so as not to introduce contaminants into the extract which could be detrimental.

The nitrogen—phosphorus selective detector, for example, is susceptible to severe interference by residues of silylating reagents and from phosphate plasticizers contained in plastic syringes and blood collection tubes which leach into the biological sample and are extracted. Thus, the selection of the proper type of syringe and collection tubes becomes a necessary part of the assay development program and should be evaluated with forethought so as not to jeopardize the clinical studies for which the assay is intended.

GLC analysis has been extensively used in the determination of compounds of the 1,4-benzodiazepine class which has yielded pharmaceuticals which are widely used in clinical practice as antianxiolytics, muscle relaxants, hypnotics, and anticonvulsant agents [10]. The benzodiazepines undergo extensive biotransformation in man and other animal species usually resulting in the presence of one or more pharmacologically active metabolites [11], which have to be resolved from the parent drug for accurate quantitation in biological fluids (Fig. 6). Since the clinically effective therapeutic doses for these compounds are usually low (generally less than 1 mg/kg in adults for single oral doses), analytical methods for their quantitation in biological media have to be both very sensitive and specific.

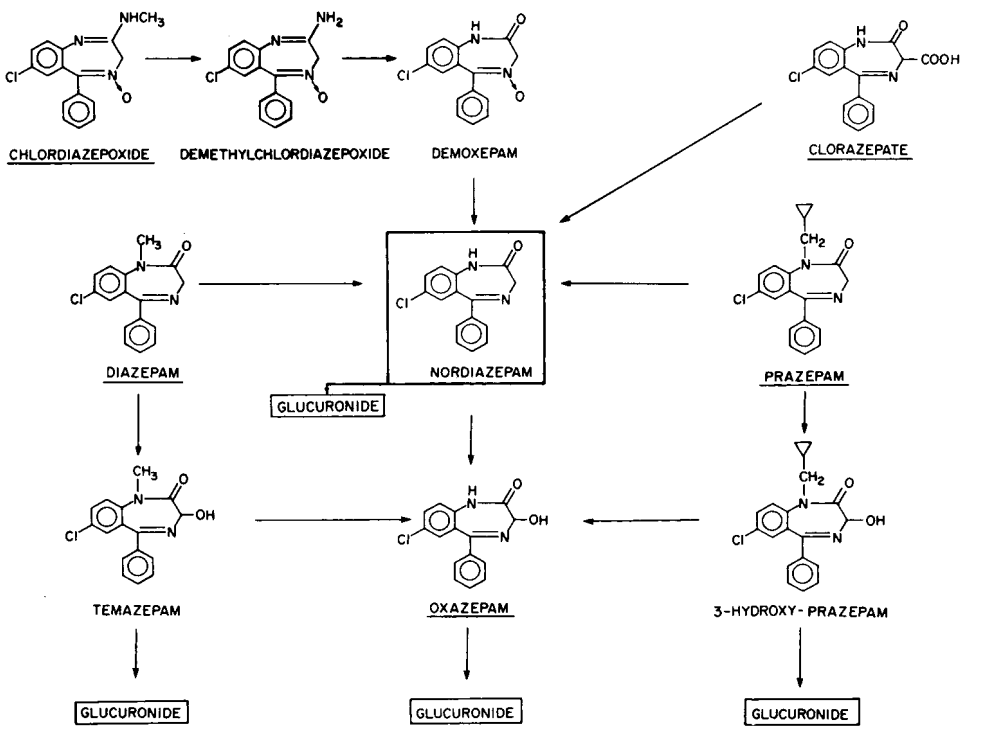


Fig. 6. Biotransformation of benzodiazepines to metabolites common to several drugs.

The majority of the GLC assays reported [12] for the determination of specific benzodiazepines in biological fluids use electron-capture detection due to the presence of an electronegative group in the 7 position of the molecule (usually a halogen or nitro group). A halogen in the 2′ position of the 5-phenyl ring, and a carbonyl group in position 2 of the 1,4-benzodiazepine ring also contribute to ECD response.

Midazolam, [I], an imidazo-1,4-benzodiazepine (pK_a 1.7, 6.15) is currently being developed orally as short acting hypnotic and parenterally as a pre-operative anesthesia inducing agent administered by intravenous infusion. Clinically active doses are of the order of 10 mg per 70 kg (ca. 0.143 mg/kg). The compound undergoes extensive "first pass" metabolism by oxidation (Fig. 7),

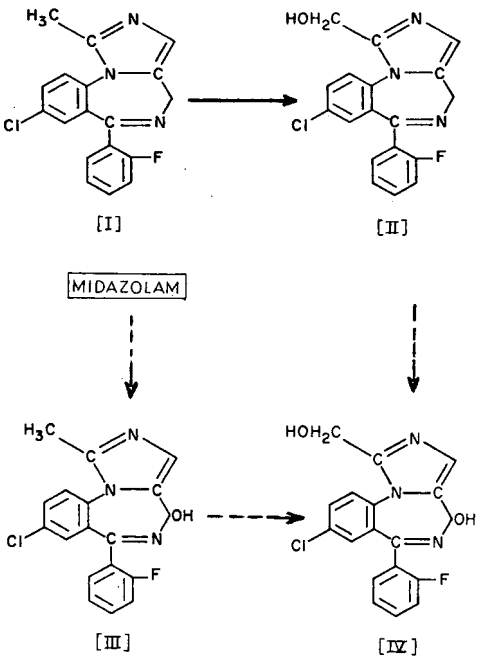


Fig. 7. Biotransformation of midazolam in man.

yielding the 1-hydroxymethyl analogue [II] as the major plasma metabolite which is eliminated as a glucuronide/sulfate conjugate.

EC—GLC analysis of plasma for [I]- and [II]-OTMS (Fig. 8) (sensitivity limit 5—10 ng/ml) enabled the elucidation of the pharmacokinetic profile of [I] and [II] in man. The elimination half-life ($t_{1/2}\beta$) of [I] and [II] is about 2 h, with a significant percentage of the dose eliminated in urine as [II]-glucuronide accounting for 40—45% following intravenous [13], and 60% of the dose following oral administration [14] in a 24- 48 h excretion interval. The major metabolite [II] is extracted from urine after glucuronidase incubation; the residue is silylated and analyzed by EC—GLC. The high sensitivity of [II]-OTMS to ECD enables sufficient sample dilution such that in the quantitation of [II]-OTMS, the bulk of the endogenous interfering peaks and the minor metabolites [III] and [IV] are diluted out ensuring its accurate quantitation (Fig. 9).

The properties of a drug molecule that render it amenable to EC—GLC analysis can be readily extended to the development of highly sensitive and specific GC—CI—MS assays obtained in either the positive ion (PI) or negative ion (NI) modes of analysis. The benzodiazepines lend themselves excellently to GC—CI—MS analysis [15].

Gas chromatography—chemical ionization—mass spectrometry

GC—CI—MS analysis is rapidly establishing itself as the method of choice for quantitation of drugs since greater sensitivity and specificity can be realized due to the milder reaction conditions used in the ionization source. The high abundance of either positive $[MH]^+$ or negative $[M-H]^-$ molecular ions gener-

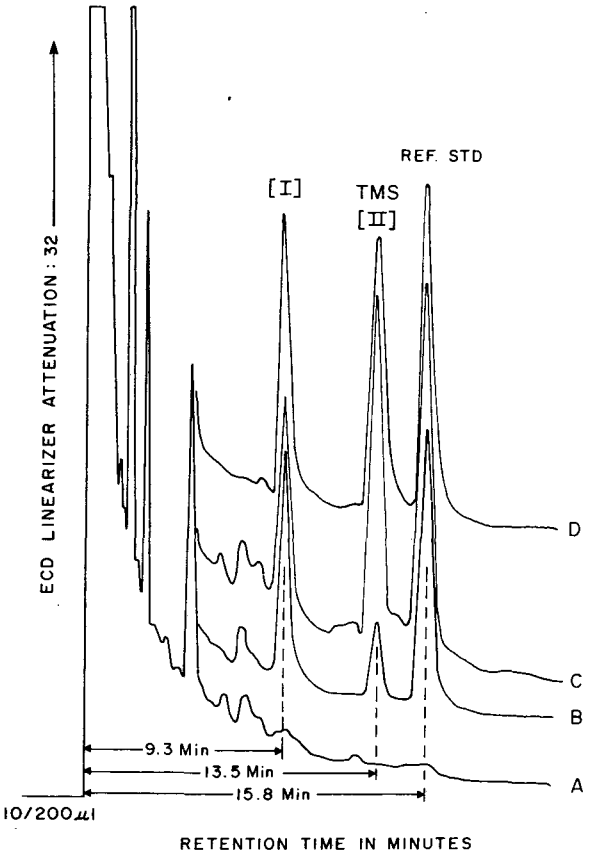


Fig. 8. Chromatograms of (A) control human blood extract; (B) control blood extract containing added authentic standards of midazolam [I] and 1-hydroxymethyl analogue [II]; (C) subject post-dose blood extract (10 mg i.v. infusion); and (D) authentic standards of compounds [I] and [II]-OTMS.

ated yield a stronger signal (hence, greater sensitivity) and the ions formed are characteristic of the parent molecule, which coupled with selected ion monitoring (SIM) imparts greater specificity of analysis [16].

GC—NCI—MS analysis of the 1,4-benzodiazepin-2-ones has inherently high sensitivity associated with negative ion formation by electron capture in the CI source which can be 100—1000 times greater than that obtainable by positive chemical ionization (PCI—MS) methods. This was demonstrated for the 7-nitro anticonvulsant, clonazepam which was analyzed by both GC—PCI—MS [17] and GC—NCI—MS [18]. The $[M-H]^-$ ion monitored at m/z 314 using the ^{15}N, ^{18}O stable isotope analogue as the internal standard (m/z 321) (Fig. 10), yielded a sensitivity limit of 100 pg/ml in the NCI mode compared to 1000 pg/ml in the PCI mode monitoring the $[MH]^+$ ion at m/z 316.

The success of GC—NCI—MS is also attributable to the development of quantitative micro chemical derivatization reactions which can convert compounds containing either an aromatic -OH or aliphatic -COOH, $-NH_2$ group to electron-capturing electrophores using either pentafluorobenzaldehyde, penta-

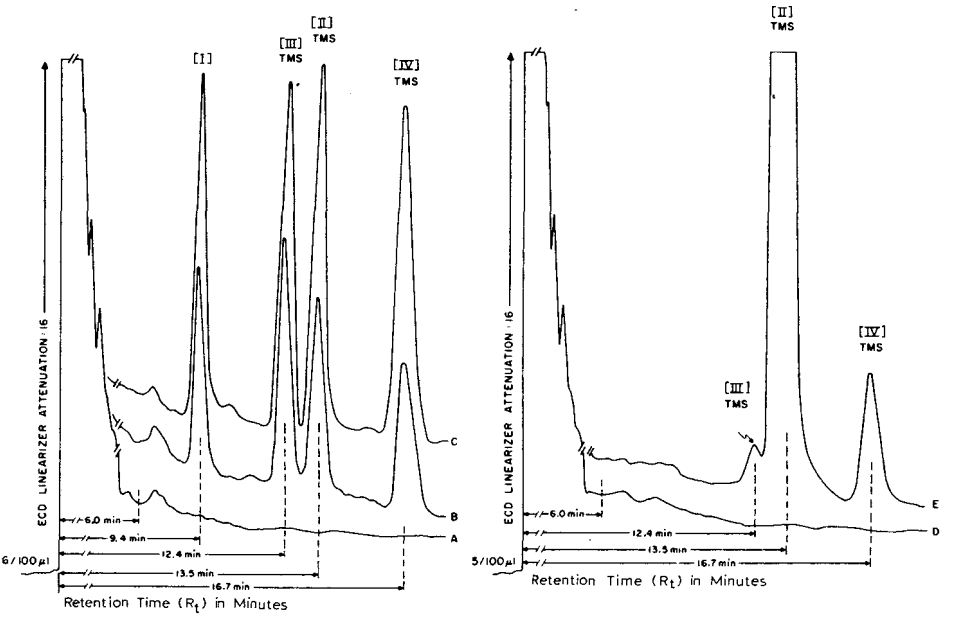


Fig. 9. Chromatograms of the EC—GLC analysis of human urine extracts for midazolam, an imidazo-1,4-benzodiazepine, and its major metabolite. A = control (0 h); B = control urine with added authentic standards; C = authentic standards; D = directly extractable fraction (0—12 h) post dose; E = extractable fraction post glusulase incubation in 0—12 h pooled voidance.

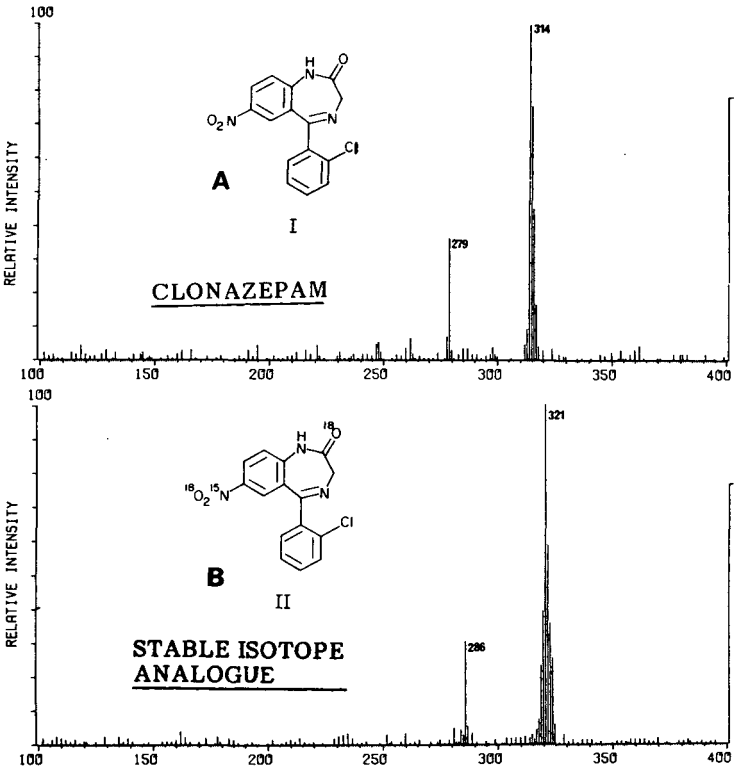
fluorobenzyl bromide or benzoyl chloride which can be quantitated with picogram (10^{-12} g) sensitivity [19].

Analysis by HPLC

HPLC is a most effective means of determining solvent extractable drugs with a minimum of clean-up, if any. Usually, the residue of the sample extract is dissolved in the mobile phase itself, and an aliquot injected directly for analysis using either normal phase or reversed-phase chromatography.

HPLC is uniquely suited to the analysis of thermally unstable compounds (e.g. the benzodiazepines chlordiazepoxide and its metabolites), and amphoteric zwitterionic compounds (antibiotics) which are difficult to extract, at best. Such compounds can be analyzed by the direct injection of an aliquot of the biological sample. Plasma and serum can be analyzed directly following protein precipitation with acetonitrile, injecting an aliquot of the protein-free filtrate after partitioning with *n*-hexane as a clean-up step to remove colloidal lipids. Urine is filtered to remove salts and colloidal materials, extracted with diethyl ether or *n*-hexane as a clean-up step (if needed), and an aliquot diluted in the mobile phase and analyzed directly using reversed-phase HPLC.

Amoxicillin [a β-lactam antibiotic (pK_a = 2.4, 9.6) structurally related to ampicillin], and its benzyl-penicilloic acid, were analyzed directly in urine, by HPLC using fluorometric detection following postcolumn derivatization with fluorescamine [20] (Fig. 11).



	$[MH]^+$	$[M-H]^-$
m/z	316	314

Sensitivity Limit:

Clonazepam $[MH]^+$ = 1 ng/ml of plasma

Clonazepam $[M-H]^-$ = 100 pg/ml of plasma

Fig. 10. Methane GC—CI—MS analysis of clonazepam, a 7-nitro-1,4-benzodiazepin-2-one using positive ion (PI) and negative ion (NI) modes.

Analysis in the fluorescence mode (excitation 385, emission 490 nm) circumvented the need for extensive clean-up due to the selectivity of the detection system for the compounds of interest with minimal interference from endogenous materials.

The clinical utility of the assay was demonstrated in the determination of the urinary excretion profile of amoxicillin and its benzyl-penicilloic acid metabolite (inactive), in a human following the oral administration of a single 250-mg dose. Virtually, quantitative recovery of the dose was achieved in a

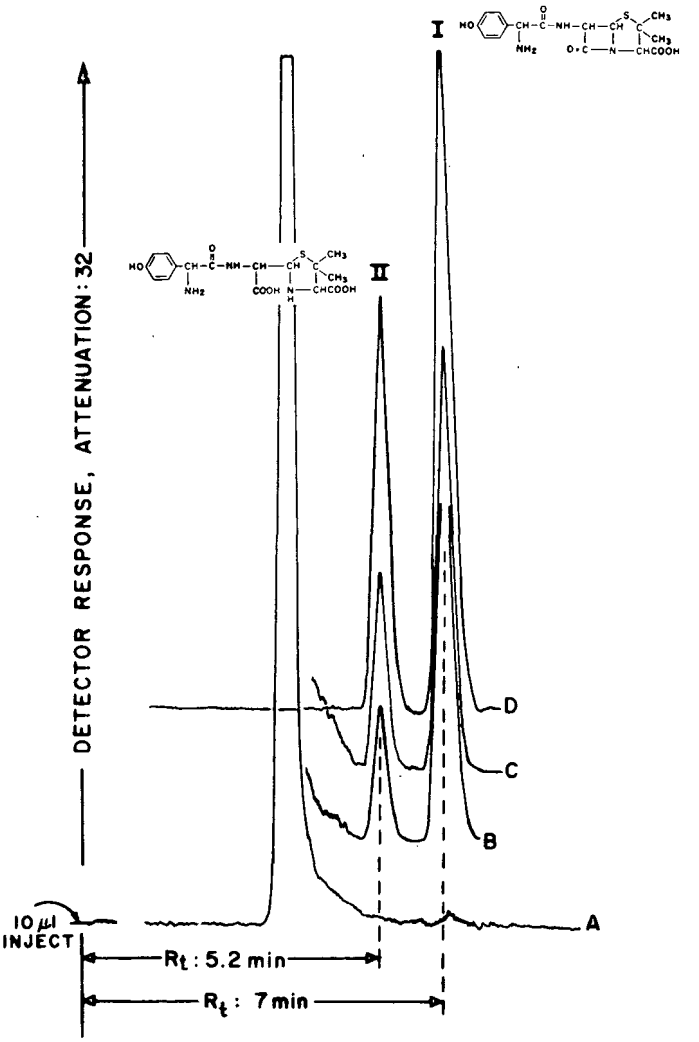


Fig. 11. Chromatograms of the HPLC analysis of amoxicillin [I] and its benzylpenicilloic acid [II] in human urine by fluorometric detection following postcolumn derivatization with fluorescamine. A = diluted control urine; B = post oral dose (250 mg); C = control urine with added authentic standards; D = authentic standards.

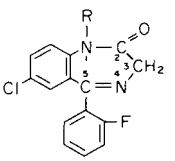
12-h pooled urine collection, making the method a valid non-invasive means of assessing the bioavailability of oral dosage forms [20].

High sensitivity and specificity can be achieved by tandem monitoring of the column effluent using either UV–fluorescence or UV–electrochemical detection. The use of an electrochemical detector for the determination of benzodiazepines in the reduction mode using a dropping mercury electrode (DME) [21] using the functional group specificity of the $>C_5=N_4-$ azomethine group attests to the utility of this technique.

Tandem detection for HPLC analysis can also be used to advantage in the

elucidation of the kinetics of enzymatic hydrolysis of a major (key) metabolite in urine and its specific quantitation as the aglycone.

Flurazepam dihydrochloride, [I] · 2HCl, a hypnotic of the 1,4-benzodiazepine class, undergoes extensive biotransformation in man (Fig. 12). The major urinary metabolite N_1-hydroxyethyl-flurazepam [II]-glucuronide accounts for 30–55% of an orally administered dose in a 72-h excretion period (>25% in a 24-h period).



	R-Group		HPLC (RR_t) N.P.	R.P.
FLURAZEPAM	$-(CH_2)_2-N-(C_2H_5)_2$	[I]	0.88	0.96
Mono-desethyl-flurazepam	$-(CH_2)_2-NH-C_2H_5$	[I-A]	0.55	0.75
Di-desethyl-flurazepam	$-(CH_2)_2-NH_2$	[I-B]	2.43	0.65
N-1-hydroxyethyl-flurazepam	$-CH_2-CH_2OH$	[II]	1.00	1.00
N-1-desalkyl-flurazepam	$-H$	[III]	0.89	1.14
N-1-desalkyl-3-hydroxy-flurazepam	$-H$	[IV], 3)CHOH	3.75	0.81
INTERNAL STANDARD for HPLC	$-(CH_2)_2-N-(CH_3)_2$	[V] 2'-Cl	1.56	1.88 (Diazepam)
[II] - glucuronide			-	0.45

Fig. 12. Chemical structures and relative retention times (RR_t) of flurazepam and its metabolites determined by normal phase (N.P.) and reversed-phase (R.P.) HPLC analysis.

Initial studies using reversed-phase HPLC showed the presence of intact [II]-glucuronide in urine directly injected. Upon enzymatic incubation over discrete time intervals ranging from 15 to 150 min followed by direct injection of the incubate (the enzyme was instantly inactivated by the addition of 500 μl of methanol), it was shown that with the progressive diminution of the [II]-glucuronide peak (t_R = 15.0 min), the peak for deconjugated [II] (free base) (t_R = 28 min), increased in height enabling the direct analysis of the hydrolysis of the glucuronide to yield the aglycone [II] which is quantitated against an authentic standard [22].

The tandem detection of the column effluent first by UV at 254 nm followed by differential pulse amperometry (DPA) ensured specificity for the components of interest. The chromatogram monitored by DPA (Fig. 13A) shows two extra components (probably minor metabolites), which are otherwise masked in the solvent front of the chromatogram monitored by UV at 254 nm (Fig. 13B) further attesting to the utility of DPA as a specific HPLC detector for the reduction of the $>C_5 = N_4$- azomethine group common to all 1,4-benzodiazepines.

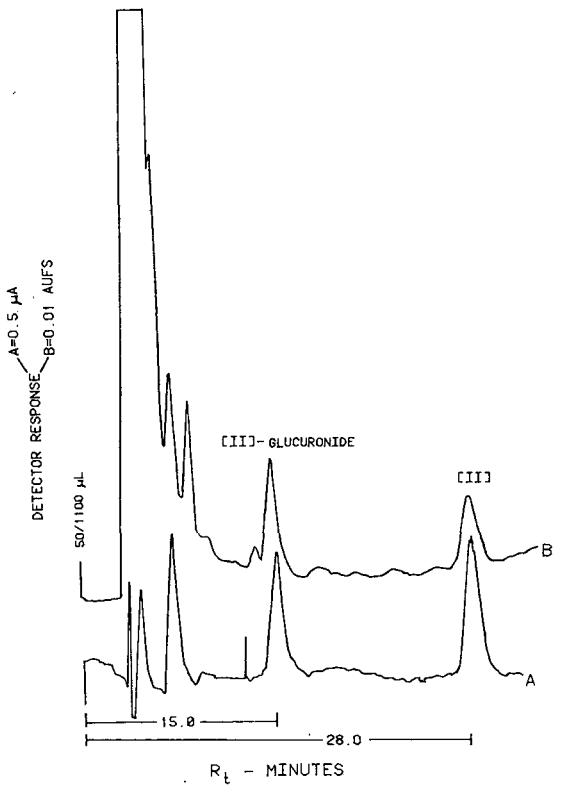


Fig. 13. Chromatograms of reversed-phase HPLC analysis of the metabolite, N_1-hydroxyethylflurazepam [II] in 1—2 h post-dose urine incubated for 30 min, monitored by tandem detection by (A) differential pulse amperometry (DPA) and (B) by UV at 254 nm. Column, μBondapak C_{18}; mobile phase, 0.0075 *M* acetate buffer (pH 3.5)—methanol (1 : 1); flow-rate, 0.9 ml/min at 1500 p.s.i.

The enzymatic hydrolysis kinetics of [II]-glucuronide yielding [II] (free base) are shown in Fig. 14 and show the progressive diminution of the peak for [II]-glucuronide and the increase in the peak height for [II] (free base) with time. The formation of [II] appeared to plateau after 180 min (3 h) with no significant increment after overnight incubation (16 h) which was then used for expediency in sample throughout.

The urinary excretion profile of [II] was determined by normal-phase (after extraction), and reversed-phase HPLC analysis in a volunteer following the administration of a single 30-mg oral dose of [I] · 2HCl (Dalmane®) (Fig. 15). The total amount of [II] excreted in the 0—48 h interval represented about 10.4 mg of flurazepam · 2HCl (parent drug) equivalent to 35% of the administered dose, demonstrating the feasibility of this approach as a facile non-invasive means of determining pharmacokinetic parameters of a drug [22].

Analysis by thin-layer chromatography

TLC analysis enables rapid development of analytical chromatographic parameters for eventual use in HPLC analysis. Preliminary separation of drug/

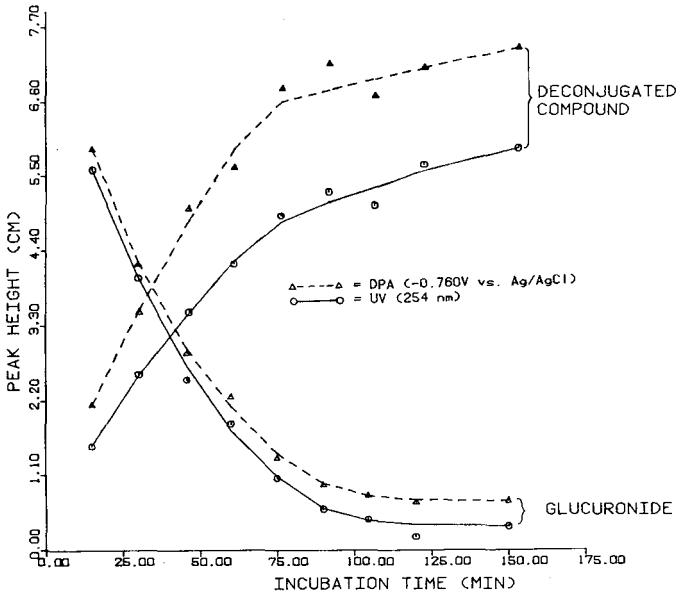


Fig. 14. Kinetics of the enzymatic hydrolysis in urine of [II]-glucuronide to yield N_1-hydroxyethylflurazepam [II] (free base).

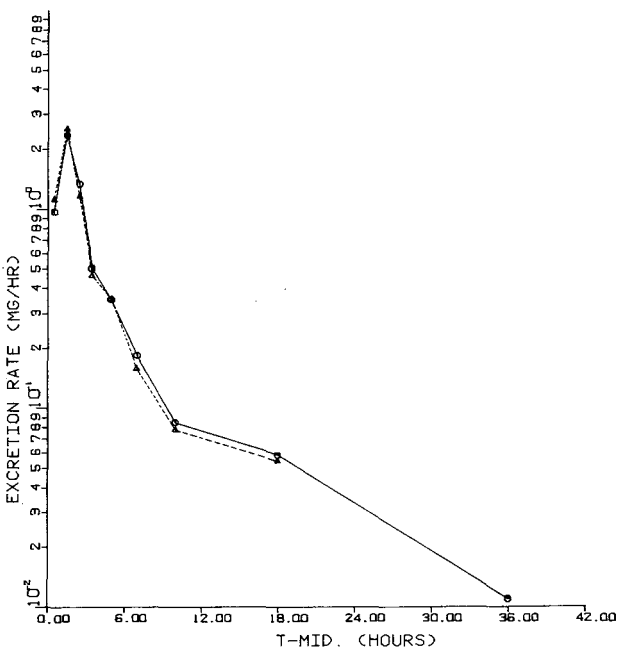


Fig. 15. Urinary excretion profile of hydroxyethylflurazepam [II] determined by normal-phase (Δ - - - Δ) and reversed-phase (o —— o) HPLC analysis.

metabolites using radiolabelled compound enables qualitative identification via radiochromatographic scanning or autoradiography to isolate metabolites from either an in vitro 9000 *g* microsomal incubation or from in vivo biotransformation studies. The separated compounds can be eluted from the silica gel and analyzed by a variety of selective techniques, e.g., spectrophotometry/fluorometry, polarography, GLC, GC—MS, nuclear magnetic resonance (NMR), not only for quantitation but also for structure elucidation.

Quantitative analysis by in situ spectrophotometry/fluorometry has been extensively utilized, especially since the advent of HPTLC using small sample aliquots (1 μl or less) applied to the chromatoplate, rapid development and densitometric analysis. The sensitivity and specificity of the technique are especially useful in the fluorescence mode and were used in the analysis of flurazepam and its major metabolites in plasma (Fig. 16). The compounds were first hydrolyzed to their *o*-aminobenzophenones which were then cyclized in base (dimethylformamide—potassium carbonate) to the highly fluorescent 9-acridanone derivatives. These were extracted, separated by TLC and quantitated by in situ spectrofluorodensitometry (Fig. 17), and applied to the determination of plasma concentrations of flurazepam and its major metabolites: hydroxyethyl-[II] and N-desalkyl[III], following a single 30-mg oral dose (Fig. 18) [23].

Some of the advantages of TLC analysis include the ability of analyzing the sample by either one- or two-dimensional solvent ascending chromatography, rapid development of the separation (HPTLC) and relatively low cost of the separations per se. This is unfortunately offset by the high cost of the spectrodensitometer required for quantitation.

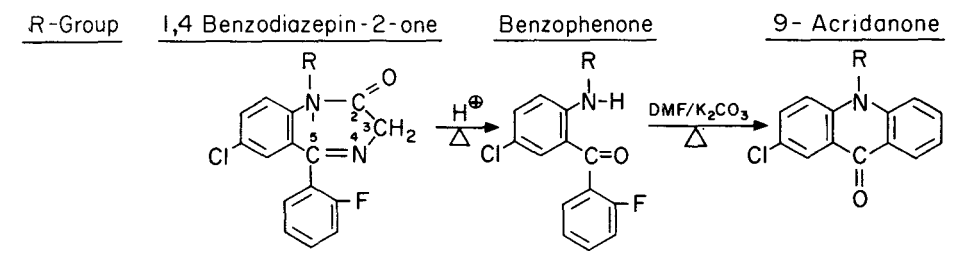


Fig. 16. Chemical reactions of N-1-alkyl-substituted 2'-fluoro-1,4-benzodiazepin-2-ones.

NON-CHROMATOGRAPHIC (DIRECT) ANALYTICAL TECHNIQUES

Absorptiometric and luminescence methods

Spectrophotometric (UV—Vis) and luminescence emission (fluorescence and phosphorescence) analysis [24] have been used extensively in drug analysis. They possess good sensitivity but lack high specificity since spectral characteristics per se cannot usually differentiate the parent drug from any metabolites

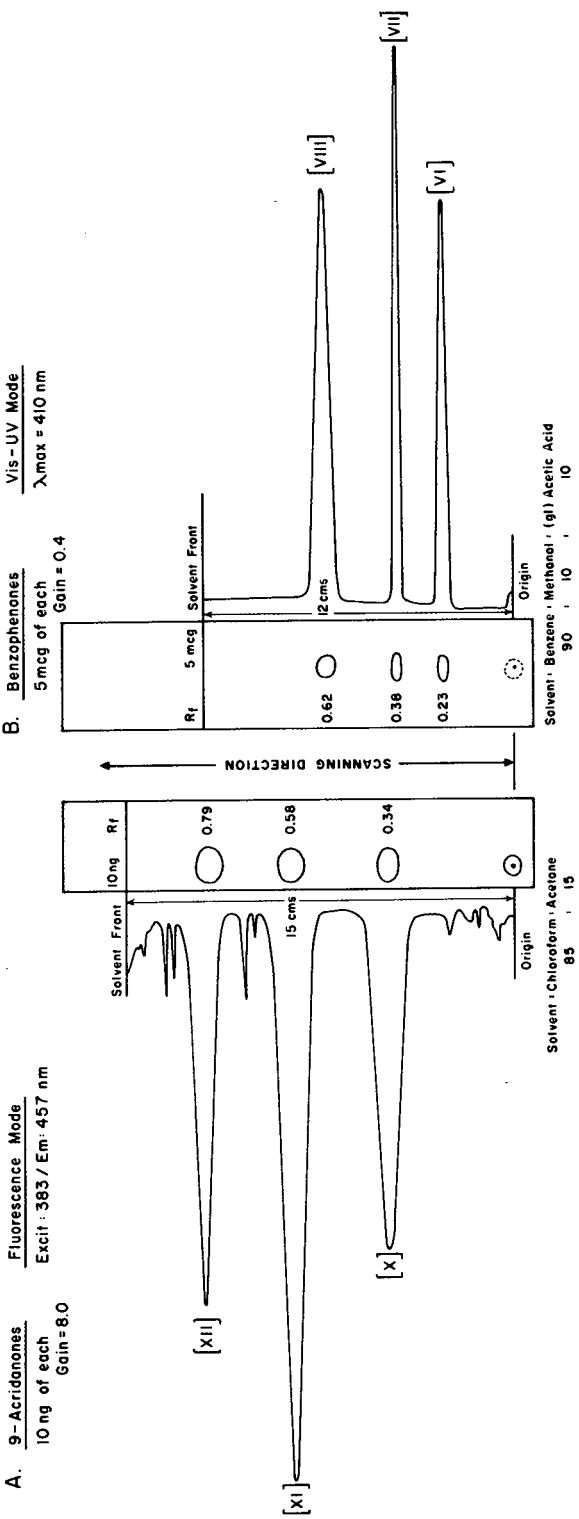


Fig. 17. Thin-layer chromatograms and densitometric scans of (A) the 9-acridanones determined in the fluorescence mode and (B) the benzophenones determined in the Vis—UV mode.

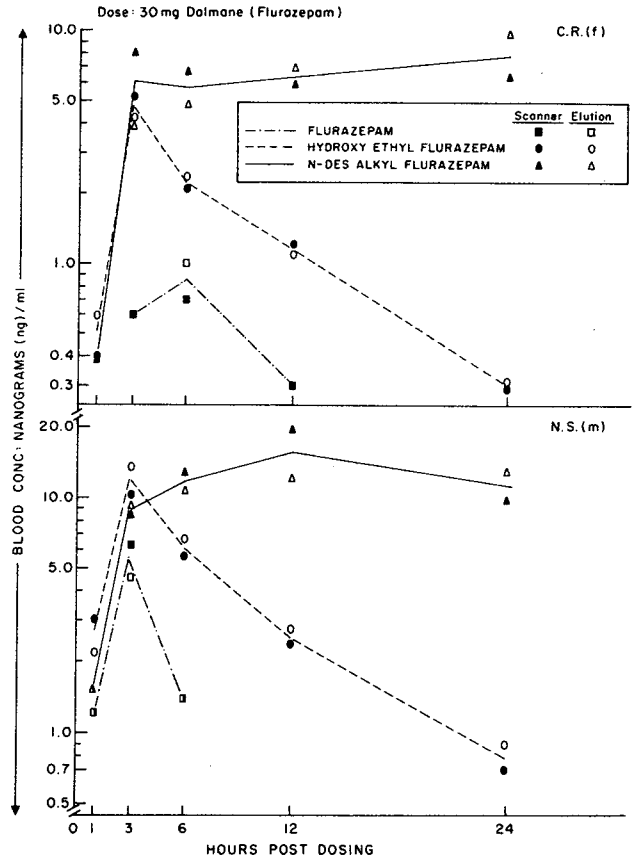


Fig. 18. Blood levels of flurazepam and its major blood metabolites in man determined by fluorometry after elution vs. scanning fluorodensitometry.

present unless used in conjunction with either differential (selective) extraction techniques [1] and/or a chromatographic separation step, e.g. column chromatography, TLC or HPLC.

Differential pulse polarography

Electrochemical methods have better specificity by virtue of the functional group(s) in the molecule involved [25].

Polarographic methods have been used to advantage for the determination of the excretion of urinary metabolites of the 1,4-benzodiazepines, due mainly to the facile reduction of the azomethine ($>C_5=N_4-$) group common to these compounds [26]. The DPP analysis of bromazepam (an antianxiolytic agent) and its major metabolites in urine, viz., 3-hydroxybromazepam [II] and 2-amino-3-hydroxy-5-bromobenzoylpyridine [V] which are present mainly as glucuronide/sulfate conjugates, with small amounts of the intact drug [I] and 2-amino-5-bromobenzoylpyridine [IV] is based on selective extraction of the unconjugated from the conjugated fractions prior to polarographic analysis.

The residues of the respective extracts were analyzed directly by DPP in 1.0

M phosphate buffer (pH 5.5) which yielded two distinct peaks resulting from the reduction of the azomethine (>C_5 = N_4-)group of the benzodiazepin-2-one and the carbonyl (>C = O) group of the benzoylpyridine component in each fraction (Fig. 19).

This fortuitous set of conditions enabled the development of a specific assay without a chromatographic separation step utilizing selective extraction and the different functional groups involved in the two compounds to analytical advantage. The sensitivity was in the order of 50—100 ng of compound per ml of urine analyzed. The recovery of single 12-mg oral doses in adults (equivalent to 0.12—0.18 mg/kg) in a 72-h excretion period ranged from 58–77% of the administered doses mainly as the glucuronide conjugates of the hydroxylated metabolites.

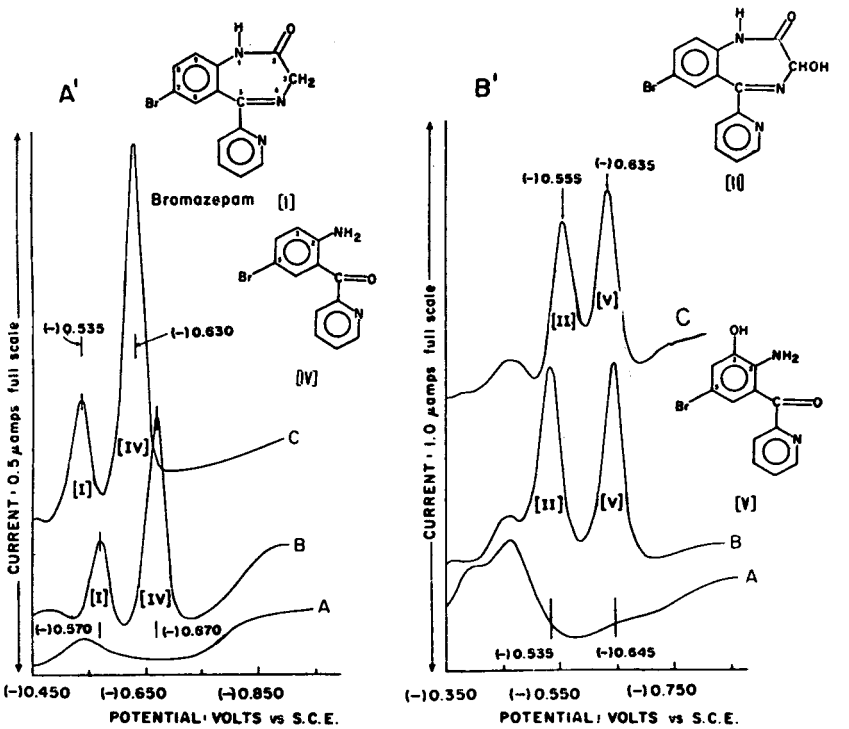


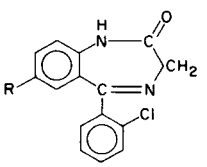
Fig. 19 Differential pulse polarograms of (A') bromazepam [I] and the aminobromobenzylpyridine metabolite [IV] and (B') the 3-hydroxy metabolites [II] and [V] obtained in 1.0 *M* phosphate buffer (pH 5.5) as the supporting electrolyte. (A) Control urine blank, (B) authentic standard mixture, (C) authentic compounds recovered from urine.

Radioimmunoassay

RIA is a most useful clinical diagnostic tool, especially in monitoring drugs in pediatric therapy where small sample volumes are necessary. Radioimmunoassays have recently been developed which are both very sensitive and specific for the parent drug in the presence of its major metabolites and/or other drugs administered concomitantly. Radioimmunoassays for benzodiazepines [27], e.g. clonazepam, are particularly useful in monitoring pediatric patients on

anticonvulsant therapy which usually involves multiple drug regimens and the need for small (microliter) sampling techniques.

Ingenuity is required in the chemical synthesis of the hapten to ensure specificity to the major portion of the parent molecule in order that the antibody produced can distinguish it in the presence of its major metabolites as shown in Fig. 20.

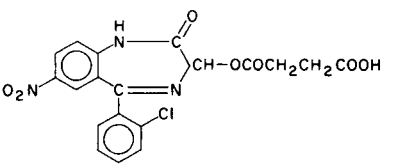


R

NO_2 = Clonazepam

NH_2 = 7-Amino-clonazepam

CH_3CONH = 7-Acetylamino-clonazepam



HAPTEN

7-Nitro-5-(2'-chlorophenyl)-2H-1,4-benzodiazepin-2-one-3-oxy-hemi succinic acid

Fig. 20. Chemical structure of a specific hapten synthesized for the radioimmunoassay of clonazepam.

The RIA has high sensitivity equal to that of EC—GLC and is in the range of 0.5—1.0 ng of drug per ml of plasma; it can be performed on 100 μl or less of plasma either directly in it or following a simple extraction step with an organic solvent. The excellent correlation between the clonazepam RIA and EC—GLC methods obtained from patients receiving clonazepam alone, and concomitant anticonvulsants attest further to the specificity of the two assays and is shown in Fig. 21.

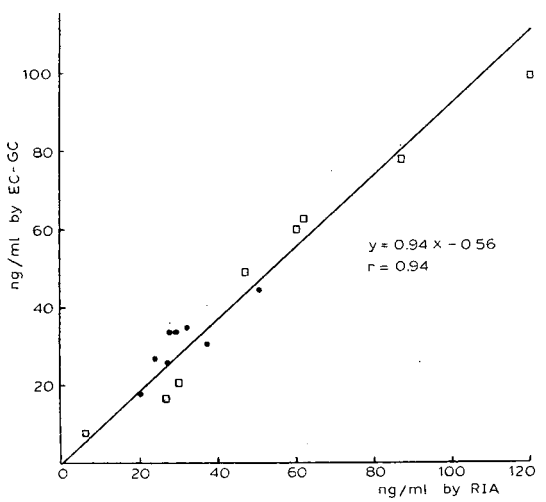


Fig. 21. Correlation between plasma clonazepam concentration determined by RIA and EC—GC in a series of patients receiving clonazepam alone and concomitant anticonvulsants.

CONCLUSION

The chemical structure and the pharmacokinetics of a compound govern not only the sensitivity and specificity requirements of the assay, but also the most suitable biological specimen for its quantitation. The criteria to be used in sample preparation should aim to optimize all of the above factors in the overall development of a reliable and validated method for the compound suitable for use in clinical therapeutic monitoring [28].

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STRATEGY OF GLYCEROLIPID SEPARATION AND QUANTITATION BY COMPLEMENTARY ANALYTICAL TECHNIQUES

PLENARY LECTURE

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SUMMARY

Although improved systems for chromatographic resolution continue to be developed there is good reason to believe that no single method will be capable of complete separation of all lipid mixtures including the geometric, positional and stereochemical isomers in each molecular species. Furthermore, the chromatographic systems giving the highest resolution usually yield the least complete recoveries of components and require separate procedures of quantitation. It is therefore necessary to develop appropriate strategies that yield the required resolution as a result of consecutive application of complementary analytical techniques. At the present time, the original combination of thin-layer and gas—liquid chromatography has been joined by the combination of thin-layer and liquid, and liquid and gas—liquid chromatography with both liquid and gas—liquid chromatography being frequently coupled to mass spectrometry with computerized data processing. Internal standardization with hydrogen flame ionization provides a simple quantitative detection for gas chromatography, while mass spectrometry serves a similar purpose in liquid chromatography, although a much more extensive calibration may be required for quantitation. Special advantages for both separation and quantitation of most neutral lipid mixtures are derived from enzymic and chemical modification of the samples prior to chromatography. With imaginative work-up of samples, superior qualitative and quantitative results can frequently be obtained by appropriate combination of chromatographic techniques of limited resolving power.

INTRODUCTION

The glyceryl esters of fatty acids possess the most complex structure of any natural products of comparable molecular weight. Their derivatives are widely distributed in nature and frequently account for the bulk of the mass of cell membranes and lipoproteins. The biochemical and metabolic requirements of the structural complexity, however, remain unknown. Since the individual molecular species of the glycerolipids exhibit marked physico-

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chemical differences in their bulk and monolayer properties, it is widely believed that the glycerolipids serve some physico-chemical function in the cell. Much effort is therefore being expended in identifying and quantitating the molecular make-up of the lipid phase of various cellular components in the hope that this information will lead to elucidation of the mechanism of their physiological action and the recognition of the need for the extreme diversity in their structure.

A complete identification of glycerolipids requires resolution of molecular species differing not only in molecular weight, degree of unsaturation and the overall polarity of the molecules as obtained by conventional methods of chromatography, but also a differentiation among double bonds of different positional distribution and geometric configuration, as well as a determination of the positional and stereoisomeric placement of the fatty acids on the glycerol molecules [1]. No single method of analysis exists that would be capable of resolving all of these molecules and there is good reason to believe that such methods will not be developed in the foreseeable future. It is therefore necessary to derive and utilize appropriate combinations of analytical techniques, which allow the separation and quantitation of a maximum number of components. In recent years the power of these separations has been further increased by combining the chromatographic techniques with mass spectrometry and computerized data processing, and by preceding the separations with appropriate chemical and enzymic transformations. Although no universally applicable routines have emerged, the advantages demonstrated for the separation and quantitation of model mixtures have suggested potential practical applications for work with specific glycerolipids and their mixtures of natural origin. In the following we have reviewed some of the more recent developments in the utilization of the combined techniques of analysis in the separation and quantitation of glycerolipids of model and natural mixtures with special reference to the utilization of the combined liquid chromatographic—mass spectrometric—computer (LC—MS—COM) system.

MATERIALS AND METHODS

The various experimental materials employed in the present illustrations were available in the laboratory from previous studies referred to in the text. The gas—liquid chromatographic (GLC) analyses were performed on a Hewlett-Packard Model 5880 capillary gas chromatograph, the high-performance liquid chromatography (HPLC) on a Hewlett-Packard Model 1084B liquid chromatograph and the mass spectrometry (MS) was done on a Hewlett-Packard 5985B quadrupole mass spectrometer equipped with an LC—MS interface based on the Baldwin—McLafferty split chemical ionization (CI) approach. The stereospecific analyses of triacylglycerols were performed as described by Myher and Kuksis [2]. The final products of the chemical and enzymic transformations were purified by conventional thin-layer chromatography (TLC) but in some instances the individual glycerolipid classes were further resolved by $AgNO_3$-TLC. The operating conditions for the polar capillary GLC of diacylglycerols were as described by Myher and Kuksis [3], while

the non-polar capillary GLC conditions for the examination of the double enzyme digestion products of glycerophospholipids were as given by Kuksis et al. [4]. The GC—MS of the diacylglycerols was performed as reported by Myher et al. [5]. The HPLC conditions of triacylglycerol resolution were modeled on the work of El Hamdy and Perkins [6] and of Herslof and Pelura [7] with the acetone—acetonitrile and acetonitrile—propionitrile systems, respectively. For the LC—MS application a gradient of 20—90% propionitrile in acetonitrile proved the most effective. The general conditions of operation and the performance of the Hewlett-Packard LC—MS interface have been described by Kenyon et al. [8]. In the present study, the MS scans were limited to masses above 200, because the lower mass range was contaminated with ions from impurities in solvents. The greatest offender was propionitrile, especially at the higher concentrations of gradient. To remove the contaminant ions (e.g. *m/z* 252, 391) a scan was made of propionitrile at 90% concentration, where no glycerolipids were being eluted, and the intensities of these ions were subtracted from all scans containing the ions belonging to the glycerolipids. There was no loss or distortion of the glycerolipid ions as verified by detailed examination of the mass spectra before and after the subtraction.

RESULTS AND DISCUSSION

The combination of complementary analytical techniques has been recognized as a useful strategy in lipid separation and quantitation in the past [9, 10] and in specific instances superior results have been obtained [11—13]. The need for strategy in glycerolipid analyses arises from the extreme complexity of natural glycerolipid mixtures, the limited resolving power of individual analytical techniques, the small amounts of sample usually available for analysis, a requirement for a realistic sample turn-around time, and a desire to minimize the cost of the analysis.

The more effective combinations of complementary analytical techniques include a systematic preliminary work-up of the sample by chemical and enzymatic means, which can also be applied to any subfractions of the sample derived following a chromatographic separation. The chemical work-up usually facilitates the separation of lipid classes, while the enzymic hydrolysis leads to a segregation of positional and stereochemical isomers of the glycerolipids. Combinations of complementary chromatographic techniques comprise the well established TLC—GLC routines and the more recently developed couplings of TLC—LC and LC—GLC. In recent years extensive use has been made of the combination of various MS methods with chromatography, of which the most valuable have been the GC—MS and LC—MS. Although these techniques possess an enormous analytical power, especially when combined with computerized data processing, there remain applications which cannot be adequately handled by unidimensional MS. There is a need for MS—MS combinations [14], which must be projected for future application in glycerolipid analysis, although the cost of the initial introduction of the system may be high.

Chemical modification

A number of chemical transformations are generally performed on the total lipid extract as well as on any subfractions of it derived by chromatography, which are not considered as specific combinations of complementary analytical techniques. These include saponification, transmethylation of fatty acids and silylation of various free hydroxyl and carboxyl groups prior to chromatography. However, Grignard degradation of triacylglycerols [15] must be recognized as a special analytical maneuver, which leads to the random release of diacylglycerols necessary for subsequent stereospecific analyses of triacylglycerols via both phospholipase A_2 and phospholipase C. Likewise, synthesis of both phosphatidylphenols [16] and phosphatidylcholines [2] as intermediates in the stereospecific analysis of the triacylglycerols via both phospholipase A_2 and phospholipase C must be considered as complementary steps in the total analytical scheme of determination of stereochemical structure of both diacylglycerols and triacylglycerols.

Specific enzymic hydrolyses

In many instances it has proved informative to subject the glycerolipid sample to a specific enzymic hydrolysis prior to chromatographic analysis. The advantages of using phospholipase C to release the diacylglycerol moieties from the glycerophospholipids are well recognized [17]. It should be noted, however, that phospholipase C is stereospecific and that it attacks the *sn*-3-phosphatides much more readily than the *sn*-1-phosphatides [18]. It can be used to distinguish between the enantiomeric glycerophosphatide types in a mixture and can serve to provide quantitative estimates of the relative proportions of the enantiomers by subsequent isolation and quantitation of the degraded and undegraded phosphatides [2]. A similar resolution of the enantiomeric glycerophospholipids can be obtained by means by phospholipase A_2, except that in the latter instance the identity of the molecular species is lost because of the destruction of the diacylglycerol moiety of the *sn*-3-phosphatide [19]. We have taken advantage of the stereospecificity of phospholipase C in devising a method of stereospecific analysis of triacylglycerols via an intermediary formation of *rac*-1,2-diacylglycerols and *rac*-1,2-diacylglycerophosphorylcholines [2].

Fig. 1 outlines the chemical and enzymic transformations in the generation of the *sn*-1,2- and *sn*-2,3-diacylglycerols. These transformations

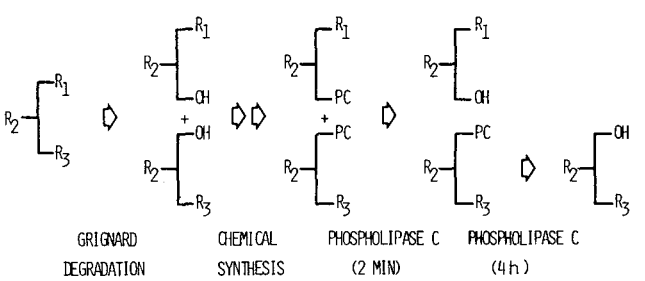

Fig. 1. Resolution of *sn*-1,2- and *sn*-2,3-diacylglycerol moieties of triacylglycerols by complementary chemical and enzymic transformations [2].

allow a direct examination of the pairing of the fatty chains in the various diacylglycerol moieties that are randomly generated from the triacylglycerols by Grignard degradation. Although this knowledge does not yet permit a direct reconstitution of the original triacylglycerol structure, it does allow the testing of the validity of the various random hypotheses about the structure of natural triacylglycerols on the basis of the predicted association of the fatty acids in these positions [20].

In the past phospholipase A_2 has been used to specifically release the fatty acids from the *sn*-2-position of *sn*-3-phosphatides and Brockerhoff [16] had utilized this reaction as the basis for his stereospecific method of analysis of natural triacylglycerols via the intermediate formation of *rac*-phosphatidylphenols. This enzyme destroys the *sn*-1,2-diacylglycerol moiety of the triacylglycerol molecules and traps the *sn*-2,3-diacylglycerol moiety in an enzymically inaccessible form.

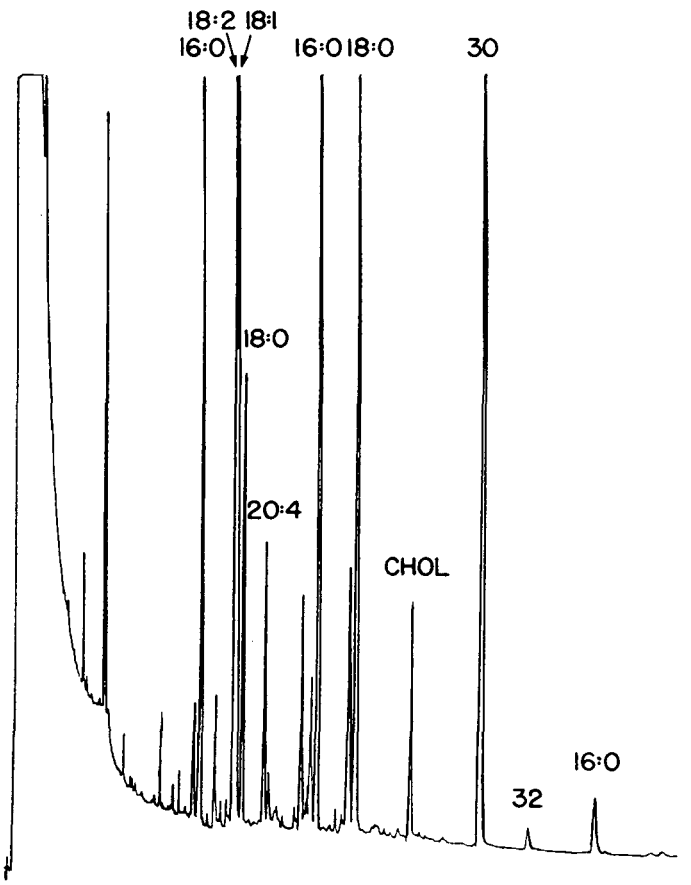

Fig. 2. Analysis of egg yolk phosphatidylcholine (PC) by consecutive digestion with phospholipase A_2 and phospholipase C, and capillary GLC [4]. First series of peaks (Peaks 16:0, 18:2, 18:1 and 20:4), fatty acids released from *sn*-2-position of PC; second series of peaks (Peaks 16:0 and 18:0), fatty acids retained in the *sn*-1-position as monoacylglycerols; CHOL, cholesterol; Peaks 30 and 32, triacylglycerols with 30 and 32 acyl carbons.

We have recently combined the action of phospholipase A_2 and of phospholipase C in a double enzyme digestion routine for the analysis of the structure of phosphatidylcholines in natural mixtures [4]. An initial digestion with phospholipase A_2 converts the diacylglycerophospholipids into the corresponding lysophospholipids with a release of the fatty acids from the *sn*-2-position. A subsequent digestion of the reaction mixture with phospholipase C converts the lysophosphatides into the corresponding monoacylglycerols. A trimethylsilylation of a total lipid extract of the final reaction mixture transforms the fatty acids into the trimethylsilyl (TMS) esters to represent the composition of the *sn*-2-position and the monoacylglycerols into the di-TMS ethers to represent the *sn*-1-position of the diacylglycerol moiety of the glycerophospholipid. All of these components can be completely recovered by means of GLC on both packed and capillary columns containing non-polar liquid phases or polar liquid phases as shown in Fig. 2.

We have also attempted to adopt the double enzyme digestion for an improved determination of plasma total lipid profiles. In the past we had depended upon phospholipase C to convert the choline phosphatides and sphingomyelins into the corresponding mono- and diacylglycerols and free ceramides, which could then be resolved from the free cholesterol, cholesteryl esters and triacylglycerols by high-temperature GLC [21]. In this routine, however, the diacylglycerols and ceramides largely overlap when run as the TMS or *tert.*-butyldimethylsilyl (t-BDMS) derivatives. A preliminary digestion with phospholipase A_2 converts the phosphatidylcholine into the lysophos-

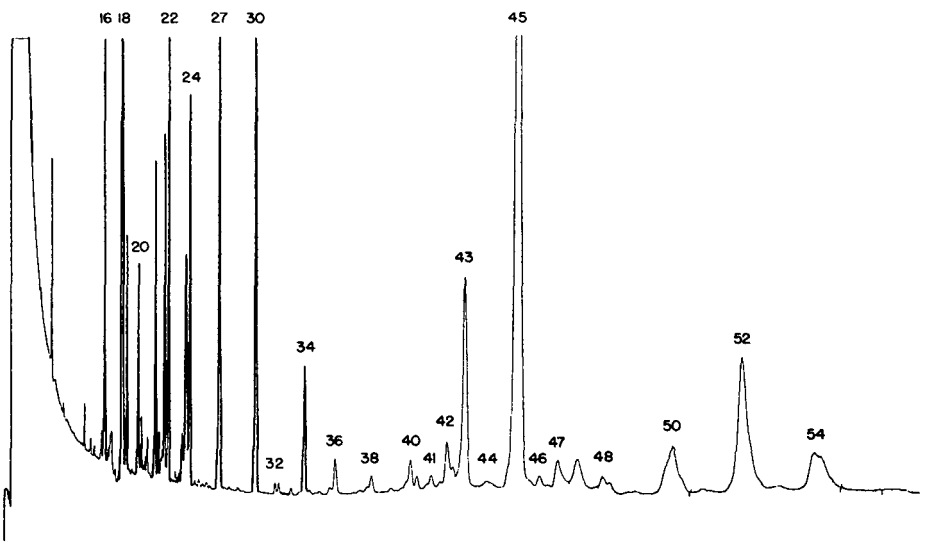

Fig. 3. Analysis of plasma total lipids by consecutive digestion with phospholipase A_2 and phospholipase C, and capillary GLC [4]. First series of peaks (Peaks 16, 18 and 20), fatty acids released from the *sn*-2-position of PC; second series of peaks (Peaks 20, 22 and 24), monoacylglycerols retaining the fatty acids in the *sn*-1-position of phosphatidylcholine; Peak 27, cholesterol; Peak 30, tridecanoylglycerol; Peaks 32, 34, 38, 40 and 42, ceramides with a total of 32—42 carbons; Peaks 41, 43, 45 and 47, cholesteryl esters of fatty acids with 14—20 acyl carbons; Peaks 44, 46, 48, 50, 52 and 54, triacylglycerols with a total number of 44—54 acyl carbons.

phatidylcholine, which can then be further converted along with the sphingomyelins into the corresponding monoacylglycerols and ceramides, which are now completely resolved as the TMS derivatives, as shown in Fig. 3. The liberated fatty acids overlap with the small amounts of plasma free fatty acids and the released monoacylglycerols overlap with the traces of plasma free monoacylglycerols, which do not significantly influence the estimates of plasma total phosphatidylcholines. The ceramides provide a direct estimate of the plasma total sphingomyelins, as well as of their relative composition or carbon number distribution. The free sterols, steryl esters and triacylglycerols remain unaffected during these transformations and are eluted with their characteristic retention times. Theoretically the remaining overlaps could be avoided by combining a double enzyme digestion with a double chemical derivatization [4]. In this procedure the plasma free fatty acids, lysophosphatidylcholines, monoacylglycerol free sterols and sphingomyelins are first converted into the corresponding t-BDMS derivatives. The reaction mixture is then subjected to hydrolysis with phospholipase A_2, which converts the diacylglycerophosphatidylcholines into the lysophosphatidylcholines and free fatty acids. A subsequent digestion with phospholipase C could then be used to remove the phosphorylcholine moieties from the lysophosphatidylcholines and the t-BDMS ethers of sphingomyelins, and the reaction products resolved by GLC following a further derivatization with the TMS reagent.

TLC—GLC

TLC is the most widely applied method of separation of natural glycerolipids and glycerophospholipids. Improved separation of glycerolipid classes has recently been realized by means of high-performance thin-layer chromatography (HPTLC) [22, 23], while improved quantitation of the resolved fractions has been achieved by the use of the Iatroscan system [24]. A combination of the TLC resolution with a subsequent GLC examination of the recovered components, however, can provide further information about the fatty acid composition of the glycerolipid classes. If the transmethylation and GLC analysis are performed in the presence of an internal standard, excellent quantitative measurements of the lipid classes may also be obtained along with the qualitative composition [25]. The TLC method is especially well suited for purification and isolation of the small amounts of material necessary for capillary GLC of the fatty acid methyl esters [26]. Furthermore, TLC may be performed with modified adsorbents, of which the most popular are those containing silver nitrate, which allows the separation of saturated and unsaturated fatty acid esters, as well as of certain geometric isomers. Although the latter method is amenable to work with intact glycerophospholipids [27, 28], more efficient resolutions of the saturated and unsaturated species are obtained following a removal of the polar head groups by phospholipase C [17, 29]. Although most of the present knowledge of the molecular species structure of glycerophospholipids has been obtained by the latter method [30], it is not entirely satisfactory, as the exact combinations of the fatty acids must be calculated from the molecular proportions of the major fatty acids assuming some type of random distribution. Much more certain are the combinations of the fatty acids derived from a high temperature GLC of the

diacylglycerols of uniform double bond content [12, 31]. Since free diacylglycerols tend to isomerize on the $AgNO_3$-TLC plates and yield separate peaks for the *sn*-1,2- and *sn*-1,3-isomers, it is necessary to block the free hydroxyl groups with acetyl [31] or t-BDMS [5] groups. Unlike the TMS ethers, the t-BDMS ethers are stable to moisture and can be purified by TLC prior to GLC. The blocking of the free hydroxyl groups also permits the resolution of the alkyl acyl, alkenyl acyl and diacyl species of the diradylglycerols, that may be present in some of the original glycerophospholipid classes [11].

Combinations of $AgNO_3$-TLC and GLC are also suitable for the investigation of the structure of natural triacylglycerols. High-temperature GLC on both packed [32] and capillary [33] columns allows a carbon number or molecular weight resolution of triacylglycerols of uniform number of double bonds. Transmethylation of the various argentation fractions yields the fatty acid composition as well as a quantitative proportionation of the individual subfractions. The method has been extensively utilized in a preliminary characterization of natural triacylglycerols [34, 35]. A more complete determination of the triacylglycerol structure requires partial degradation of the acylglycerol molecules prior to further resolution. Hammond [36] has outlined a theoretical approach to a complete determination of the molecular species of triacylglycerols by means of combined argentation TLC—preparative GLC and stereospecific analysis via phospholipase A_2. This method, however, is too complicated for practical execution and no natural fats have been analyzed in this manner as yet.

GC—MS

Another popular combination of complementary analytical systems is GC—MS. MS in the form of single ion monitoring provides a specific and sensitive detection for the effluents of the GLC columns, while the total ion current output compares favourably to the output of a hydrogen flame ionization detector. The MS identification of the molecular species within a complex mixture of diacylglycerols is in general limited to determination of carbon and unsaturation number. The technique also requires time-consuming data processing [37] and extensive calibration [38, 39].

When working with deuterium-labelled molecular species of the glycerolipids, it is necessary to perform the GC—MS assays following a preliminary $AgNO_3$-TLC separation. However, recent advances in the development of polar capillary columns may improve the GC—MS application to the natural diacylglycerol moieties of the glycerophospholipids or of triacylglycerols. Preliminary studies have shown that the capillary columns coated with SP-2330 (Silar 9C) produce very little bleed at column temperatures which yield essentially complete resolution of the molecular species of natural diacylglycerols (250°C). Fig. 4 shows that the resolution of diacylglycerols on these columns is sufficiently complete and reproducible and that the need for MS examination of the column effluents is largely eliminated [3]. However, unknowns and deuterium-labelled species must still be identified and quantitated by MS.

Likewise, GC—MS is effective in the characterization of natural triacyl-

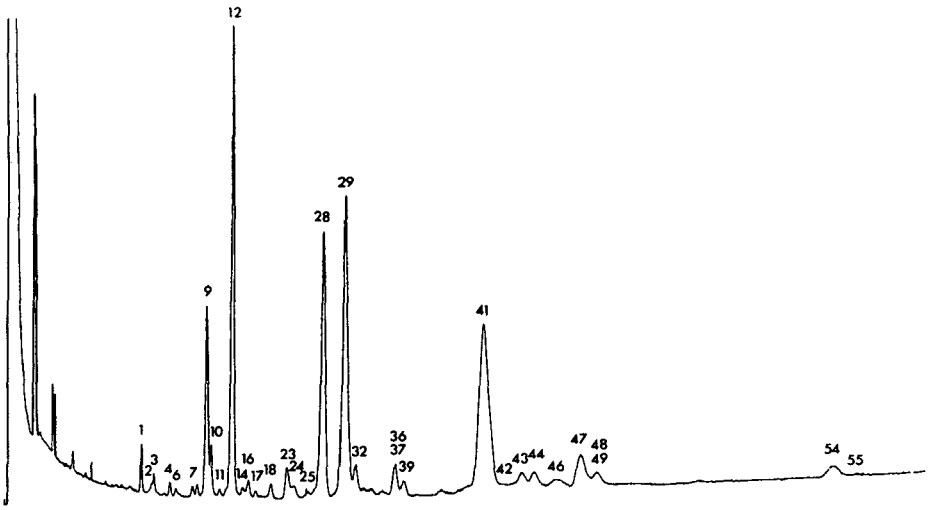

Fig. 4. Capillary GLC of TMS ethers of *sn*-1,2-diacylglycerols of rat liver phosphatidylcholines. The major peaks are: Peak 9, 16:0 18:1w9; Peak 12, 16:0 18:2w6; Peak 28, 18:0 18:2w6; Peak 29, 16:0 20:4w6; Peak 41, 18:0 20:4w6; and Peak 54, 18:0 22:6w3.

glycerols only following $AgNO_3$-TLC. The triacylglycerol fractions of uniform degree of unsaturation can then be admitted to the mass spectrometer as uniform carbon numbers, the fatty acid and diacylglycerol content and composition of which can then be readily recognized in the ammonia ionization mode [40].

Like capillary GLC, GC—MS has been greatly advanced by the development of the flexible quartz capillary column [41], which practically eliminates costly breakages and permits simple exchange of columns as the experimental needs may dictate. The flexible quartz capillaries are readily interfaced with MS instruments without the need of ineffective gas separators. At the present time, however, the flexible quartz capillaries are not available in a complete selection of liquid phases.

LC—TLC

In recent years LC in the form of HPLC has undergone a spectacular development and now provides a favourable alternative choice for the resolution of glycerolipids. While normal-phase HPLC yields essentially the same separations as TLC, reversed-phase HPLC provides separations that were previously possible only by a combination of $AgNO_3$-TLC and GLC. Thus, reversed-phase HPLC resolves natural triacylglycerols on the basis of both molecular weight and degree of unsaturation or polarity [42]. In fact, the reversed-phase HPLC columns are capable of a nearly complete resolution of all molecular species of the neutral acylglycerols, including many critical pairs and triplets [6, 7]. It is also possible to resolve all the major molecular species of the common glycerophospholipids by means of reversed-phase HPLC on C_{18} columns using phosphate buffers and methanol or choline chloride and methanol as the eluting solvents [43, 44].

Since the solutes resolved by HPLC are readily collected, it is possible to

subject them to further chromatographic resolution as such or following a chemical or enzymic transformation. In those instances where the critical pairs of the acylglycerols overlap during the reversed-phase HPLC, a subsequent $AgNO_3$-TLC resolution of the collected peaks may yield evidence of additional components. Bezard and Ouedraogo [45] have noted that certain natural triacylglycerol mixtures are more effectively resolved by HPLC following a preliminary segregation by argentation TLC. Furthermore, since the solutes of interest in the HPLC separation are frequently detected by UV absorption, the collected material may contain components which do not absorb in the UV. It is therefore prudent to subject the collected fractions to TLC to ensure their purity and to resolve any overlapping solutes.

LC—GLC

One of the major shortcomings of the HPLC method of analysis is the absence of a universal detector for lipids. Although the presence of unsaturated fatty acids and their esters may be detected by short-wavelength UV light (190—210 nm), the absorptivity is relatively low and variable, and quantitative analysis is impractical because of the need for extensive calibration. It has therefore proved practical to use the UV absorption only as a guide for peak collection and to perform the quantitative determinations of the solutes by GLC. In addition, complex mixtures of lipids may not be reliably identified by the retention times of standards, hence the peaks must be collected and identified by GLC as the methyl esters of the component fatty acids, which then also serve for their quantitation. Although laborious, the combination of HPLC and GLC provides the most effective means of resolving, identifying and quantitating the molecular species of intact natural glycerophospholipids [44]. Furthermore, in combination with capillary GLC it is possible to identify and quantitate the molecular species differing in the positional distribution and geometric configuration of the double bonds of the component fatty acids which remain unresolved by HPLC. There is no record as yet of the behaviour of the plasmalogens during reversed-phase HPLC of the glycerophospholipids. In a few instances the molecular species of glycerophospholipids have been first dephosphorylated and the released diacylglycerols converted into UV-absorbing derivatives prior to HPLC [46]. This maneuver greatly improves the sensitivity of detection and the accuracy of quantitation of the molecular species, but does not alter the extent of resolution or of the peak identification, which still requires assistance from GLC or MS.

Likewise, only small gains are made in the resolution of the glycerolipid species by converting the original glycerophospholipids into UV-absorbing derivatives. Proper identification of the peaks requires their collection and GLC examination. Consecutive application of reversed-phase HPLC and GLC has also proven to be effective in the resolution and identification of the molecular species of triacylglycerols [6, 7, 45]. Thus, by collecting the effluent from the HPLC columns and examining it by GLC for the fatty acid composition it has been possible to demonstrate that mixtures of acetone—acetonitrile (63.5:36.5) [6] and acetonitrile—propionitrile (50:50 or 30:70) [7] yield excellent resolution of certain critical pairs of triacylglycerols. Since positional isomers and enantiomers are not resolved by any of the HPLC systems, a

complete identification of the triacylglycerol species requires collection of peaks and stereospecific analysis as indicated above.

HPLC—MS

A combination of HPLC with MS is especially useful in glycerolipid work because of the low chromogenicity of the saturated fatty acid esters in the short-wavelength range of UV (190—210 nm), and the general low sensitivity of refractometric assay of all lipids. The mass spectrometer serves as a more sensitive detector and as a further analyzer of the components. The simplest technique for coupling HPLC to MS is the direct introduction of a fraction of the column effluent into the mass spectrometer [47]. The system employed in the present studies includes a provision for extra cryogenic pumping within the mass spectrometer in which the solvent vapour is trapped via cooling with liquid nitrogen. In this system the quantity of solvent introduced into the mass spectrometer is only about 1/100th of the effluent from the liquid chromatograph (1.5 ml/min). The mass spectrometer is operated in the chemical ionization mode, with the HPLC effluent serving as the reagent gas. The less extensive fragmentation in the chemical ionization mode provides increased sensitivity of detection, which is most important in view of the unfavourable effluent split.

Since the natural triacylglycerols are readily resolved by HPLC but not easily detected or identified by the UV and refractometry detection systems, we selected triacylglycerols for the combined LC—MS examination. The acetone—acetonitrile and acetonitrile—propionitrile solvent systems, which have thus far been shown to give the best resolution of triacylglycerols, also appeared to be excellent reagents for chemical ionization. They yielded readily

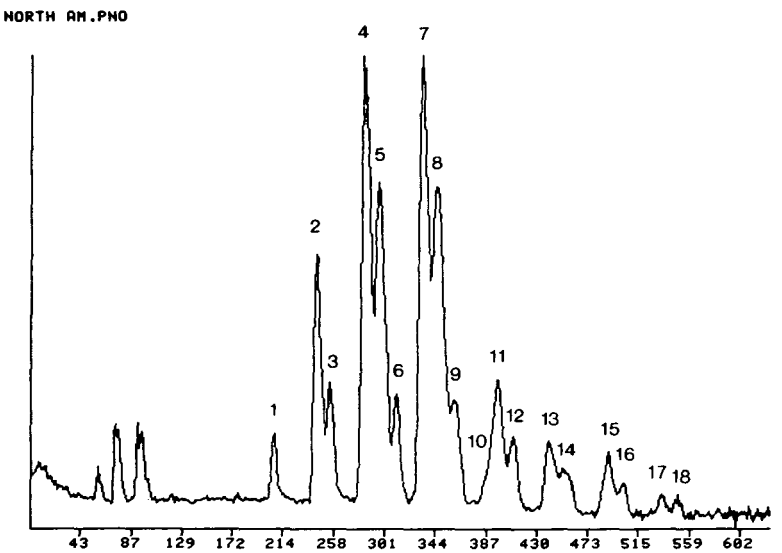

Fig. 5. LC—MS of peanut oil triacylglycerols. The major peaks are: Peak 2, 18:1 18:2 18:2; Peak 4, 18:1 18:1 18:2; Peak 5, 16:0 18:1 18:2; Peak 7, 18:1 18:1 18:1; and Peak 11, 18:1 18:1 18:0. LC—MS conditions: 30—90% propionitrile in acetonitrile in 60 min. Flow-rate, 1.5 ml/min.

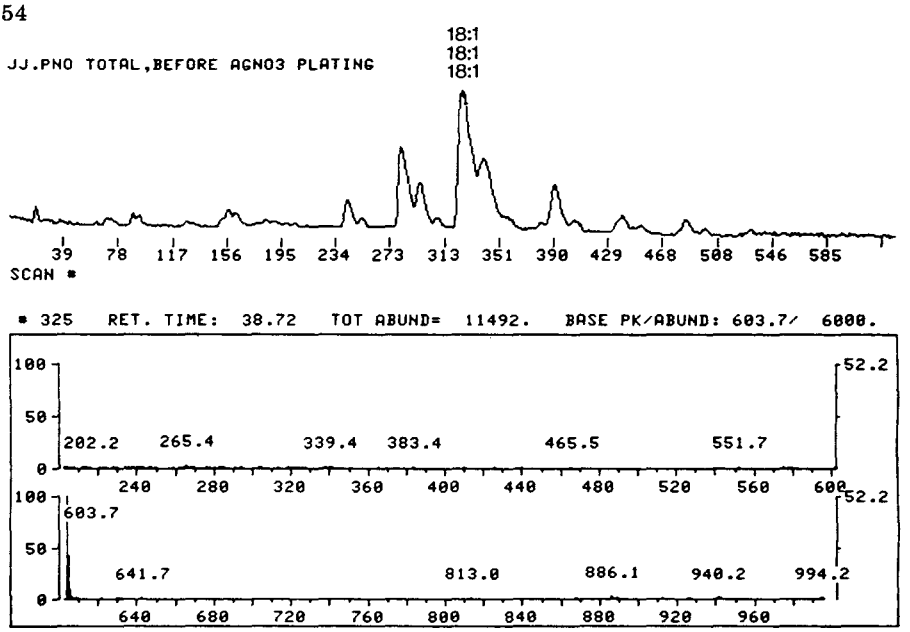

Fig. 6. Chemical ionization spectrum of Peak 7 in Fig. 5. Upper panel, total ion current profile; lower panel, mass spectrum of trioleoylglycerol. LC—MS conditions as in Fig. 5.

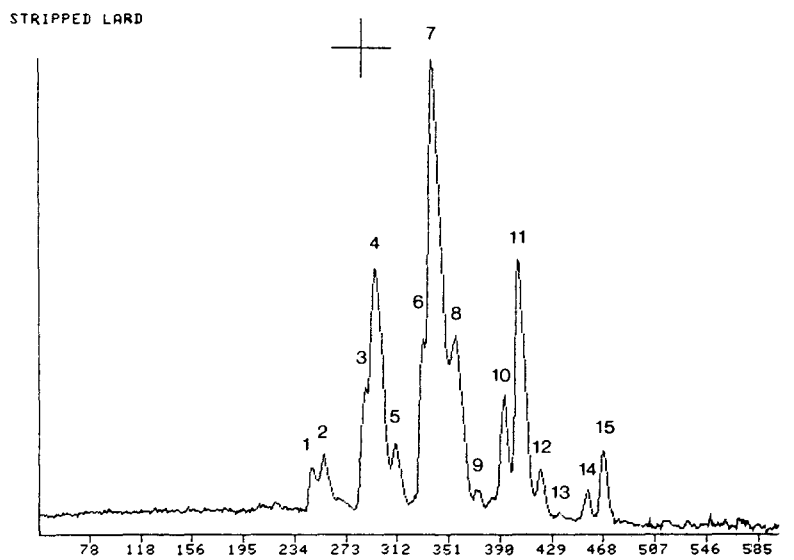

Fig. 7. LC—MS of stripped lard. The major peaks are: Peak 4, 16:0 18:1 18:2; Peak 6, 18:1 18:1 18:1; Peak 7, 16:0 18:1 18:1; Peak 8, 16:0 16:0 18:1; Peak 10, 18:0 18:1 18:1, Peak 11, 16:0 18:0 18:1; Peak 12, 16:0 16:0 18:0; Peak 14, 18:0 18:0 18:1; and Peak 15, 16:0 18:0 18:0. LC—MS conditions as in Fig. 5.

detectable protonated molecular ions as well as characteristic mass ions for the component diacylglycerols. We observed that a 20—90% gradient of propionitrile in acetonitrile provided sharper peaks and more complete recoveries of the saturated species than any of the isocratic solvent systems. Further-

more, this gradient system appears to be universally applicable to diacylglycerol and triacylglycerol mixtures. Fig. 5 shows the total ion current profile obtained for peanut oil triacylglycerols. The numbered peaks represent either individual triacylglycerols or small groups thereof. The three unnumbered peaks in the front represent small amounts of contaminating diacylglycerols. Fig. 6 shows the mass spectrum of one of the major peaks (Peak 7) identified as essentially pure trioleoylglycerol. It yields a small ion for the protonated parent molecule (*m/z* 886) and a single ion for dioleoylglycerol (*m/z* 603). The presence of significant amounts of trioleoylglycerol in the peanut oil was confirmed by LC—MS of the triene band isolated by $AgNO_3$-TLC.

The LC—MS method is equally well suited for analyses of animal fats. Fig. 7 shows the elution pattern obtained for a sample of stripped lard using the propionitrile gradient in acetonitrile. The numbered peaks represent individual triacylglycerols or simple mixtures of them. Peak 6 is made up of pure trioleoylglycerol as indicated by the single prominent diacylglycerol ion at *m/z* 603 representing dioleoylglycerol. Fig. 8 shows that a much smaller peak, Peak 12 in the original pattern, can be identified as largely 16:0 16:0 18:0 triacylglycerol. The presence of this species is indicated by the formation of nearly equal intensities of diacylglycerols corresponding to 16:0 16:0 (*m/z* 551) and 16:0 18:0 (*m/z* 579) ions, although theoretically a ratio of 2:1 would have been anticipated.

Fig. 9 shows the total ion current profile of human plasma triacylglycerols as obtained by LC—MS in the acetonitrile—propionitrile gradient. The numbered peaks correspond to individual triacylglycerols or small groups thereof. Fig. 10 shows that Peak 13 is made up essentially of pure 16:0 18:1 18:0 triacylglycerol as indicated by the ions *m/z* 579 (16:0 18:0), *m/z* 577 (16:0 18:1) and *m/z* 605 (18:1 18:0), in roughly 1:1:1 proportion.

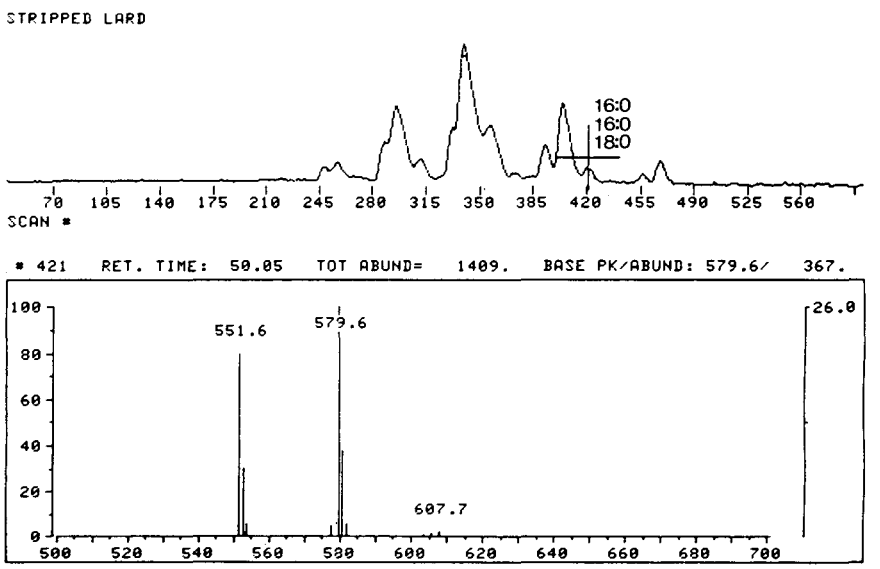

Fig. 8. Chemical ionization spectrum of Peak 12 in Fig. 7. Upper panel, total ion current profile; lower panel, mass spectrum of dipalmitoylmonostearoylglycerol. LC—MS conditions as in Fig. 5.

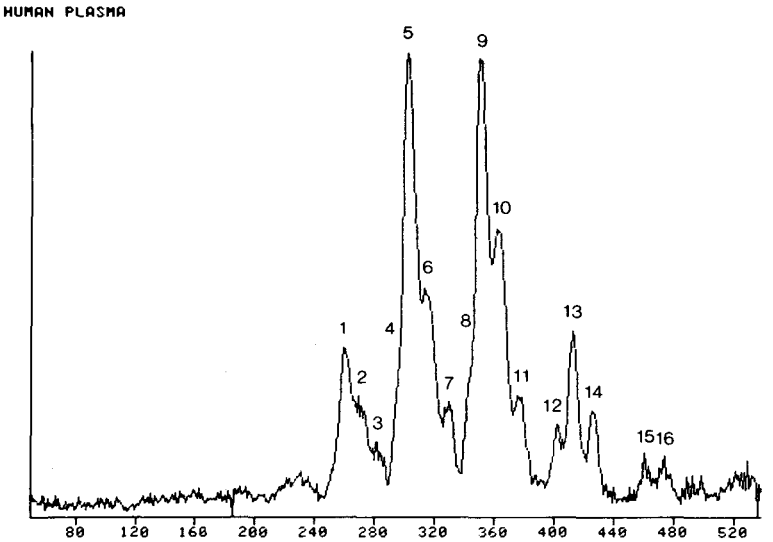

Fig. 9. LC—MS of human plasma triacylglycerols. The major peaks are: Peak 1, 16:0 18:2 18:2; Peak 5, 16:0 18:1 18:2; Peak 6, 16:0 16:0 18:2; Peak 9, 16:0 18:1 18:1; and Peak 10, 16:0 16:0 18:1. LC—MS conditions as in Fig. 5.

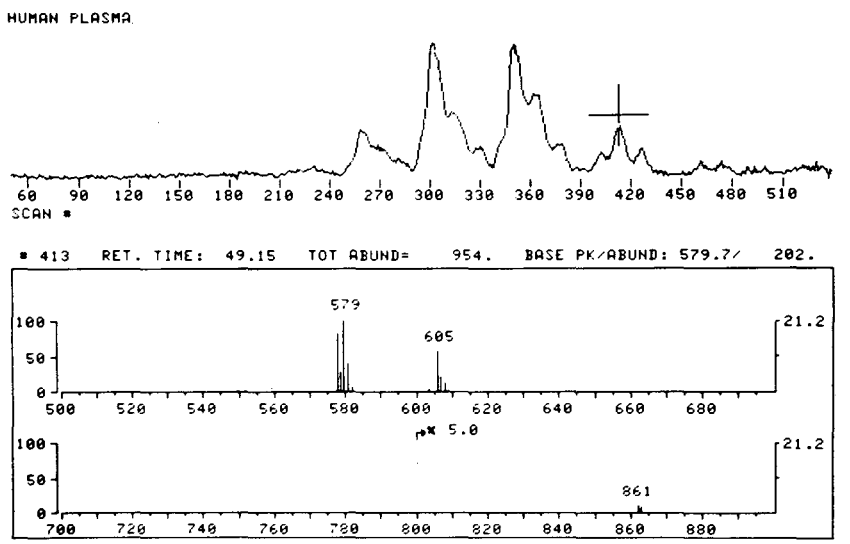

Fig. 10. Chemical ionization spectrum of Peak 13 in Fig. 9. Upper panel, total ion current profile; lower panel, mass spectrum of palmitoyloleoylstearoylglycerol. LC—MS conditions as in Fig. 5.

We have attempted to apply HPLC to the determination of plasma total lipid profile, but these efforts thus far have not been as successful as those obtained by high-temperature GLC. Fig. 11 shows the LC—MS profile of human plasma lipids after treatment with phospholipase C. In the acetonitrile—propionitrile gradient there was an excellent resolution of the diacylglycerols

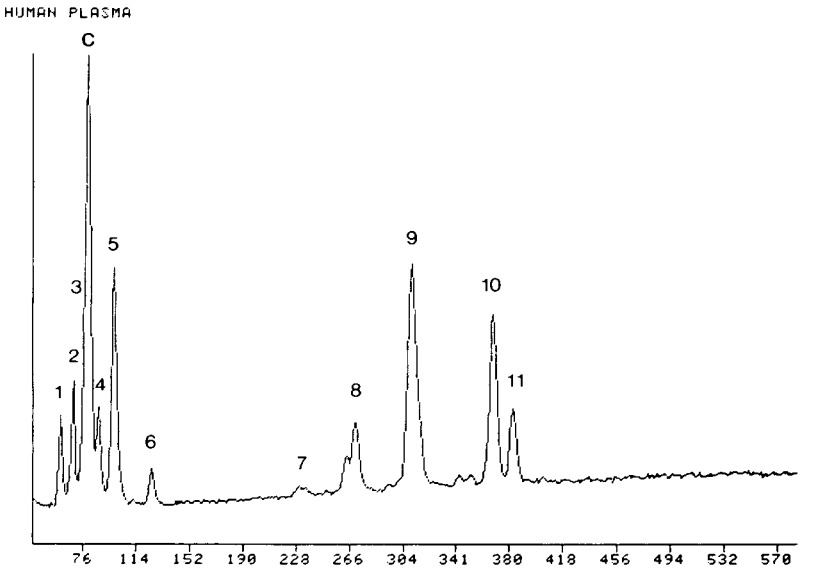

Fig. 11. LC—MS of human plasma lipids after dephosphorylation with phospholipase C. The major peaks are: Peak 1, 16:0 22:6; Peak 2, 16:0 20:4; Peak 3, 18:0 22:6 + 16:0 18:2; Peak 4, 18:0 20:4; Peak 5, 16:0 18:1 + 18:0 18:2; Peak 6, 18:0 18:1; Peak 8, cholesteryl arachidonate; Peak 9, cholesteryl linoleate; Peak 10, cholesteryl oleate; Peak 11, cholesteryl palmitate; and C, cholesterol. LC—MS conditions as in Fig. 5.

released from the plasma phosphatidylcholines by the phospholipase C digestion, and of the cholesteryl esters. Thus, Peaks 1—6 represent the diacylglycerols 16:0 22:6, 16:0 20:4, 18:0 22:6 + 16:0 18:2, 18:0 20:4, 16:0 18:1 + 18:0 18:2, and 18:0 18:1 as the major components. When run in the free form, the diacylglycerols 16:0 18:2 and 18:0 22:6 overlap partially with free cholesterol. In the acetate form, the cholesterol peak overlaps with that of 18:0 20:4 diacylglycerol species. There was also an overlap using the t-BDMS ethers of diacylglycerols and of free cholesterol. Peaks 8—11 represent the cholesteryl esters of arachidonic, linoleic, oleic and palmitic acids, respectively. The cholesteryl esters overlap with the triacylglycerols, which are seen as minor peaks interspersed among the major cholesteryl ester peaks. An overlap of triacylglycerol and cholesteryl ester peaks during HPLC has been previously noted [48, 49]. The triacylglycerol peaks can be best seen by subtracting the ions due to the cholesteryl esters from the total ion current profile. Fig. 12 shows that such a differential plot readily identifies the triacylglycerols as minor Peaks 1—13. Although this plasma sample is different from that analyzed above, it is seen that the same triacylglycerol pattern can be recognized. The removal of the ions due to cholesterol results also in the removal of the free cholesterol peak from the diacylglycerol profile.

Another complex mixture of triacylglycerols which we have subjected to LC—MS—COM analysis is provided by bovine milk fat. Fig. 13 shows the LC—MS profiles of the most volatile 2.5% of butterfat, and of the whole butterfat triacylglycerols. There is an excellent correspondence between the

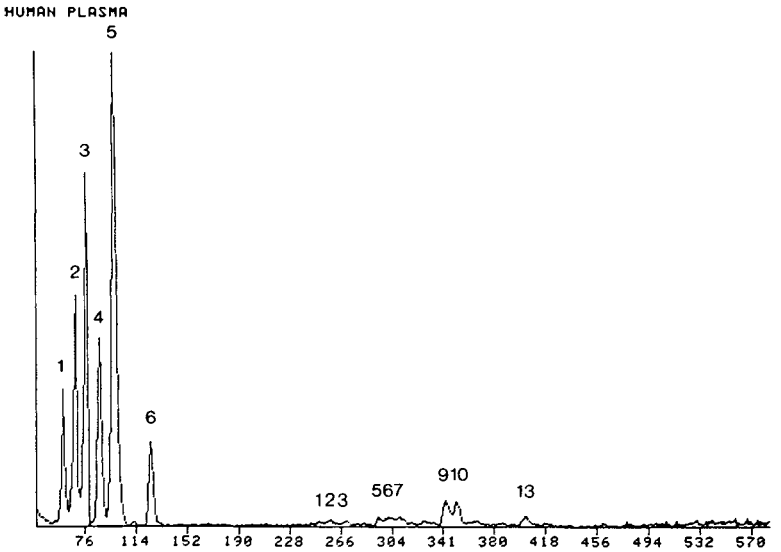

Fig. 12. Differential mass spectrum of plasma lipids as obtained by subtracting the ions for free cholesterol and cholesteryl esters from the total ion current profile in Fig. 11. First set of peaks (Peaks 1—6), diacylglycerols; second set of peaks (Peaks 1—13), triacylglycerols. LC—MS conditions as in Fig. 5.

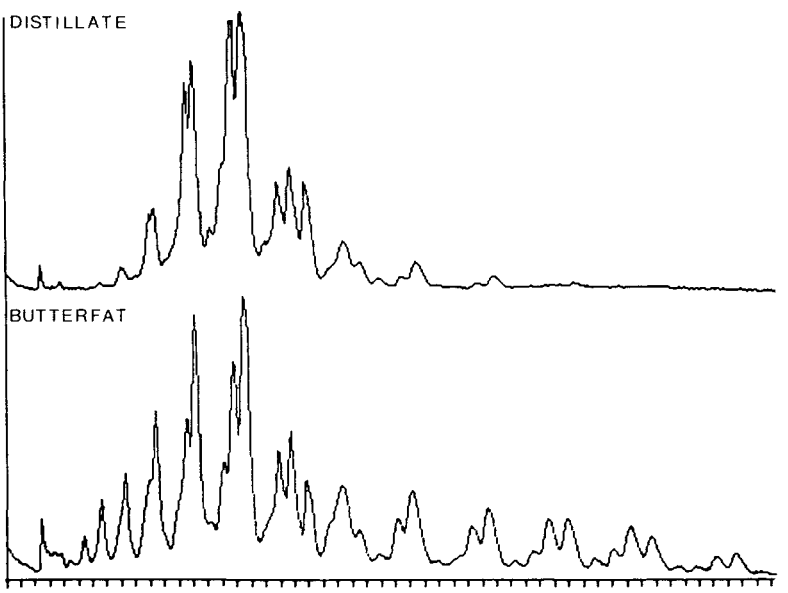

Fig. 13. LC—MS of the most volatile 2.5% distillate and of total bovine milk fat triacylglycerols. LC—MS conditions as in Fig. 5.

distillate peaks and the peaks in the total fat, although the actual sources of the triacylglycerols are not the same. Fig. 14 shows that the second half of the first major peak in the whole fat corresponds to 16:0 14:0 4:0, as the main component. We have identified all the peaks in the distillate and in the whole

butterfat and have verified that the corresponding HPLC peaks possess comparable compositions of molecular species. The composition of the peaks, however, is much more complex than the simple HPLC elution profiles would indicate. Fig. 15 shows the LC—MS—COM plot of the composition of the butterfat, by displaying the mass distribution for the ions of the component diacylglycerols and for some triacylglycerols corresponding to each triacylglycerol peak in the HPLC profile. Although the pattern is too complex to make any quantitative distinction, the qualitative distribution provides a good indication of the complexity of the species. The mass ranges have been given for each peak in a forward and backward direction so as not to obscure any of the masses present.

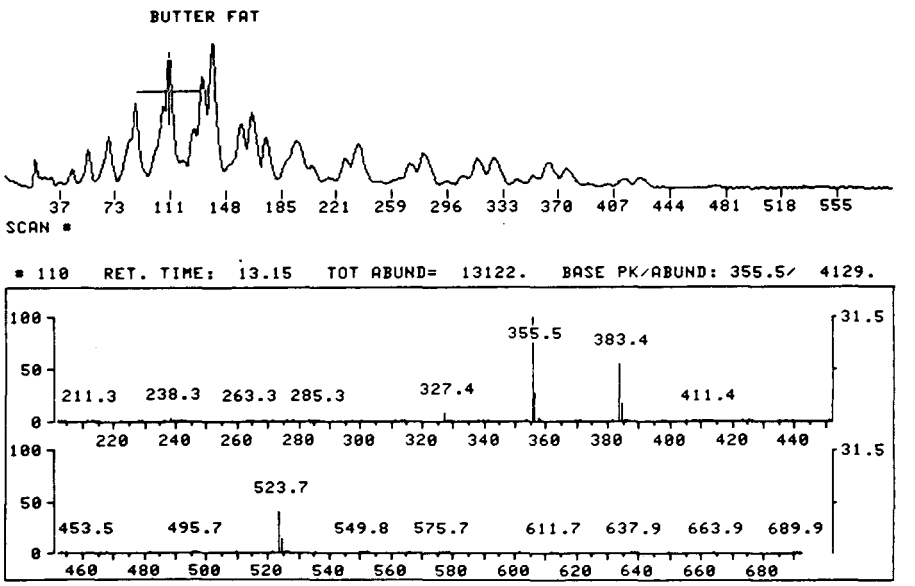

Fig. 14. Chemical ionization spectrum of one of the major peaks in the total bovine milk fat shown in Fig. 13. Upper panel, total ion current profile; lower panel, mass spectrum of palmitoylmyristoylbutyroylglycerol. LC—MS conditions as in Fig. 5.

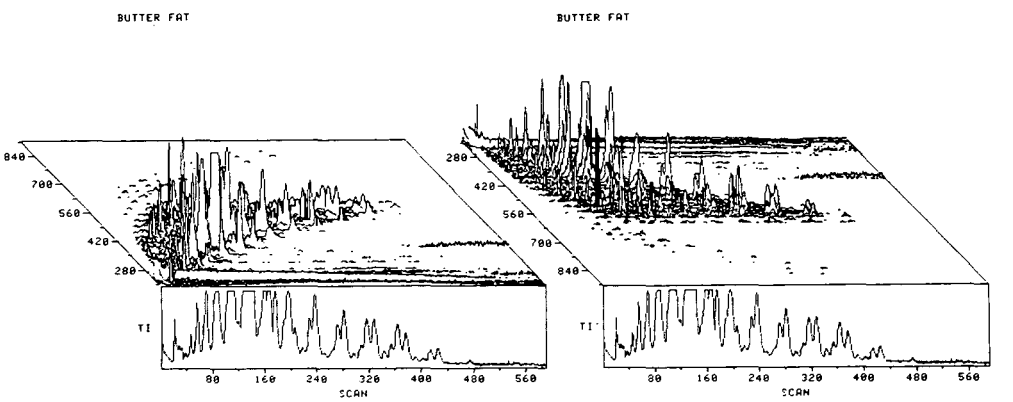

Fig. 15. Three-dimensional computer plot of the LC—MS data obtained for total bovine milk fat triacylglycerols in Fig. 13.

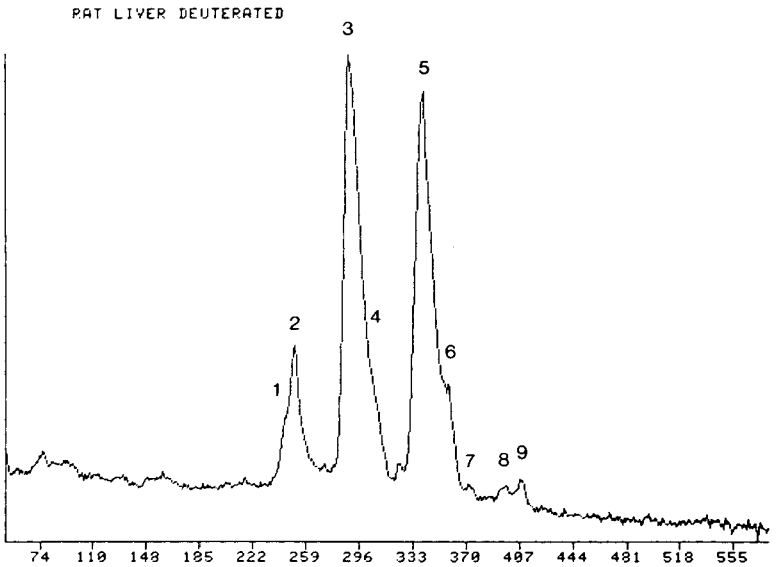

Fig. 16. LC—MS of deuterated rat liver triacylglycerols. The major peaks are: Peak 2, 16:0 18:2 18:2; Peak 3, 16:0 18:1 18:2; Peak 5, 16:0 18:1 18:1; and Peak 6, 16:0 16:0 18:1. LC—MS conditions as given in Fig. 5.

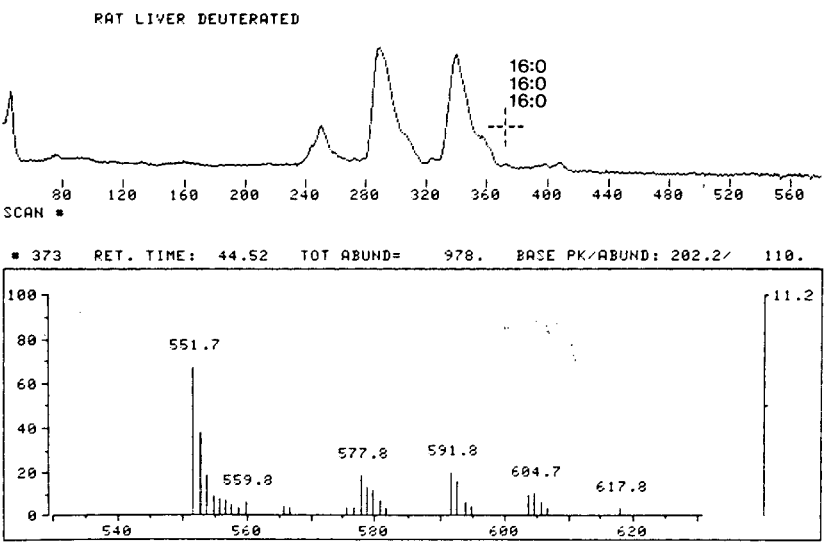

Fig. 17. Chemical ionization spectrum of Peak 7 in Fig. 16. Upper panel, total ion current profile; lower panel, mass spectrum of deuterated tripalmitoylglycerol. Note the presence of deuterium in the ions *m/z* 552—599. LC—MS conditions as in Fig. 5.

Like GC—MS, LC—MS is apparently well suited for the measurement of the stable isotope content and distribution among the various molecular species of triacylglycerols. Fig. 16 shows the total ion current profile of rat liver triacylglycerols obtained from an animal subjected to an overnight infusion of perdeuterated ethanol. Again the numbered peaks represent individual

triacylglycerol species or small groups thereof. Fig. 17 shows that a minor peak can be identified as tripalmitoylglycerol, with smaller amounts of 16:0 18:1 16:0 and a 17:0-containing species trailing from the preceding peak. Clearly the palmitic acid residues contained deuterium as indicated by the elevated intensities at *m/z* 552—559.

Fig. 18 shows the mass spectrum of one of the major triacylglycerol peaks. It can be identified as largely 16:0 18:1 18:2, as indicated by the corresponding ions for the diacylglycerols. Again, the palmitic acid component contains deuterium, but the presence of deuterium in any of the fatty chains in the 18:1 18:2 diacylglycerol moiety is less certain, as the increase in the mass could have arisen from a presence of deuterium in the glycerol moiety of the molecule. Although some of the ambiguity of the distribution of the deuterium among the triacylglycerol species can be eliminated by a preliminary $AgNO_3$-TLC, a complete clarification of the problem is not possible. For this purpose an MS—MS analysis of the peaks would be desirable [14]. In such a system, selected ions of the parent molecule or of any derived fragments could be admitted to another mass spectrometer and the composition of these ions determined by a further fragmentation. In such a case no doubt would remain about the true origin of any of the fragments in the mass spectrum of the total mixture.

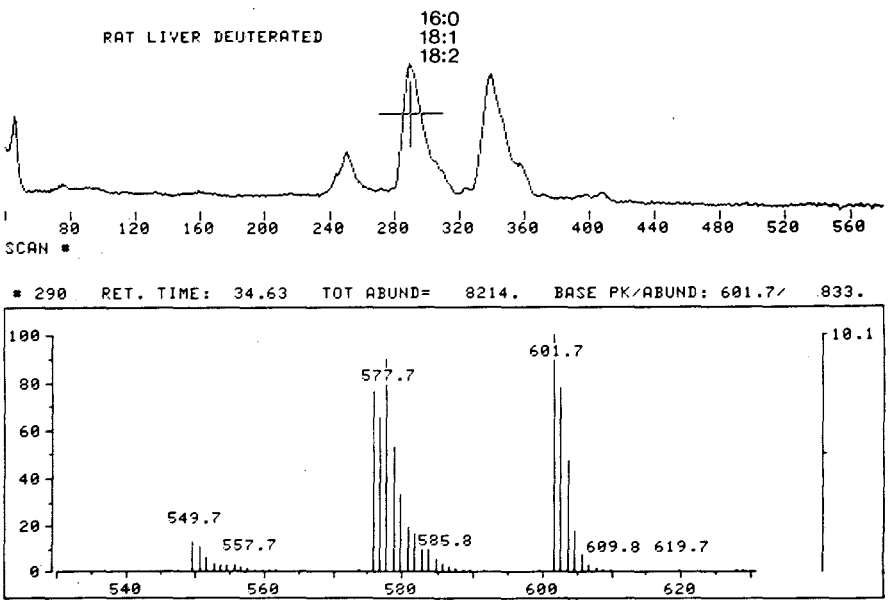

Fig. 18. Chemical ionization spectrum of Peak 3 in Fig. 16. Upper panel, total ion current profile; lower panel, mass spectrum of deuterated palmitoyloleoyllinoleoylglycerol. LC—MS conditions as in Fig. 5.

Quantitation

The most effective quantitation of neutral glycerolipids is obtained by means of hydrogen flame ionization detection, which gives a response directly proportional to the carbon content of the fatty esters over a wide range of

concentrations [50]. This method has been extensively employed in the quantitation of fatty acids and their glycerol and long-chain alcohol esters using both non-polar and polar, packed and capillary GLC columns. It can be applied to the determination of the relative and absolute amounts of the lipid components in the original sample or in any fraction thereof derived by the combined application of complementary analytical techniques, in which GLC provides the final step. An endless belt technique of hydrogen flame ionization detection of solutes in HPLC effluent has also been described and can give highly reproducible results under appropriate conditions of analysis [51]. The Iatroscan system is designed to provide a flame ionization response for the components resolved in the TLC or Chromarod systems, and for some homologous series a close relationship has been found between the mass concentration of the solutes and the peak areas recorded by the flame ionization detector [24]. However, many other mixtures undergo a highly variable decomposition on the absorbent surfaces and yield flame ionization responses that require extensive calibration for quantitative work [52]. A comparable measurement of the mass of the eluted components in a chromatographic system is provided by the total ion current of the mass spectrometer, but this response varies considerably with the molecular weight, unsaturation and chemical stability of the solutes and requires extensive calibration of individual molecular species [5].

The intact glycerophospholipids are best quantitated by their phosphorus content following a perchloric acid digestion to inorganic phosphorus, if necessary, in the presence of the silica gel from the TLC plate [53]. Colorimetric means of quantitation are also available for the fatty acid, glycerol and nitrogenous base moieties of the glycerolipids [54]. The latter methods utilize the Beer—Lambert principles of colorimetric analysis and do not require special discussion here. Of interest is, however, the post-column utilization of some of these methods for the detection and quantitation of the solutes emerging in the effluent of the HPLC columns [55].

In connection with the HPLC separation and detection of lipids must be mentioned the possibility of their detection in the short-wavelength UV range (205—210 nm), which is largely due to the double bonds in the fatty acid molecules [56]. This method of detection and quantitation of the lipid esters requires extensive calibration with the corresponding molecular species before the recorded peak areas may be effectively utilized for calculation of the masses of the solutes. In contrast, the UV-absorbing derivatives of fatty acids and diacylglycerols (e.g. esters of UV-absorbing alcohols and acid) can be quantitated directly on the basis of the molar extinction coefficient of the reagent, which is not significantly affected by the nature of the molecular species of the fatty chains [46, 56]. Extensive calibration is also not necessary for quantitative measurements of lipids in the HPLC column effluents by means of refractometry [42].

Relative composition

A simple GLC elution pattern provides the relative peak area composition of the fatty ester mixture [57]. The individual areas of all components are determined, and from the mean the relative peak areas are established.

$$\%X = 100\ A_x/(A_1 + A_2 + x \ldots + A_n)$$

where A_x is the area of a given peak X and A_1 to A_n the sum of all the peak areas. High-quality packed columns or short capillary columns, which allow complete recoveries of all components, when combined with hydrogen flame ionization detection generally yield area responses, which agree closely with the weight concentration of the fatty esters in the mixture and the correction factor is 1. It is important to verify that the estimates of the relative composition hold over the entire range of concentrations encountered in the samples, not just for the concentrations found in a commercial sample that happens to be used for calibration of the system. This can be tested by means of appropriate dilution of the various components of the standard mixture by adding known amounts of pure components, as well as by injecting different concentrations of the total sample in the presence of an internal standard of a fixed concentration. For fatty esters comprising different homologous series and for analytical systems not giving complete recoveries of all components, it is important to correct the peak area for differences in the detector response. This can be done by means of correction factors (F), which yield corrected individual and total peak areas,

$$\text{Corr. Area} = A_1F_1 + A_2F_2 + \ldots + A_nF_n$$

where A_1 to A_n are the peak areas and F_1 to F_n are the corresponding response correction factors. The correction factors are determined experimentally by reference to a standard known to be completely recovered ($F = 1$), as follows

$$F_x = (A_r/A_x)\ (W_x/W_r)$$

where A_x and A_r are the areas of the solute and reference peaks, respectively. W_x and W_r are the concentrations of the solute and the reference compounds.

It should be noted that in these corrections no distinction is being made between differences in the detector response due to loss or decomposition of the solute on the column and an inherent difference in the total ion yield in the hydrogen flame ionization detector. The weight response can be easily converted into a relative molar response by dividing it out by the molecular weight of each fatty ester and normalizing the result.

Absolute composition

In addition to the relative composition of the sample, it is frequently necessary to ascertain the total amount of the fatty ester in the sample or in a subfraction of it. This can be accomplished by means of either the internal or external standard method. In the internal standard method the concentration of the fatty esters in the mixture is derived by adding to the unknown sample a known amount of an internal standard, which is completely recovered and which does not overlap with any of the solutes in the chromatogram [57]. The concentration of any one of the unknowns can be obtained by relating

its area to the area of the internal standard, and the total weight of the sample to the sum of all the areas.

$$C_x = (C_s/A_s)\, A_x F_x$$

where C_s and A_s are the concentrations and area of the standard and A_x and F_x are the peak area and response factors of the measured compounds. If the volume containing the unknowns is also known, the results can be expressed as weight units per 100 volume units, as an example. For analytical systems, which possess a limited linear response to increasing or decreasing solute concentration, the response relative to the internal standard is plotted against solute concentration over a range of solute concentration with a constant addition of internal standard, and the concentration of the unknown is obtained from the calibration curve. It is recommended, however, that an attempt is first made to adjust the analytical system in such a way that the analyses can be completed within the linear range of the response, as the errors outside this range can be variable (adjust sample size or column length).

External standards are used when it is not possible to use internal standards, when peaks are poorly resolved or when there are too many peaks. A calibration curve is prepared using a range of the external standard concentration by injecting accurately controlled volumes in the column and plotting the response against the concentration of the standard. As the injection volume remains constant, absolute amount of the standard is directly proportional to the concentration. Injection of an equal volume of sample containing the unknown can then be used to obtain its concentration from the graph. Reproducible volumes may be injected with a conventional Hamilton syringe by first drawing up 1 μl of the solvent followed by 1 μl of the standard solution. Emptying the entire contents of the syringe into the injector results in a quantitative delivery of 1 μl of standard solution. It is usually impractical to prepare precise calibration curves by injecting increasing volumes from a constant standard concentration. The external standard technique has been extensively employed in the calibration of the total ion current and of single ion response in the mass spectrometer, where a representative internal standard cannot be used because of the need for closely reproducing both molecular weight and chemical properties of the unknown. In GC—MS and in LC—MS isotope-labelled internal standards may be employed to provide an exact match of the recoveries and ionization intensities for the measured and reference species.

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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF TOTAL 4-HEPTANONE, A NEW MARKER IN DIABETES MELLITUS

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SUMMARY

Total 4-heptanone is the sum of a β-oxocarboxylic acid, 2-ethyl-3-oxohexanoic acid, and its decarboxylation product, 4-heptanone.

The β-oxocarboxylic acid is found in serum and in urine, and is detected by gas chromatography—mass spectrometry in the form of its methyl ester or its O-methyloximated acid methyl ester. The ketone is detected within the profile of volatile metabolites in serum and in urine. However, the analytical procedure includes some ketone coming from in vitro decarboxylation.

Total 4-heptanone is measured by gas chromatography—mass fragmentography. The method includes a quantitative transformation of the β-oxocarboxylic acid into the ketone. A comparative study with 270 patients with diabetes mellitus, 28 healthy individuals and 143 non-diabetic hospitalized patients showed that the urinary excretion of total 4-heptanone is increased in diabetes mellitus. The mean values are 1073 μg per 24 h for diabetics, compared with 207 μg per 24 h and 246 μg per 24 h for healthy individuals and non-diabetic patients, respectively. Diabetic ketoacidosis and fasting conditions decrease the total 4-heptanone.

INTRODUCTION

Ketone bodies are, besides glucose and pH value, the classical parameters to indicate in a diabetic patient a decompensation in the sense of hyperglycemia and insulin deficiency. The ketone bodies, which are formed by ketogenesis after increased lipolysis and fatty acid oxidation, are normalized when the patient is restabilized by therapy. In addition to the regular ketone bodies, acetoacetic acid, acetone and β-hydroxybutyric acid, higher-molecular-weight ketone bodies are found in blood and urine, especially the ketones themselves which are analytically best detectable [1—4]. We could show that the levels of the higher-molecular-weight ketone bodies, 2-pentanone, 2-penten-3-one and 2-heptanone, as well as the β-oxocarboxylic acid which acts as precursor for 2-pentanone, also rise during increased ketogenesis and normalize when the diabetic patient is under good therapeutical control.

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On the other hand, we observed that two other ketonic substances, 4-heptanone and its precursing β-oxocarboxylic acid, 2-ethyl-3-oxohexanoic acid, behave differently. They are not normal when the glucose levels of diabetic patients are well controlled, and therefore reflect an abnormality in the metabolism of diabetics even if by therapy the patients are normalized. This paper describes mainly the results of a broad study on the urinary total 4-heptanone (ketone plus its precursor) in diabetic patients in comparison with healthy individuals and non-diabetic hospital patients.

EXPERIMENTAL

Apparatus

The analyses were performed on a Model 900 gas chromatograph (Bodenseewerk Perkin-Elmer, Überlingen, G.F.R.), a Model 3700 gas chromatograph (Varian, Darmstadt, G.F.R.) and a combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian-MAT, Bremen, G.F.R.). The gas chromatograph and the mass spectrometer were interfaced by a 30 cm × 0.1 mm I.D. platinum capillary.

Detection of 2-ethyl-3-oxohexanoic acid

2-Ethyl-3-oxohexanoic acid was detected in a fraction of the organic acids in serum and in urine in the form of its methyl ester or in the form of its O-methyloximated acid methyl ester. The method for extraction, derivatization and pre-fractionation leading to the O-methyloximated acid methyl ester derivative was described previously [5]. To obtain the methyl ester with the free carbonyl group left, the method was modified such that the reaction of the serum or urine sample with O-methyl hydroxylamine hydrochloride was omitted.

Fraction 2 of the pre-fractionated sample contained the 2-ethyl-3-oxohexanoic acid derivative and was analyzed by gas chromatography—mass spectrometry (GC—MS) under the following conditions: 25 m glass capillary column coated with OV-17 (Bodenseewerk Perkin-Elmer); carrier gas, helium at 4 ml/min; column temperature, 40°C for 10 min, then programmed at 2°C/min; injector block temperature, 250°C; sample size, 1 μl; ionization by electron impact; ionization energy, 70 eV; accelerating voltage, 3 kV; multiplier voltage, 2.5 kV; emission current, 300 μA; ion source temperature, 220°C; interface temperature 220°C; resolution 700; recording mode, automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15—380 and stored on magnetic tape.

Detection of total 4-heptanone

4-Heptanone was detected in the profile of volatile substances. The gas phase extraction and adsorption technique used in the work-up of the sample, and the GC procedure were described for urine [2] and serum [3].

Quantitative determination of total 4-heptanone in urine

Total 4-heptanone was determined by gas chromatography—mass fragmentography (GC—MF). A 6.4 mg/l solution of 3-heptanone in water containing 0.1%

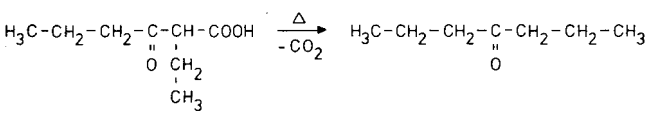
of Triton X-100 was used as the internal standard.

Urine samples (4-ml; usually in series of 20 samples) were heated in glass-stoppered and clamped vials for 90 min in a water-bath at 90°C to complete the decarboxylation of 2-ethyl-3-oxohexanoic acid to form 4-heptanone. After cooling, 0.2 ml of the internal standard solution was added to each of the samples resulting in internal standard concentrations of 305 μg/l. The mixtures were extracted with 1 ml of cyclohexane by shaking for 1 min. The extracts were separated from the aqueous phases, and 1-μl aliquots of the extracts were subjected to GC—MF analysis under the following operating conditions. Two different columns were used with equal suitability: column A, 100 m × 0.5 mm I.D. stainless-steel column, coated with Emulphor ON-870, at an oven temperature of 80°C; column B, 25 m × 0.2 mm I.D. fused silica column, coated with OV-1701 (Scientific Glass Engineering, Weiterstadt, G.F.R.), at an oven temperature of 40°C, injector block temperature 150°C. Mass spectrometric conditions were as described above, except for resolution (400) and recording mode (the mass spectrometer was focused on the molecular ion *m/e* 114).

The calculation of the concentrations of total 4-heptanone was based on the ratios of the peak heights of 4-heptanone and internal standard, and on calibration graphs obtained from eight aqueous standard solutions with 4-heptanone concentrations between 15 and 3200 μg/l. The amount of total 4-heptanone excreted in 24 h was calculated by multiplying the concentration with the 24-h urine volume.

RESULTS AND DISCUSSION

We define total 4-heptanone as the sum of 2-ethyl-3-oxohexanoic acid and 4-heptanone. The ketone is formed from the labile β-oxocarboxylic acid by decarboxylation according to the reaction



It must be anticipated that this decarboxylation partly occurs in vivo, analogous to the decarboxylation of acetoacetic acid to form acetone. In vitro, heat enhances this reaction.

The β-oxocarboxylic acid is found in urine and in serum. It can be detected in fraction 2 of the organic acids either in the form of its methyl ester with the carbonyl group left underivatized, or in the form of the methyl ester with the carbonyl group being derivatized to form the O-methyloxime. By computer—MF, especially the methyl ester is detected with good sensivity and specificity. Its detection is based on the intense ion *m/e* 71, supported by the molecular ion *m/e* 172 and the fragment ions *m/e* 129 and *m/e* 141 (Fig. 1). The method is suitable for the qualitative detection and identification of 2-ethyl-3-oxohexanoic acid. To a certain degree, semiquantitative information can be derived. A quantitative determination of the acid is restricted by its lability. The extent of decarboxylation during the work-up procedure is

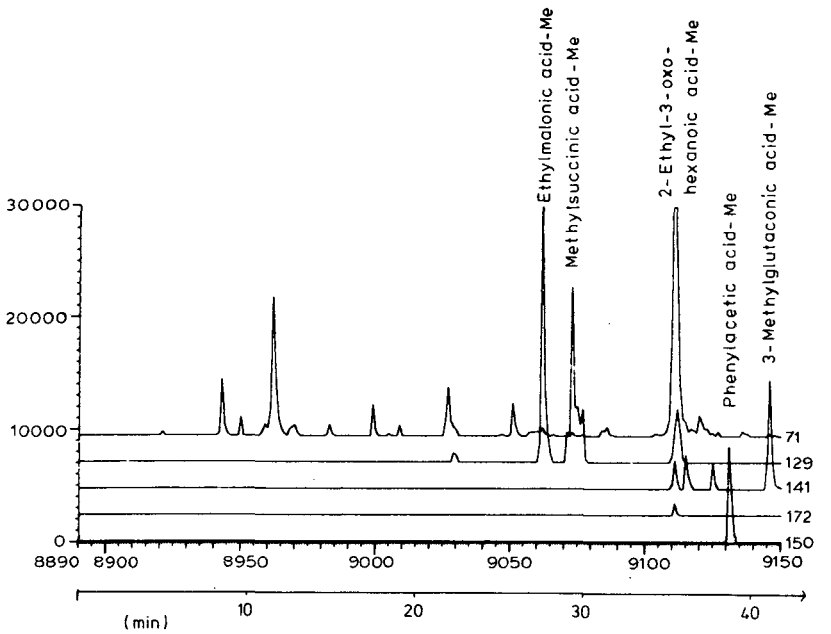


Fig. 1. Computer mass fragmentogram of fraction 2 of the organic acids in urine of a patient with diabetes mellitus, for the detection of the methyl ester of 2-ethyl-3-oxohexanoic acid (traces of the ions *m/e* 71, 129, 141, 172 and 150).

difficult to control. Even with the O-methyloximated derivative a correct quantification appears doubtful.

The amount of ketone which occurs in vivo in urine and in serum is difficult to detect, since by decarboxylation of the β-oxocarboxylic acid in vitro, the amount of the ketone will rise. The 4-heptanone which is detected within the profile of volatile metabolites in urine (Fig. 2) and serum (Fig. 3), corresponds only to some extent to the original ketone. The greater part is produced during the analytical procedure by decarboxylation of the β-oxocarboxylic acid. Nevertheless, when the analytical procedure is strictly standardized, the profiles of volatile metabolites allow the reproducible detection and recognition even of small variations and shifts in the concentration of 4-heptanone.

However, since there is no indication that the β-oxocarboxylic acid and the ketone have different physiological significance, it is justified to quantify the total 4-heptanone. By heating the sample, 2-ethyl-3-oxohexanoic acid is quantitatively decarboxylated. The reaction kinetics (Fig. 4) demonstrate that after 90 min at 90° C the decarboxylation process is completed. Fig. 5 shows the recording of the GC—MF determination of total 4-heptanone in three different samples. The extracts are injected every 8 min. Interfering substances are not observed.

We determined the total 4-heptanone in the urine of 270 patients with diabetes mellitus. The control groups consisted of 28 healthy individuals and 143 hospitalized patients with no obvious metabolic abnormalities. For each of the patients and the healthy individuals, the measurements were made in

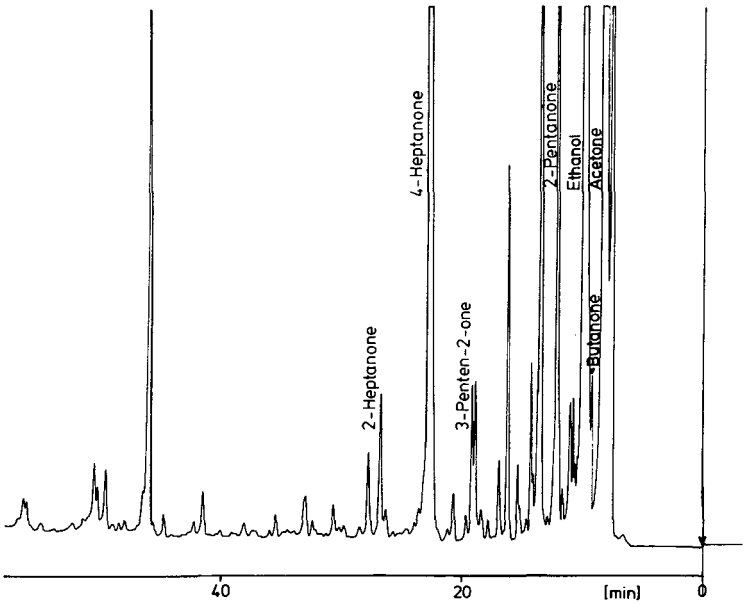


Fig. 2. Profile of the volatile metabolites in urine of a patient with diabetes mellitus, for the detection of 4-heptanone.

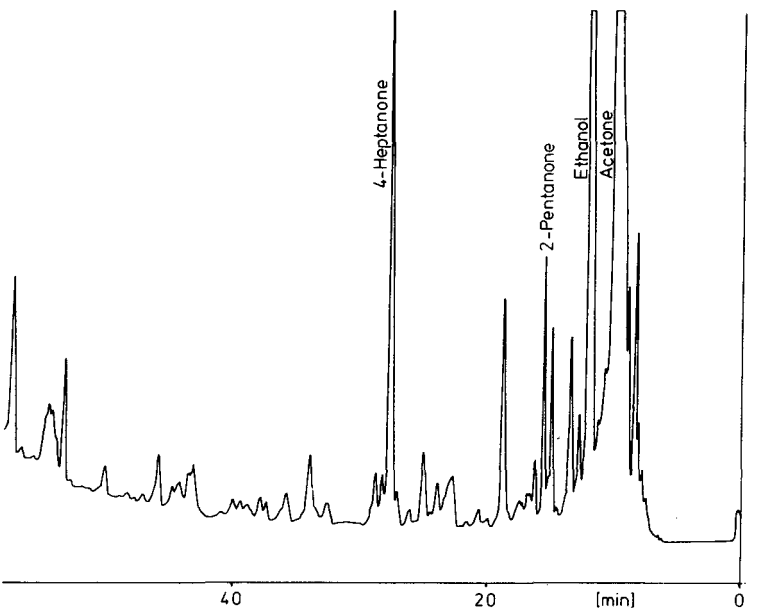


Fig. 3. Profile of the volatile metabolites in serum of a patient with diabetes mellitus, for the detection of 4-heptanone.

three consecutive 24-h urines. Of the diabetic cases 234 were classified as diabetes mellitus type I (insulin-dependent) and diabetes mellitus type II (not insulin-dependent).

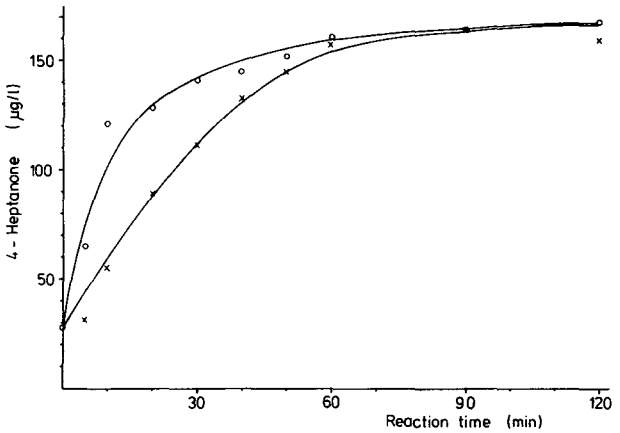


Fig. 4. Kinetics of the formation of 4-heptanone by heat decarboxylation of 2-ethyl-3-oxo-hexanoic acid in urine. (×) 90°C; (○) 99°C.

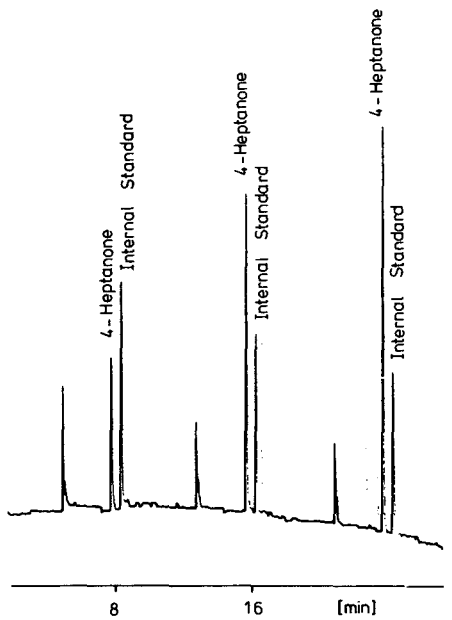


Fig. 5. GC—MF trace of the determination of total 4-heptanone in three urine samples, *m/e* 114.

The results are summarized in Table I. Whereas the mean values of urinary total 4-heptanone in healthy individuals and non-diabetic hospitalized patients are very similar (207 µg per 24 h and 246 µg per 24 h, respectively), the mean value is increased by a factor of 4 to 5 in diabetics (1073 µg per 24 h). Comparing diabetes mellitus type I and type II, we find that, on average, type I diabetics excrete more total 4-heptanone than type II diabetics. Elevated total 4-heptanone values are also found in patients with steroid-induced diabetes. Patients without overt diabetes mellitus, but abnormal glucose tolerance, already show increased total 4-heptanone (Table I).

TABLE I

TOTAL 4-HEPTANONE IN URINE

Group	Mean excretion (μg per 24 h)	Range of excretion (μg per 24 h)
Healthy individuals (n = 28)	207	60—470
Non-diabetic patients (n = 143)	246	40—810
Diabetic patients (n = 270)	1073	15—5590
Type I diabetes (n = 109)	1215	19—5590
Type II diabetes (n = 125)	978	15—4640
Steroid-induced diabetes (n = 10)	1020	610—2000
Abnormal glucose tolerance (n = 22)	448	50—1500
Fasting, non-diabetics (n = 14)	35	5—75
Fasting, diabetics (n = 8)	86	25—170

Characteristic of the excretion of total 4-heptanone in the group of the diabetic patients is the fact that the range is very broad. As well as in Table I, the distribution is illustrated in Fig. 6. From a detailed analysis of the clinical situation of the diabetic patients we could draw some conclusions.

During ketoacidotic periods total 4-heptanone decreases significantly and turns to its high level after the ketoacidosis is overcome. Total 4-heptanone also decreases when obese diabetic patients are put on fasting conditions (Table I). The same observation is made when obese but otherwise healthy individuals are put on fasting conditions (Table I). Not only complete fasting conditions result in a decrease of total 4-heptanone. A reduced diet already leads to a drop in the urinary total 4-heptanone. In conjunction with a study in which diabetic and non-diabetic patients were fed exclusively by infusion of glucose and fructose for 48 h, receiving only 25 kcal/kg body weight in 24 h, the total 4-heptanone decreased on average to half of the starting value in the control group (Fig. 7) and to much less than half in the diabetic group (Fig. 8). We con-

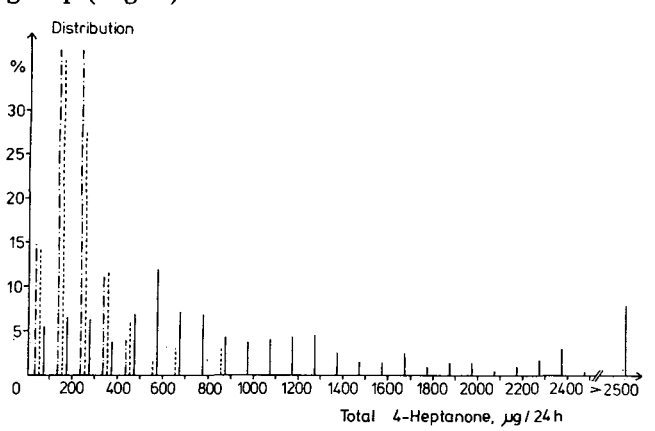


Fig. 6. Distribution of the urinary excretion of total 4-heptanone. (• — •) Healthy individuals, n = 28. (— — —) Patients without diabetes mellitus, n = 143. (———) Diabetic patients, n = 270.

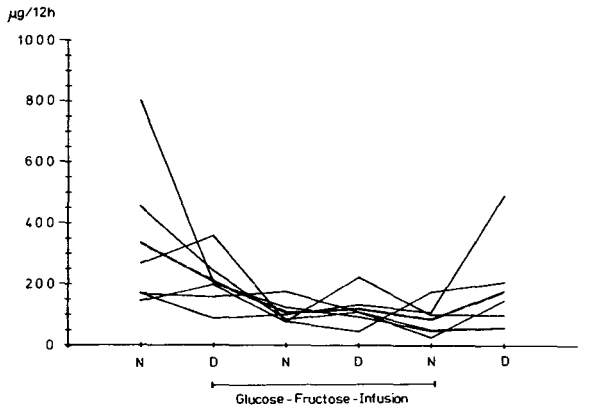


Fig. 7. Total 4-heptanone in urine of a control group being fed by infusion of glucose and fructose, 25 kcal/kg body weight in 24 h. N = night, D = day.

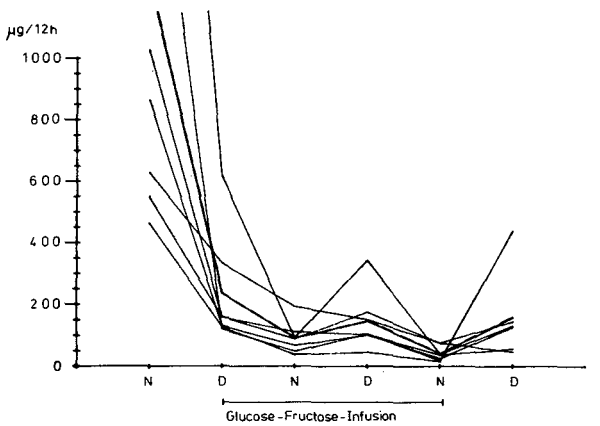


Fig. 8. Total 4-heptanone in urine of diabetic patients under the same conditions as the control group in Fig. 7.

clude from these observations that total 4-heptanone decreases in all those physiological and pathophysiological situations in which lipolysis and fatty acid oxidation is increased and fatty acid synthesis is decreased.

This behaviour of total 4-heptanone has to be considered in the comparison of diabetic patients with control persons. Consistent results are obtained when for the determination of total 4-heptanone the patient is on a balanced diet and not in a hyperglycemic and ketoacidotic period. Most of the 4-heptanone values of the diabetic patients falling in the normal range must be understood as pseudonormal since, at the time when the urine was collected, the patients were either ketoacidotic or under fasting conditions or on a reduced diet.

In diabetic patients who are on a balanced diet and are not ketoacidotic, the extent of increase of the total 4-heptanone covers a broad range. The distribution curve shows a maximum for values between 500 and 600 µg per 24 h (Fig. 6). However, many values are higher and reach several thousand µg per 24 h. It is observed that patients with very high total 4-heptanone are in many cases patients who are therapeutically difficult to control and whose blood

glucose levels are very unstable.

On the basis of the large number of diabetic patients and control persons included in the described study, we conclude that an increased urinary excretion of total 4-heptanone is inherently connected with diabetes mellitus and is an additional marker for the disease. On the other hand, total 4-heptanone is a sensitive indicator for the interplay between fatty acid oxidation and fatty acid synthesis.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOLOGICALLY IMPORTANT PYRIMIDINE DERIVATIVES WITH ULTRAVIOLET—VOLTAMMETRIC—POLAROGRAPHIC DETECTION

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SUMMARY

High-performance liquid chromatographic separation of a number of biologically important pyrimidine derivatives was studied in the reversed-phase system. Good results were obtained using a C_{18} alkyl-bonded silica column and an aqueous citrate—phosphate mobile phase of pH 3.5. All eluted components are detected with the UV absorbance detector at 254 nm, whereas the voltammetric detector with a polymeric carbon-paste electrode detects only derivatives containing oxidizable functional groups (amino, mercapto) and the polarographic detector with a mercury electrode only those with reducible groups (nitro, aza). The signal of the electrochemical detectors is proportional to the number of electroactive groups in the solute molecule. The use of two or three detectors in series thus improves the resolution of complex mixtures and facilitates identification.

INTRODUCTION

Pyrimidine derivatives, especially uracil, cytosine and thymine, belong among the biologically most important substances. Many other derivatives, e.g. fluoro- or aza-, are contained in pharmaceuticals and are monitored from the point of view of their metabolism in living organisms. For these reasons, much attention has been devoted to their analysis.

Gas chromatography (GC) has often been used for the separation and determination of pyrimidine bases (e.g. refs. 1—4). However, these determinations require derivatization and thus high-performance liquid chromatography (HPLC) is more advantageous, permitting direct and rapid analyses that are also sometimes more sensitive (for example, the HPLC determination of 5-fluorouracil in plasma is twenty times more sensitive than the GC determination [5]). For a detailed survey of HPLC of pyrimidine derivatives see the review [6].

As pyrimidine derivatives contain functional groups of various properties in a

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single molecule (basic and acidic groups, groupings of various polarities), a great variety of chromatographic systems can be employed for their separation. Ion-exchange has been used for HPLC separation of pyrimidine and purine bases, both on cation [7—9] and anion [10—13] exchangers, with isocratic or gradient elution. For an anion-exchange separation, the effect of the composition and pH of the mobile phase and of the type and concentration of the counter-ion on the retention behaviour has been studied [9—11]. Separations on silica gel, using a non-polar mobile phase (dichloromethane—methanol—aqueous salt solution) [14—16], and on a macroporous styrene—divinylbenzene copolymer [17] have also been described.

A great contribution to the analysis of pyrimidines was the introduction of reversed-phase systems (e.g. refs. 18—30). Various chemically bonded phases have been employed. A comparative study [31] has shown that the best results are obtained with the Spherisorb ODS-2 phase. Pyrimidine and purine bases have been determined in serum and plasma (e.g. refs. 18, 21, 22, 25, 29), in hydrolysates of nucleic acids (e.g. ref. 20), with isocratic [31] or gradient [18—21, 32] elution. As counter-ions, dodecyl sulphate [27], 11-aminoundecanoic acid [26] and several quaternary ammonium salts [23, 33] have been used. The effect of the temperature, pH and the composition of the mobile phase, and of the counter-ion concentration on the separation has been studied [23, 27, 28, 30].

UV photometric and sometimes also fluorescence [21] detection has been used. For identification of pyrimidines and purines, the ratio of the absorbances at 254 and 280 nm, or a combination of the UV photometric and fluorescence detectors have been employed [18, 21].

The papers published so far deal almost exclusively with nucleobases and 5-fluorouracil. As a number of other derivatives may be encountered in the production of pharmaceuticals and in the study of their chemical reactions and metabolism, we studied the conditions for separation of a number of derivatives containing a great variety of functional groups. In separations of complex mixtures occurring mainly in biological samples, difficulties may arise concerning the resolution and identification of the components [18]. Therefore, we investigated the combination of UV detection with electrochemical detection, as the latter is characterized by considerable selectivity (e.g. ref. 34).

EXPERIMENTAL

Apparatus

An LC-XP liquid chromatograph (Pye Unicam, Cambridge, Great Britain) with a Separon C_{18} column, 25 cm × 0.25 cm I.D., 10 μm particle size (Laboratorní Přístroje, Prague, Czechoslovakia), a TZ-4200 double-line recorder (Laboratorní Přístroje) and a Varian-Techtron line recorder were used for the measurements. To evaluate the elution curves, a Model 3390A Hewlett-Packard reporting integrator was employed. Samples were injected through a 20-μl loop.

For the detection, an LC-UV photometric detector (Pye Unicam) and two electrochemical detectors were used. The voltammetric detector was of our own construction [35—37], operated in the wall-jet system with three electrodes and a polymeric carbon-paste working electrode (for the preparation of

the Alkapren paste see ref. 36). The polarographic detector was similar to that marketed by Princeton Applied Research, U.S.A., but with the eluate fed horizontally to the mercury-drop electrode. The electrochemical detectors were operated with prototype instruments from Laboratorní Přístroje, permitting d.c., damped d.c., sampled d.c. and differential pulse voltammetric measurements in a three-electrode system, with effective background current compensation. The potentials are related to saturated silver chloride electrode. During the measurement, the three detectors were connected in series — first the UV photometric detector, followed by the voltammetric and then the polarographic detector. The detectors were interconnected by pieces of stainless-steel capillary, 15 cm × 0.2 mm I.D.

Chemicals

The studied pyrimidine derivatives were obtained from Fluka, Buchs, Switzerland (6-azathymine), Calbiochem, Los Angeles, CA, U.S.A. (5-bromouracil, 6-azauracil, 5-iodouracil), Sigma, St. Louis, MO, U.S.A. (5-methylcytosine, 5,6-dihydrothymine), Lachema, Brno, Czechoslovakia (2,4,5-triamino-6-hydroxypyrimidine, 5-bromocytosine, cytosine, 5,6-dihydrothymine, uracil, 2-amino-4,6-dihydroxypyrimidine, 2,4-diamino-6-hydroxypyrimidine, 6-azacytosine), Hoffmann-La Roche, Basle, Switzerland (5-fluorouracil), Koch-Light, Colnbrook, Great Britain (thymine). 2-Mercapto-, 4-mercapto- and 2,4-dimercaptouracil, 2-mercapto-6-azathymine and 6-aminouracil were synthesized by Dr. Černohorský of the Department of Clinical Biochemistry (Prague, Czechoslovakia). 4,5,6-Triaminouracil, 6-amino-4-hydroxy-2-mercaptopyrimidine, 4,5-diamino-6-hydroxy-2-mercaptopyrimidine and 4,6-diamino-2-mercaptopyrimidine were provided by Dr. Žilka (ÚVVVR, Prague, Czechoslovakia).

Standard solutions of these substances in the mobile phase were always prepared immediately before the measurement.

The mobile phase consisted of 300 ml of 0.1 *M* citric acid and 160 ml of 0.1 *M* Na_2HPO_4 [38]; the pH was adjusted by appropriate addition of phosphoric acid and sodium hydroxide. It was deaerated by pre-purified nitrogen and degassed in vacuo. The mobile phase flow-rate was 1 ml/min.

All the measurements were carried out at laboratory temperature.

RESULTS AND DISCUSSION

Column separation

The reversed-phase system with a C_{18} alkyl-bonded silica column was selected on the basis of the good results reported in the literature (see Introduction). The phosphate–citrate mobile phase was chosen because of efficient separations attained with this system previously [38] and because citrate was reported as an optimal counter-ion in anion-exchange separation of nucleobases and nucleotides [11]. In this system, the capacity ratios were measured for the studied substances in dependence on the pH and are given in Table I and Fig. 1.

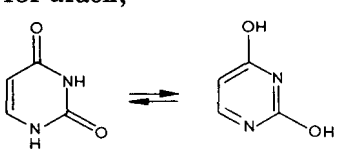
It is very difficult to explain the retention behaviour of pyrimidines on the basis of their structure, because of the simultaneous presence of acidic (—OH, —SH) and basic ($—NH_2$) ionizing groups and other substituents (—F, —Br, —I, etc.).

TABLE I

CAPACITY RATIOS OF VARIOUS PYRIMIDINE DERIVATIVES AND THE DETECTOR RESPONSE (AVERAGES OF THREE DETERMINATIONS)

Compound	Detector response		Capacity ratio			
	Voltammetric	Polarographic	pH 2.5	pH 3.5	pH 4.5	pH 6.0
2,4-Dihydroxypyrimidine (uracil)	—	—	0.99	1.13	1.25	1.26
2-Amino-4,6-dihydroxypyrimidine	+	—	0.29	0.65	0.60	0.34
4,5-Diamino-6-hydroxypyrimidine	+	—	0.22	0.58	1.15	1.24
2-Hydroxy-4-aminopyrimidine (cytosine)	—	—	0.43	0.66	0.79	1.04
2,4,5-Triamino-6-hydroxypyrimidine	+	—	0.13	0.28	0.30	0.65
2,4-Diamino-6-hydroxypyrimidine	+	—	0.88	1.72	1.50	1.39
2,4-Dihydroxy-6-aminopyrimidine	+	—	1.04	1.18	1.21	1.12
4,5,6-Triaminopyrimidine	+	—	0.87	1.58	1.43	2.12
2,4-Dihydroxy-5-methylpyrimidine (thymine)	—	—	3.36	3.73	4.35	4.60
5-Methylcytosine	—	—	1.02	1.78	2.28	3.49
5-Nitrouracil	—	+	1.12	1.50	1.73	1.75
2-Mercaptouracil	+	—	1.61	1.83	2.18	2.23
2,4-Dimercaptouracil	+	—	1.60	1.81	2.17	2.20
4-Mercaptouracil	+	—	2.73	3.12	3.72	3.85
2-Mercapto-4-hydroxy-6-aminopyrimidine	+	—	1.95	2.23	2.49	2.34
2-Mercapto-4,6-diaminopyrimidine	+	—	1.98	3.30	3.28	4.31
6-Azacytosine	—	+	0.58	0.70	0.73	0.75
6-Azauracil	—	+	0.75	0.88	0.95	0.95
6-Azathymine	—	+	2.30	2.77	3.17	3.65
2-Mercapto-6-azathymine	+	+	3.19	3.86	4.69	—
5-fluorouracil	—	—	1.01	1.03	1.37	1.37
5-Bromouracil	—	—	3.25	3.47	4.53	4.31
5-Iodouracil	—	—	5.25	4.94	7.49	—
5-Bromocytosine	+	—	1.90	3.90	5.36	—
5,6-Dihydrouracil	—	—	0.87	1.04	0.54	1.15
5,6-Dihydrothymine	—	—	3.23	3.05	3.74	3.72

Pyrimidines occur in two tautomeric forms, lactam and lactim; for example, for uracil,



lactam lactim

In reversed-phase systems, substances that are not ionized have high retention times; the k' value decreases with increasing ionization. The pyrimidines in which the lactam form predominates in aqueous solutions exhibit lower retention times. This phenomenon has been explained by self-association (vertical stacking) of the lactim form [24], leading to hydrophobization of the molecules and thus to longer retention times. Any substituent supporting the formation of the lactam form (e.g. —OH, —SH) causes a decrease in the capacity

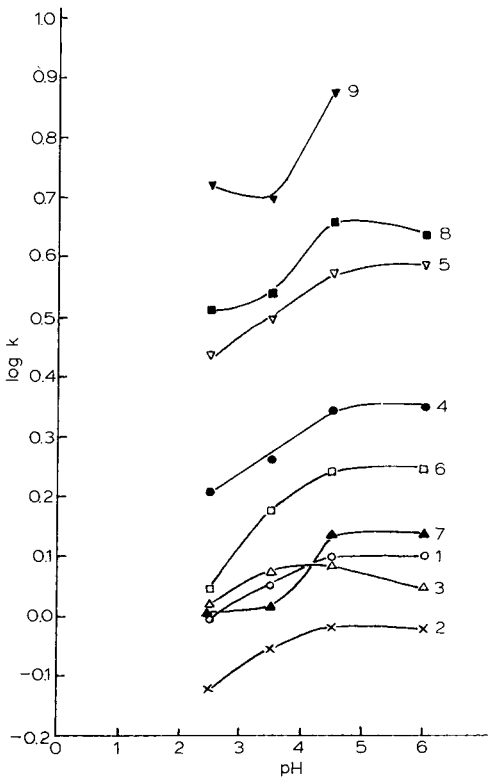


Fig. 1. Dependence of the logarithm of the capacity ratio (k') for some uracil derivatives on the pH of the mobile phase. 1 = Uracil, 2 = azauracil, 3 = 6-aminouracil, 4 = 2-mercaptouracil, 5 = 4-mercaptouracil, 6 = 5-nitrouracil, 7 = 5-fluorouracil, 8 = 5-bromouracil, 9 = 5-iodouracil.

ratio. The retention order of variously substituted pyrimidines increases in the series OH < H < NH_2 < CH_3, the greatest effect being exerted by the substituent in position 5. The presence of the methyl group in position 5 more than doubles the retention time, as it enhances the formation of the lactim form and thus also supports the stacking process (see k' for uracil and thymine, cytosine and 5-methylcytosine in Table I).

Fluorine, as an electron-withdrawing substituent in position 5, has little effect on the retention (see k' for 5-fluorouracil and uracil in Fig. 1). In the series of 5-fluoro-, 5-bromo- and 5-iodouracil, the retention order is inversely proportional to the electronegativity of the halogen atoms (Fig. 1). 6-Aza- and 5,6-hydrogeno- substitution lead to only small changes in the retention behaviour.

In agreement with Miller et al. [31], the optimum pH for the separation was found to be 3.5, since at higher pH values the elution curves exhibited progressively increasing tailing and sometimes even doubled peaks were obtained. The effect of pH on the capacity ratios is complicated, due to the simultaneous presence of acidic (—OH, —SH) and basic (—NH_2) groups and it cannot be directly correlated with the individual ionization constants; the effect of the

position of the substituent on the heterocycle is definitely more pronounced (cf. the great difference between k' values for 2-mercapto- and 4-mercapto-uracil and the elution order uracil (pK_a 9.38; 12.0) < 5-nitrouracil (pK_a 5.3; 11.7) < 2-mercaptouracil (pK_a 7.74; 12.7) in Fig. 1; the pK_a values were taken from Brown [39].

The effect of addition of dodecyl sulphate to the mobile phase on the separation was also tested. However, the retention times changed very little and increased peak tailing has been observed.

Detection

UV photometric detection at 254 nm is generally sufficiently sensitive for all pyrimidine derivatives. However, as follows from Table I, certain combinations of pyrimidine derivatives will not be resolved on the column and there may arise problems with identification of the individual components. Electrochemical detection depends on the presence of oxidizable or reducible groupings in the solute molecule and thus is selective for certain derivatives. The half-wave potentials of the voltammetric waves vary widely (e.g. + 0.45 V for 2,4,5-triamino-6-hydroxypyrimidine, + 1.15 V for 2,4-diamino-6-hydroxypyrimidine and + 1.2 V for 2-amino-4,6-dihydroxypyrimidine [40]) and therefore the selectivity of the detection can further be varied by varying the potential applied to the working electrode. To test the possibilities of electrochemical detection, the potentials of the working electrodes were set at limiting values, given by the mobile phase composition, i.e. + 1.4 V for the carbon-paste voltammetric wall-jet detector and − 1.0 V for the mercury-drop electrode polarographic detector.

The results can be seen in Table I: the voltammetric detector responds to substances with oxidizable groups, i.e. amino and mercapto, and the polarographic detector to reducible groups, i.e. nitro and aza. It is important that the signal magnitude is proportional to the number of the electroactive groups (e.g. the detection limits are 5, 10 and 25 ng for tri-, di- and monoamino derivatives) due to the varying number of electrons exchanged per solute molecule.

The detection limits for typical solutes are given in Table II. It can be seen that, except for 4,5,6-triaminopyrimidine, the sensitivity of electrochemical detection is not greater than that of UV photometric detection. The detection limit of the polarographic detector is even considerably higher than that of the UV photometric detector. However, the combination of UV photometric detector with the electrochemical detectors can substantially improve the possibilities of resolving and identifying the components of the mixture, especially in biological fluids, in which most ballast substances absorbing in the UV region will not yield an electrochemical response. In these experiments, the detectors were connected in the series, UV—voltammetric—polarographic, by the shortest possible steel capillaries with an internal diameter of 0.2 mm. As the internal volume of the UV detector cell is 5 μl and that of the voltammetric detector ca. 1 μl, the elution curves are not appreciably broadened even in the polarographic detector recording. The voltammetric detector is partially destructive; however, the residence time of the substance inside the cell is so short that the degree of electrochemical conversion is very low. For this reason, the oxidation products generated in the voltammetric detector that might be re-reduced in the polaro-

graphic detector, thus interfering with its performance, were not actually detected. The precision of the determination with the electrochemical detectors is satisfactory for quantitative analysis (see Table II).

There is another possibility of electrochemically detecting substances eluted from a chromatographic column. If a mercury electrode is polarized at a potential corresponding to the dissolution of mercury, then diffusion-controlled anodic waves are formed in the presence of substances that form stable complexes or precipitates with mercury ions (see e.g. ref. 41). Most organic substances separated by HPLC would form complexes with mercury ions; there-

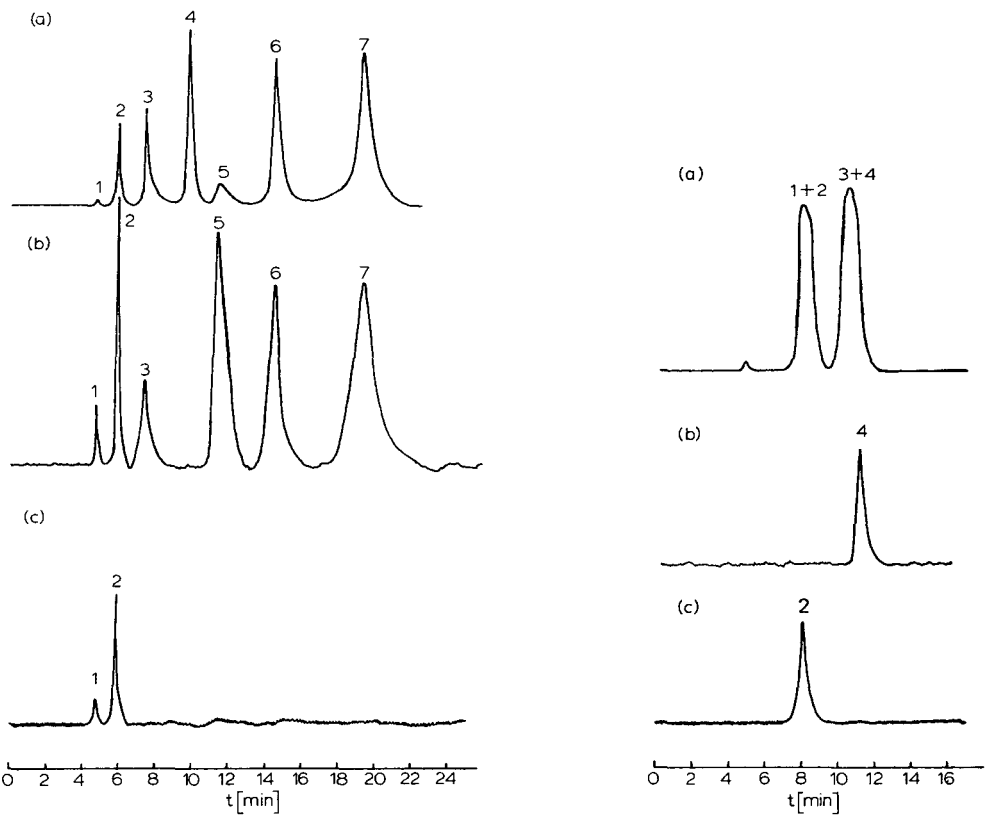


Fig. 2. Separation of several pyrimidine derivatives. (a) UV photometric detection at 254 nm (sensitivity 0.32 absorbance units/scale). (b) Voltammetric detection, +1.4 V (sensitivity 0.2 μA/scale). (c) Voltammetric detection, +0.8 V (sensitivity 0.2 μA/scale). Mobile phase pH, 3.5; for other conditions see the experimental part. 1 = Hold-up time; 2 = 2,4,5-triamino-6-hydroxypyrimidine, 1.12 μg, 3 = 2-amino-4,6-dihydroxypyrimidine, 0.88 μg, 4 = uracil, 0.56 μg, 5 = 4,5,6-triaminopyrimidine, 0.64 μg, 6 = 6-amino-4-hydroxy-2-mercaptopyrimidine, 0.40 μg, 7 = 4-mercaptouracil, 1.50 μg.

Fig. 3. Chromatograms of cytosine (1) (0.65 μg), 6-azacytosine (2) (1.2 μg), uracil (3) (0.56 μg), and 6-aminouracil (4) (0.60 μg). (a) UV photometric detection at 254 nm (sensitivity 0.32 absorbance units/scale). (b) Voltammetric detection, + 1.4 V (sensitivity 0.2 μA/scale). (c) Polarographic detection, −1.0 V (sensitivity 0.05 μA/scale). Mobile phase pH, 3.5; for the other conditions see the experimental part.

TABLE II

TYPICAL DETECTION LIMITS AND REPRODUCIBILITY OF MEASUREMENT

Detection limits were determined as three times the peak-to-peak noise values.

Substance	Detection limit (ng)			Reproducibility of measurement (relative S.D., %)				
	UV	Voltammetric	Polarographic	UV		Voltammetric		Polarographic
				Height	Area	Height	Area	Height
2-Amino-4,6-dihydroxypyrimidine	2	25	—					
2,4-Diamino-6-hydroxypyrimidine	2	10	—					
2,4,5-Triamino-6-hydroxypyrimidine	2	5	—	1.53*	1.24*	5.18*	5.92*	—
4,5,6-Triaminopyrimidine	20	7	—					
2-Mercaptouracil	10	50	—					
5-Nitrouracil	2	—	1000	1.72**	—	—	—	2.25**
6-Azauracil	2	—	800					
6-Azacytosine	5	—	400					
2-mercapto-6-azathymine	10	40	800					

*1.32 μg, seven measurements.
**2.8 μg, four measurements.

fore, this possibility was tested, setting the mercury electrode potential at + 0.5 V. There was a response to the substances eluted from the column; however, the sensitivity was too low. The obvious reason for the low sensitivity was the fact that the substance to be detected was carried away from the electrode before the chemical reaction of complexation or precipitation could proceed to a greater extent. Therefore, it seems that the use of chemical reactions with mercury ions that have a substantial importance, e.g. in stripping analysis [42], will be unimportant in flow systems because of too slow kinetics of the overall process.

The advantages following from combination of the UV photometric detection with voltammetric detection at various electrode potentials and further with polarographic detection are illustrated in Figs. 2 and 3. This selective detection will be utilized in the determination of active components based on the substances studied in pharmaceuticals and in some biological samples when following their metabolism. The selectivity of detection and improved separation will be especially advantageous in analyses of biological samples with complicated matrices.

CONCLUSIONS

The experiments carried out have shown that a reversed-phase HPLC separation of pyrimidine derivatives can be used even for very complex mixtures. The resolution and identification of the components is facilitated by combined UV–voltammetric–polarographic detection.

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CHROMBIO. 1535

SIMPLE ENZYMATIC DETECTION METHOD FOR URINARY SULFATED 7α-HYDROXY BILE ACIDS IN NORMAL SUBJECTS AND IN PATIENTS WITH ACUTE HEPATITIS

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SUMMARY

Urinary sulfated primary bile acids, 7α-hydroxy bile acids, are detected by an enzymatic method using 7α-hydroxysteroid dehydrogenase (EC 1.1.1.-, 7α-HSD) after chromatographic fractionation on Sephadex G-25.

Urinary sulfated or glucuronated bile acids are hydrolyzed by β-glucuronidase/sulfatase (EC 3.2.1.31/EC 3.1.6.1) from *Helix pomatia* and then released 7α-hydroxy bile acids are detected with 7α-HSD in the presence of β-NAD^+, diaphorase (EC 1.6.99.2, from *Clostridium kluyveri*) and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride. The absorbance of formazan formed during the enzymic reaction is measured at 500 nm.

Excretion values of 7α-hydroxy bile acids in normal subjects and in patients with acute hepatitis were compared. This enzymatic detection method for the excretion pattern of urinary 7α-hydroxy bile acids may be useful for clinical diagnosis.

INTRODUCTION

The enzymatic assay of 3α-hydroxy bile acids in serum by the use of NAD-linked 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50, 3α-HSD, from *Pseudomonas testosteroni*) is widely studied as a simple, specific and sensitive method [1—3].

The enzyme, 7α-HSD, isolated from *Escherichia coli* which uses NAD as cofactor and from *P. testosteroni* which uses NADP as cofactor has also been introduced [4, 5].

It is well known that the major bile acids in urine are chenodeoxycholic

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acid, cholic acid and their taurine or glycine conjugates. These bile acids are excreted as sulfates, being sulfated at the 3-hydroxy group [6—10].

In this paper, an enzymatic detection method for conjugated 7α-hydroxy bile acid is described. The principle of the method is as follows:

$$\text{Conjugated 7}\alpha\text{-hydroxy bile acid} \xrightarrow[\beta\text{-glucuronidase}]{\text{sulfatase}} \text{free 7}\alpha\text{-hydroxy bile acid}$$

$$\text{7}\alpha\text{-Hydroxy bile acid} + \text{NAD}^+ \xrightarrow{\text{7}\alpha\text{-HSD}} \text{7-keto bile acid} + \text{NADH}$$

$$\text{NADH} + \text{INT} \xrightarrow{\text{diaphorase}} \text{formazan}$$

EXPERIMENTAL

All the reagents used were of analytical grade, and β-glucuronidase from *E. coli* (EC 3.2.1.31) and sulfatase/β-glucuronidase (EC 3.1.6.1 and EC 3.2.1.31) from *Helix pomatia* were purchased from Sigma, St. Louis, MO, U.S.A.

All standards of steroids and bile acids, INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride), diaphorase (EC 1.6.99.2) from *Clostridium kluyveri* and 7α-HSD from *E. coli* (EC 1.1.1.-) were purchased from Sigma. 3α-Hydroxysteroid dehydrogenase (3α-HSD) from *P. testosteroni* (EC 1.1.1.50) and β-NAD^+ were purchased from Nyegaard & Co. (Oslo, Norway). Sephadex G-25 Fine and the column used were purchased from Pharmacia (Uppsala, Sweden).

Preparation of reagent

Enzyme solution for hydrolysis of conjugates: sulfatase/β-glucuronidase from *H. pomatia* (Type H-1), 200 units as sulfatase/3000 Fishman units as β-glucuronidase per 1 ml of 0.05 *M* acetate buffer (pH 5.0), and β-glucuronidase from *E. coli*, 500 Fishman units per 1 ml of 0.1 *M* phosphate buffer (pH 6.8), were prepared. All of these enzymes were used without the addition of an activator during the hydrolysis of steroid conjugates.

Preparation of enzyme reagent for color development of 7α-hydroxy bile acids: 20 mg of INT, 10 U of 7α-HSD, 100 U of diaphorase and 40 mg of β-NAD were dissolved in 100 ml of 0.2 *M* K_2HPO_4 (pH 9.0).

Reagent for color development for 3α-hydroxysteroid and 3β-hydroxysteroid was described previously [11—14].

Preparation of Sephadex G-25 column

Sephadex G-25 was swollen by heating the suspension in acetate buffer (0.05 *M*, pH 5.0) for 4 h at 90°C under constant stirring. The fines were removed by several decantations and the slurry was poured directly into the column which was then washed for 3 h with acetate buffer solution.

Preparation of urine sample

A 24-h urine specimen was collected and an aliquot of the 24-h urine was centrifuged for 3 min at 2500 *g*; 1—2 ml of supernatant of urine were directly applied to the Sephadex G-25 column (27 × 1 cm).

Sephadex chromatography

The supernatant from a 1—2-ml urine sample was applied to the column. After application of the sample, chromatographic separation was performed with acetate buffer (0.05 *M*, pH 5.0). One fraction of the effluent contains 1.3 ml; ten fractions were collected within 30 min followed by another 35 fractions.

Procedure for detection of sulfated 7α-hydroxy bile acids

To each fraction collected during chromatography was added 0.1 ml of sulfatase/β-glucuronidase solution and incubated for 20 h at 37°C. After hydrolysis of the conjugates, 1 ml of the color-developing reagent for 7α-hydroxy bile acids was added, and after incubation for 20 min at 37°C the absorbance at 500 nm was read against the first fraction of the effluent.

RESULTS

Specificity of the 7α-HSD from *E. coli* is shown in Table I. As shown in the table, this enzyme reacted only with the 7α-hydroxy group of steroids.

TABLE I

SPECIFICITY OF 7α-HYDROXYSTEROID DEHYDROGENASE FROM *E. COLI*

A 2-ml volume of 7α-HSD solution was added to a tube containing 20 μl of 2.5 m*M* of standard solutions.

Steroids	Absorbance at 500 nm (20 μl of 2.5 m*M*)
Cholic acid	0.304
Taurochenodeoxycholic acid	0.352
Glycochenodeoxycholic acid	0.295
Taurocholic acid	0.346
Glycocholic acid	0.335
Taurolithocholic acid	0.000
Androsterone	0.000
Testosterone	0.000
Dehydroepiandrosterone	0.000
Tetrahydrocortisol	0.000
Estradiol	0.000

The precision of the method using the sample of a patient with acute hepatitis was C.V. = 7.5% (five repeated assays, mean 32 ± 2.5 mg/day) and with a normal subject C.V. = 18% (mean 2.8 ± 0.5 mg/day). When glycochenodeoxycholic acid added to urine as a standard for 7α-hydroxy bile acid was analyzed in triplicate, the C.V. obtained was 5.7% at a level of 20 μg/ml with a mean recovery of 93.5%.

Excretion pattern of 7α-hydroxysteroids with normal subjects and a patient with acute hepatitis

A typical chromatogram for normal subjects showing the excretion pat-

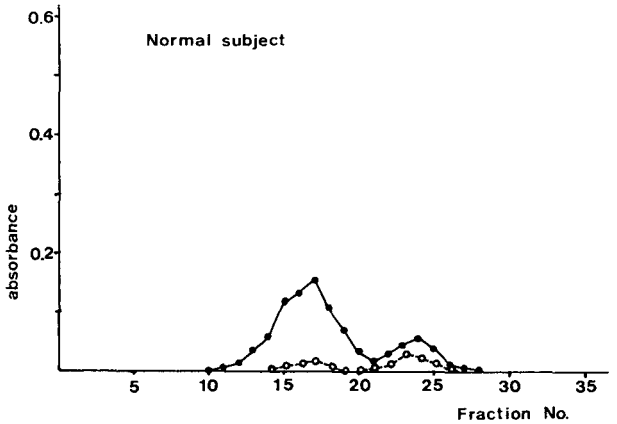

Fig. 1. Excretion pattern of 7α-hydroxy bile acids, obtained from a normal subject. (o— — — o), 7α-Hydroxy bile acids (color-developed with 7α-HSD as described in Experimental); (•——•), 3α-hydroxysteroids.

tern of 7α-hydroxysteroids and 3α-hydroxysteroids is given in Fig. 1. A typical excretion pattern for 7α-hydroxy bile acids in acute hepatitis is shown in Fig. 2.

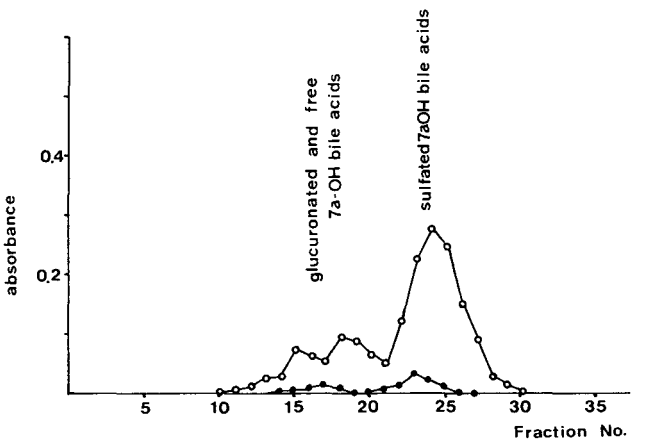

Fig. 2. Excretion pattern of 7α-hydroxy bile acids obtained from a patient with acute hepatitis. (o — — — o), 7α-Hydroxy bile acids obtained from a patient with acute hepatitis; (•——•), 7α-hydroxy bile acids in a normal subject.

In order to obtain information about the conjugated form of 7α-hydroxy bile acids, the sample was treated with β-glucuronidase from *E. coli* to hydrolyse the glucuronate, and with sulfatase/β-glucuronidase from *H. pomatia* to hydrolyse the sulfate; the results are shown in Fig. 3.

Comparison of the excretion patterns of 3α-hydroxysteroids in a normal subject and a patient with acute hepatitis is shown in Fig. 4; elevated steroid-3α-sulfate was observed in patients with acute hepatitis. In order to study the conjugated form of 3α-hydroxysteroids, the sample was treated step-

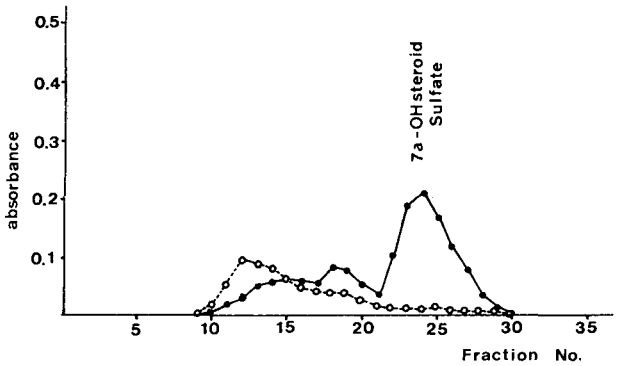

Fig. 3. Effect of β-glucuronidase on hydrolysis of conjugated 7α-hydroxy bile acids. Sample used is from a patient with acute hepatitis. (●——●), Sulfatase/β-glucuronidase; (○ — — —○), β-glucuronidase.

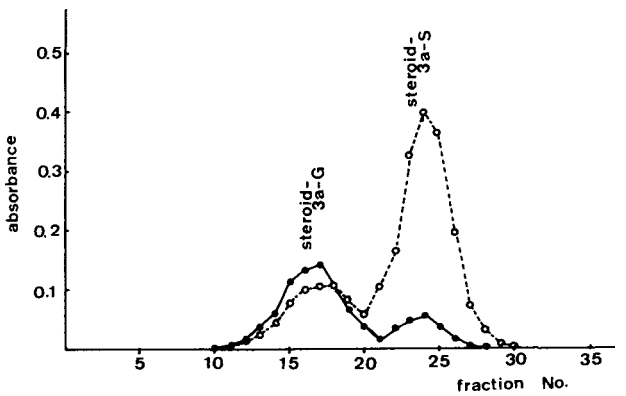

Fig. 4. Comparison of excretion patterns of 3α-hydroxysteroids between a normal subject and a patient with acute hepatitis. (○ — — — ○), 3α-Hydroxysteroids in a patient with acute hepatitis; (●——●), 3α-hydroxysteroids in a normal subject.

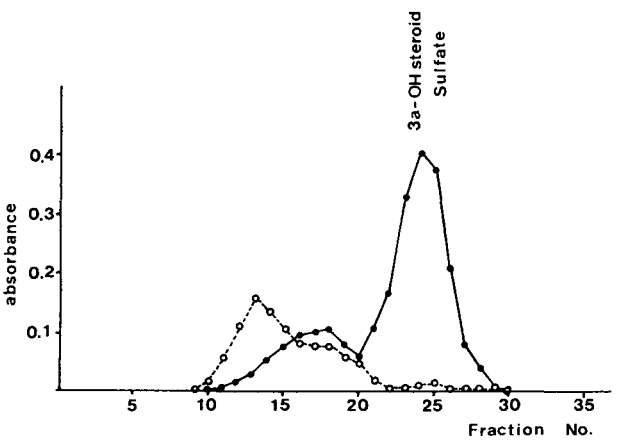

Fig. 5. Effect of β-glucuronidase on hydrolysis of conjugated 3α-hydroxysteroids. Sample used is from a patient with acute hepatitis. (●——●), Sulfatase/β-glucuronidase; (○ — — — ○), β-glucuronidase.

wise with β-glucuronidase and with sulfatase/β-glucuronidase; the result is shown in Fig. 5.

The excretion rate of total 7α-hydroxy bile acids in normal subjects was 3.5 ± 2.8 mg/day (n = 15). Excretion rates for patients with acute hepatitis varied from 7.3 to 57 mg/day (n = 6).

DISCUSSION

In this paper, typical excretion patterns of urinary 7α-hydroxy bile acids obtained for a patient with acute hepatitis and a normal subject are shown. In the chromatogram obtained with the healthy subject the amount of 7α-hydroxy bile acids was too low to promote the detector response. On the other hand, in diseases such as liver cirrhosis, which show elevated excretion of bile acids, the excretion pattern of 7α-hydroxy bile acids was distinctly increased (Fig. 6).

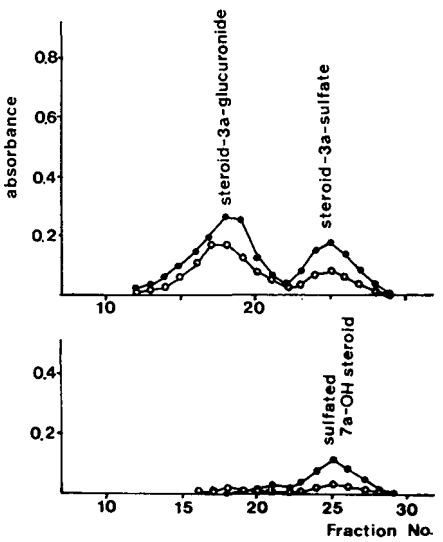

Fig. 6. Excretion patterns of 7α-hydroxy bile acids obtained from a patient with liver cirrhosis. (o——— o), Normal subject, (•———•), liver cirrhosis (compensative stage). 3α-Hydroxysteroids were color-developed with 3α-hydroxysteroid dehydrogenase as described in a previous paper [12].

The excretion patterns of other conjugated steroids obtained from patients with acute hepatitis was also investigated by the method previously reported [11, 12]. Our own data are presented in Fig. 7 for comparison. Urinary 3β-hydroxysteroids eluted at the same position as sulfated 7α-hydroxy bile acids. The 3β-hydroxysteroids are suggested to be bile acids because the neutral 3β-hydroxysteroids were not detected in any significant amount (using 3β-hydroxysteroid oxidase from *Brevibacterium sterolicum*). It is known that this enzyme reacts mainly with neutral 3β-hydroxysteroids [11, 15].

The elution position of standards of non-sulfated bile acids was also determined (Fig. 8) and compared with the chromatogram of Fig. 5. At the posi-

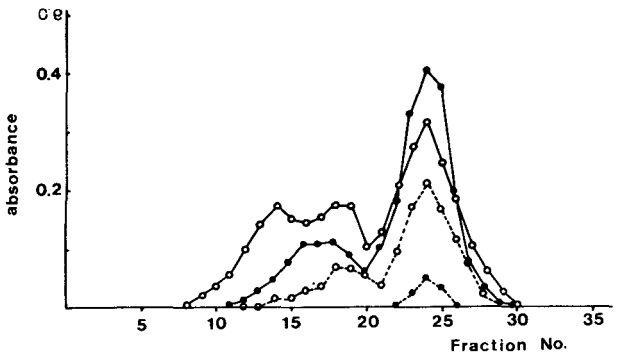

Fig. 7. Excretion patterns of some other urinary steroids obtained from a patient with acute hepatitis. (•——•), 3α-Hydroxysteroids; (○——○), 3β,17β-hydroxysteroids; (○ — — — ○), 7α-hydroxy bile acids, (•— — — •), neutral 3β-hydroxysteroids.

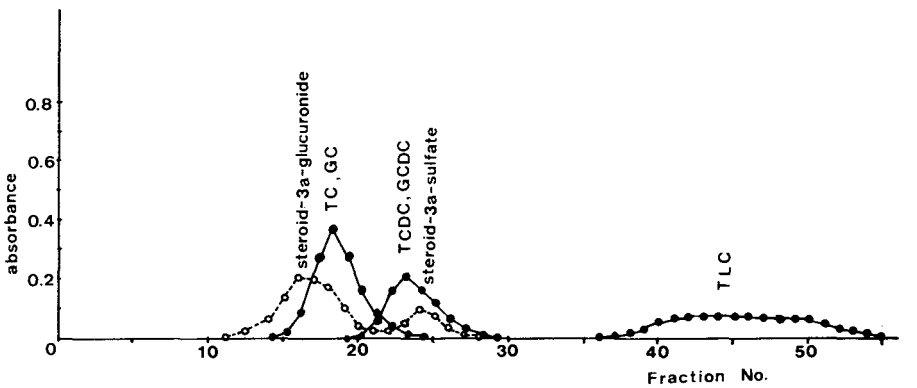

Fig. 8. Chromatogram of non-sulfated bile acids. TC = Taurocholic acid, GC = glycocholic acid, TCDC = taurochenodeoxycholic acid, GCDC = glycochenodeoxycholic acid, TLC = taurolithocholic acid. For each of TC, GC, TCDC, GCDC and TLC 100 μg per 1 ml were applied to the column of Sephadex G-25 and, after fractionation, 3α-HSD was added.

tions of taurochenodeoxycholic acid and glycochenodeoxycholic acid no peaks were seen (see Fig. 5, β-glucuronidase hydrolysis). Taurocholic acid and glycocholic acid overlapped with steroid-3α-glucuronide; therefore it was necessary to make use of the enzymatic detection via 3α-HSD without hydrolysis to distinguish between steroid-3α-glucuronide and non-conjugated (3-hydroxy) bile acids.

Urinary metabolites of bile acids are mainly 7α-hydroxy, 7-keto and 7-deoxy bile acids, the 7α-hydroxy bile acids being the parent compounds. It appears that this rather simple detection method for sulfated 7α-hydroxy bile acids can be used for the diagnosis of some liver diseases.

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CHROMBIO. 1544

MICROBORE COLUMN EXCLUSION CHROMATOGRAPHIC METHOD FOR STUDYING PROTEIN ASSOCIATION AND ITS RELATION WITH ENZYMATIC ACTIVITY

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SUMMARY

The principles of treating chromatographic data are considered to allow the determination of number of components, equilibrium and kinetic association constants of proteins by means of micro-column exclusion chromatography. The method is developed to study protein association in cases complicated by adsorption. The potentialities of the chromatographic method have been examined for the study of the mechanism of enzyme catalytic action.

INTRODUCTION

Association and biological function of proteins are closely interrelated. The interest in studying equilibrium and kinetics of protein association has increased particularly during the last years, since these reactions have been recognized to be fundamental to many metabolic control mechanisms in vivo. Of particular importance are those systems that contain two kinetically different forms of an enzyme which are capable of reversible interconversion. For example, such is the case when only one of the components has affinity to a ligand, taking the part of activator or of inhibitor in enzymatic catalysis. Biochemical aspects of correlations between enzymatic activity and protein self-association have been discussed in the review of Frieden [1], who cited as an example a number of self-interacting enzymes demonstrating the flexibility in the activity control which may be attained by means of reversible association.

The present work deals with methodological problems of studying protein association and its correlation with enzymatic activity by means of exclusion liquid chromatography. Apart from using microbore columns, the novelty in this field consists in expanding the potentialities of the chromatographic method for determination of the rate constants of these reactions and for the case of

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protein adsorption on the column. The methodological principles that are developed in this paper for protein association should also be useful for studying other reactions of biopolymers such as complex formation, ligand binding, etc.

EXPERIMENTAL

A microbore-column liquid chromatograph Kh-Zh-1305 (Special Design Office of Analytical Instrument-making, Academy of Science, U.S.S.R.) was used for the experimental study of protein association. It was equipped with a multi-wavelength spectrophotometric detector (cell volume 1 μl) and precision syringe pump (syringe volume 2 ml, flow-rate 8—4000 μl/h). Glass columns (250 mm × 1 mm I.D.) were packed with Sephadex G-75 superfine (Pharmacia, Uppsala, Sweden), or Bio-Gel P-60, <400 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.). The procedure of microbore-column filling is described in ref. 2.

The absorbance of samples was monitored in the range 220—300 nm. Wide-zone technique was used with a sample volume of 200 μl and flow-rate of 500 μl/h.

Purified samples of phospholipase A_2 (isozymes E_2 and E_3) and dihexanoyllecithin were obtained from the Institute of Bioorganic Chemistry (Academy of Science of the U.S.S.R.). Lysozyme activity A (Sojuzreaktiv, U.S.S.R.) was purified on a Sephadex G-75 preparative column with UV detection. Standard proteins for calibration were obtained from Serva (Heidelberg, G.F.R.). All other reagents (Sojuzreaktiv, U.S.S.R.) were of analytical grade, with the exception of potassium chloride, which was of reagent grade.

RESULTS AND DISCUSSION

Steric exclusion chromatography (SEC) which is the traditional method of biopolymer separation according to molecular size, appears also to be a convenient means of studying physicochemical properties of reacting systems. The reversible association does not allow the monomer and polymer zones of the protein to be resolved completely since polymer would always be formed in the monomer chromatographic zone, whereas in its own zone polymer would dissociate. Depending on the ratio of the reaction rate to the rate of chromatographic separation, different shapes of the concentration profile would be observed on the chromatogram. When the ratio is small (complete separation would be attained if it were high) no separation would be observed. Comparable rates of separation and interconversion of the components would result in partially resolved zones. On the other hand, association reaction causes the additional spreading of chromatographic zones due to the different chromatographic mobilities of the components and random distribution of the times which the macromolecule spends in its different states of aggregation. Thus the location of peaks on the chromatogram and the degree of its spreading reflect the properties of associating protein migrating along the column. A quantitative theory has been developed [3—9] which provides the possibility of determining equilibrium and kinetic constants for association reactions by studying concentration dependences of the first and the second moments of the chromatogram.

Carrying out SEC experiments on capillary microbore columns with an inner diameter of 0.5—1 mm, results in a significant reduction in the amount of protein required (100-fold when the column diameter is reduced from 5 mm to 0.5 mm). This clears the way to studying reactions of deficient proteins. Micro-column SEC also provides certain advantages when compared with the widespread sedimentation method of association studies. Among these the possibility of studying an extremely wide concentration range (down to 10^{-4} M with detection at 220 nm) is an important advantage.

Protein association should be studied in the following stages: (1) diagnostics of association; (2) determination of the number of components and the degree of association; (3) determination of equilibrium and kinetic constants; (4) studying the correlation between association parameters and functional activity of the protein. Micro-column SEC (MCSEC) appears to be useful at every level of study, though sometimes other methods should also be used to obtain additional information (sedimentation, diffusion).

The most complex problem in the analysis of associating protein is to determine the number of protein oligomers. The following types of association are especially frequent: (A) one-stage equilibrium monomer—n-mer (1); (B) multistage equilibrium — indefinite association with subsequent addition of monomer units and equal change in the free energy at every stage (2, 3).

$$nM_1 \underset{}{\overset{K_{1,n}}{\rightleftharpoons}} M_n; K_{1,n} = [M_n]/[M_1]^n,\ n \geqslant 1 \tag{1}$$

$$\begin{cases} M_1 + M_1 \overset{K_{1,2}}{\rightleftharpoons} M_2 & K_{i,i+1} = [M_{i+1}]/[M_i][M_1] \quad (2) \\ M_2 + M_1 \overset{K_{2,3}}{\rightleftharpoons} M_3 & K_{1,2} = K_{2,3} = \ldots = K_{i,i+1} \equiv K = \quad (3) \\ \ldots\ldots & \\ M_i + M_1 \overset{K_{i,i+1}}{\rightleftharpoons} M_{i+1} & = (1/C_1)(1 - \sqrt{C_i/C}) \end{cases}$$

where C is the total concentration of protein. Between these limiting cases the intermediate mechanisms are possible, among which model 4 is realized frequently.

$$qM_1 \underset{}{\overset{K_{m,n}}{\rightleftharpoons}} sM_m + rM_n \qquad n > m > 1 \tag{4}$$

$$K_{m,n} = \frac{[M_m]^s [M_n]^r}{[M_1]^q} = K_{1,m}^s \cdot K_{1,n}^r \tag{5}$$

Two-component reaction of monomer—n-mer type 1 may be distinguished with comparative ease. It is usually carried out on the basis of the Sophianopolous and Van Holde criterion [10], which predicts for two-component systems the inverse linear relationship between z-average ($\bar{V}_z$) and weight-average ($\bar{V}_w$) elution volumes of polydisperse protein.

$$\bar{V}_z = (V_1 + V_n) - V_1 V_n \bar{V}_w^{-1} \tag{6}$$

According to definition of averages

$$\bar{V}_w = \sum_1^n V_i C_i / \sum_1^n C_i \tag{7}$$

$$\bar{V}_z = \sum_1^n V_i^2 C_i^2 / \sum_1^n V_i C_i \tag{8}$$

$\bar{V}_w$ and $\bar{V}_z$ are calculated from wide-zone frontal chromatograms by means of the following formulae

$$\bar{V}_w = \int_0^{C_0} V(C)\mathrm{d}C / C_0 \tag{9}$$

$$\bar{V}_z = \int_0^{C_0} V^2(C)\mathrm{d}C / \int_0^{C_0} V(C)\mathrm{d}C \tag{10}$$

where C_0 is the total protein concentration. If criterion 6 shows the number of components $n > 2$, there is no other way than sorting out the models of association. This means varying the equilibrium constants for the reactions 2 and 3 and to draw a conclusion about the reaction mechanism on the basis of the best fit between experimental and computed dependences $V_w(C)$. The uncertainty of such an analysis may be reduced by making use of additional sedimentation data on the concentration dependence of $\bar{M}_w$ (weight-average mol. wt.) or by means of an independent method of evaluating the number of components. Unfortunately, the only method to be used for this purpose is the measurement of instantaneous electrophoretic mobility [11] based on the Doppler effect in light scattering.

When the number of components is known, it is possible to determine equilibrium association constants from the concentration dependence of the weight-average elution volume [5]. The frontal mode of chromatographic analysis should be used, since it provides a constant composition in the plateau region. A typical chromatogram, obtained under these conditions, is depicted in Fig. 1, and the position of centroid, which corresponds to the weight-average elution volume ($\bar{V}_w$), is defined by eqn. 9.

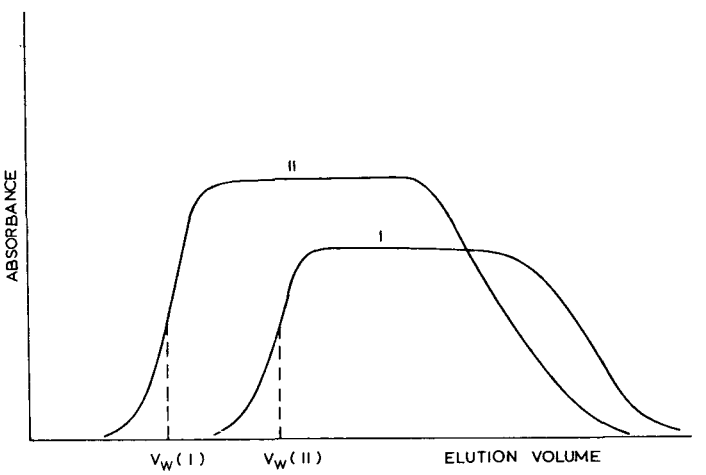

Fig. 1. Wide-zone chromatograms obtained by MCSEC. I = lysozyme (n = 2), pH = 7.0, μ = 0.2; II = α-chymotrypsin (n = 3), pH = 6.2, μ = 0.2, n = number of components.

For the reaction 1, eqns. 7 and 1 lead to the following relationship

$$\bar{V}_w(C_0) = \frac{V_1 + K_{1,n} C_1 V_n}{1 + K_{1,n} C_1} \tag{11}$$

where monomer concentration (C_1) in the plateau region is related to C_0 and $K_{1,n}$ as follows:

$$K_{1,n} C_1^n = C_n; \qquad C_1 + C_n = C_0 \tag{12}$$

The monomer elution volume (V_1) may be determined from $V_w(C)$ by extrapolation to infinite dilution, and the polymer elution volume (V_n) from the calibration curve. It should be kept in mind that individual proteins are extremely different in terms of solvation, asymmetry of the native conformation, and ability to interact with the sorbent. That is why molecular mass calibration is unreliable, whereas hydrodynamic radius calibration is not possible in some cases (for instance, in the presence of adsorption).

For swelling gels Stokes' radii (R) calibration of the distribution coefficient (K_d) is based on Ackers' equation [4]

$$R = a + b \ \mathrm{erfc}^{-1} K_d \tag{13}$$

where a and b are constants, $\mathrm{erfc}\ K_d = \frac{\sqrt{\pi}}{2} - \int_0^{K_d} e^{-t^2} dt$, $\mathrm{erfc}^{-1} K_d$ the inverse function.

For rigid gels the best calibration results are obtained with the so-called random-sphere model [12] which presents an adequate geometrical image of the structure of porous silica-based materials. According to the random-sphere model,

$$K_d = \psi^{(1+R/R_s)^3 - 1} \tag{14}$$

where ψ is the inner porosity of the sorbent, R_s the radius of elementary SiO_2 microsphere in the matrix of the silica gel. Figs. 2 and 3 show calibration relationships for globular proteins obtained on the columns with Sephadex G-75 (Fig. 2) and TSK-gel SW 3000 (Fig. 3).

When the protein under investigation is being adsorbed on the column, the problem of association study becomes rather difficult, since V_i values of oligomer elution volumes are in this case unknown. Just this very case is realized with lysozyme, the protein which is known to interact even with inert carbohydrate and polyacrylamide gels. However, with this protein it is possible to overcome these adsorption-based complications and to determine the dimerization constant by means of MCSEC. For this purpose special methods for treating the experimental data have been developed [13]. Assuming Langmuir-type protein adsorption, the equilibrium dimerization constant and two parameters of the adsorption isotherm may be found from the experimental $V_w(C)$ dependence and eqn. 6 on the basis of the best-fit values for these parameters, providing the closest correspondence of calculated and experimental $\bar{V}_w$ and $\bar{V}_z$. The result is shown in Figs. 4 and 5. It is worth mentioning that an identical value for the dimerization constant ($K_{1,2} = 0.9 \pm 0.2$ dl/g) has been obtained for both dextran and polyacrylamide gels despite the difference in adsorption isotherms for these gels. This fact, together with the good fit of our result with sedimentation equilibrium data, demonstrates the reliability of the meth-

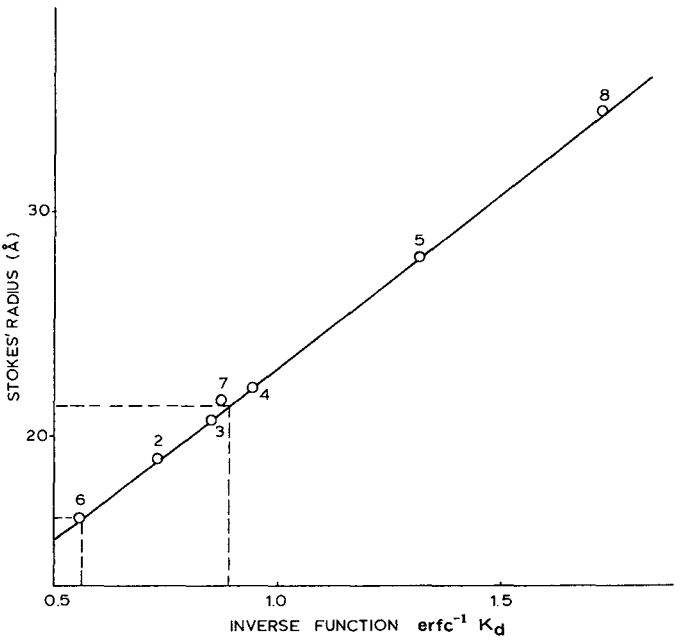

Fig. 2. Calibration dependence of distribution coefficient K_d on the Stokes' radii of proteins for microbore column with Sephadex G-75. V_i = protein elution volume; V_0 = void volume; V_p = pore volume. 1 = cytochrome *c*, 2 = ribonuclease, 3 = myoglobin, 4 = chymotrypsinogen A, 5 = ovalbumin, 6 = phospholipase A_2 (monomer), 7 = phospholipase A_2 (dimer), 8 = bovine serum albumin (BSA).

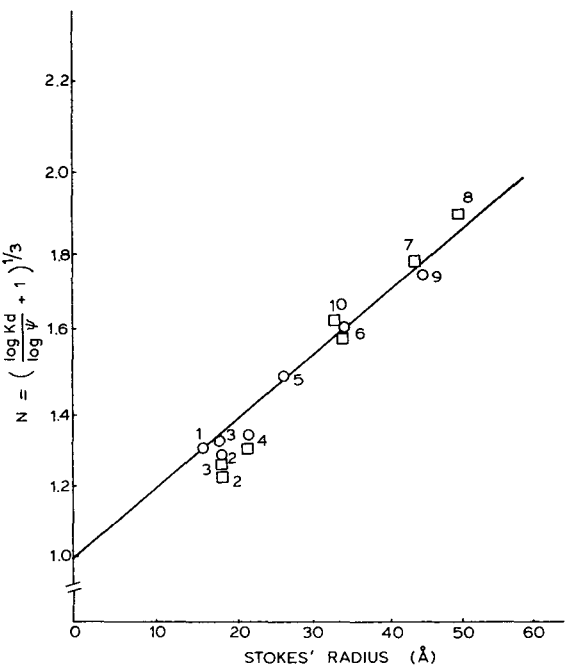

Fig. 3. Calibration dependence for TSK-gel SW 3000, based on the random-sphere model of porous structure. Experimental data are from refs. 15 and 16. R_s = 59.5 Å; ψ = 0.708; 1—5 as in Fig. 2, 6 = BSA, 7 = BSA—dimer, 8 = BSA—trimer, 9 = aldolase.

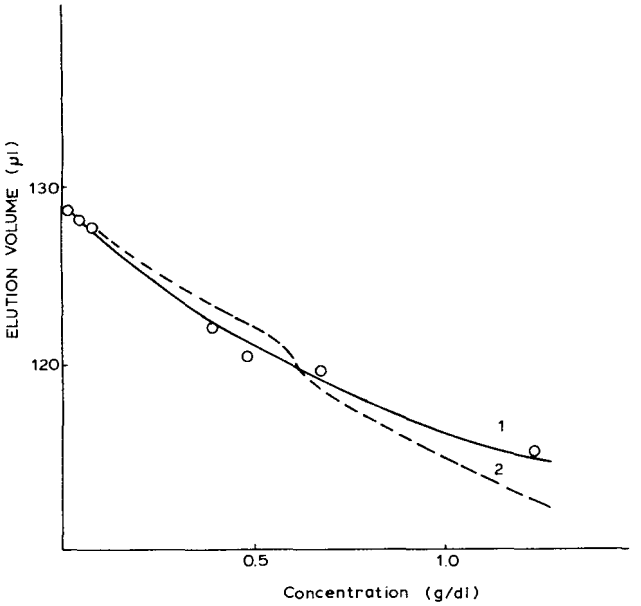

Fig. 4. Concentration dependence of V_w for lysozyme (Bio-Gel P-60 column). 1 = curve, calculated for $K_{1,2}$ = 0,9 dl/g; 2 = curve, calculated for the doubled dimerization constant. (o) Experimental data.

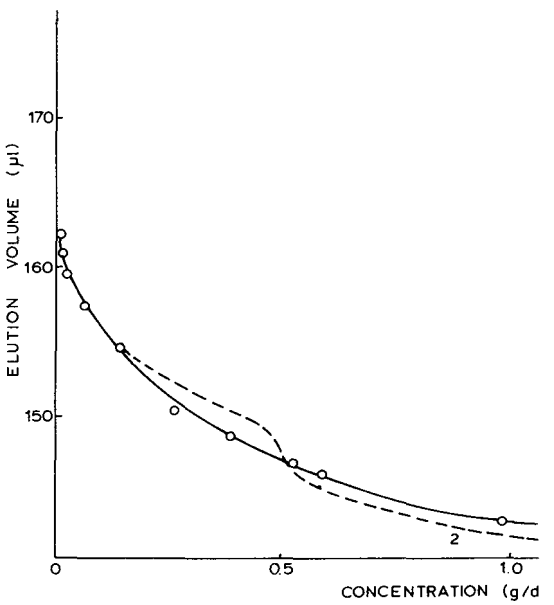

Fig. 5. Concentration dependence of V_w for lysozyme (Sephadex G-75 column). Designations correspond to those in Fig. 4.

od developed by us for chromatographic study of protein association complicated by protein adsorption.

Now consider the principles of chromatographic estimation of the rate constants for protein self-association reactions. The equilibrium mixture of monomer with the associated form of protein tends to separate when it is moving in the column, but the equilibrium composition would be re-established in partially resolved zones with a certain lag time. Therefore, the spreading of chromatographic boundary of reversibly associated protein is increased in comparison with a non-interacting mixture of the same composition. Besides longitudinal and eddy diffusion, which are usual factors of spreading, for reacting systems there is another type of spreading caused by different times for protein to move as monomer or as associate. Thus the measured dispersion coefficient $L_{V(M)}$ may be represented as the sum of the weight-average dispersion coefficient and a certain kinetic term [2].

In the simplest case of dimerization

$$L_{V(M)} = \frac{1}{N} + L_K \tag{15}$$

$$L_K = 2F(V_1 - V_2)^2 K_{1,2} C_1 / (1 + K_{1,2} C_1)^3 V_w^3 k_2 \tag{16}$$

In these equations $L_V = \frac{\sigma_V^2}{V^2}$, σ_V^2 is the volume dispersion of boundary, F is volume flow-rate, N is the number of theoretical plates, $k_2 = \frac{k_1 C_1^2}{C_2}$ is rate constant

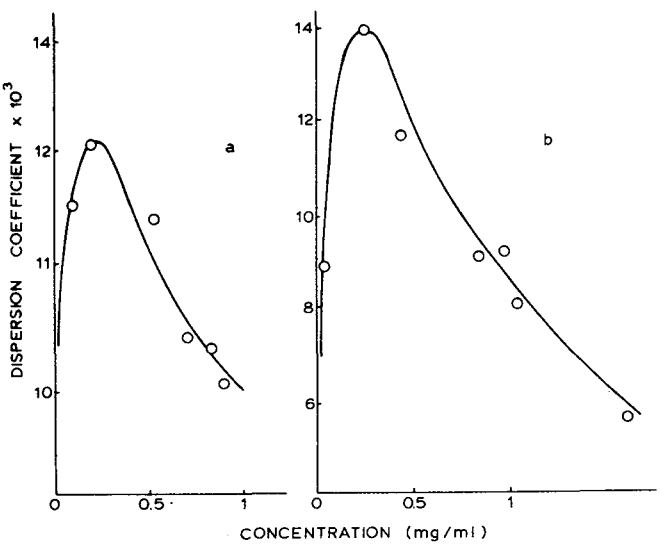

Fig. 6. Concentration dependence of the dispersion coefficient $L_{V(M)} = \sigma_V^2/\bar{V}_w^2$ for phospholipase A_2 leading boundary of chromatogram. (a) $C_{Ca^{2+}} = 0$; (b) $C_{Ca^{2+}} = 0.02$ M. Curves are the theoretical results based on the data of Table I for $N = 200$ theoretical plates. (○) Experimental points.

for the reverse and k_1 for the direct reaction. Eqn. 16 may be used for the estimation of rate constants. The results obtained for phospholipase A_2 are presented in Fig. 6 and Table I. The rate constants for dimerization of this enzyme appeared to be sensitive to the presence of activator (Ca^{2+}).

TABLE I

RATE CONSTANTS FOR PHOSPHOLIPASE A_2

Ca^{2+} concentration	$K_{1,2} \cdot 10^{-4}$ (M^{-1})	$k_1 \cdot 10^{-4}$ (M^{-1} sec^{-1})	k_2 (sec^{-1})	$\tau = k_2^{-1}$ (sec)
0	6.0 ± 0.5	4.2 ± 0.5	0.7 ± 0.1	1.5
0.02	6.3 ± 0.5	1.27 ± 0.32	0.2 ± 0.05	5

It can be seen from Fig. 6 that there is a maximum in $L_{V(M)}$ versus C_0 plot. It is explained as follows. When $C_0 \to 0$ (complete dissociation) and $C_0 \to \infty$ (complete association), $L_{V(M)} \to 1/N$, whereas the intermediate range of concentration is characterized by mixed kinetics, when association and mass transfer are equally rate-limiting.

We have considered physicochemical aspects of chromatographic study of associating proteins. To illustrate the biochemical potentialities of this method let us cite as an example the use of MCSEC to ascertain the mechanism of catalytic action of phospholipase A_2 [14]. This enzyme hydrolyzes the fatty ester bound at position 2 of 1,2-diacyl-*sn*-phosphoglycerides.

The rate of lipolysis is effectively increased when the substrate is in micellar form. By means of MCSEC, dimerization of this enzyme was investigated, whereas the activity data have shown the dimer to be the only active form of phospholipase A_2. We examined the influence of substrate (dihexanoyl-lecithin) in molecular form and in micelles on the dimerization equilibrium of phospholipase A_2, and the influence of activator (Ca^{2+}) and inhibitor (Ba^{2+}) on complex formation of the enzyme with the micelle. Fig. 7 shows the dependence of reduced elution volume on the total concentration of phospholipase A_2 isozyme E_3. The ordinate in Fig. 7 corresponds to the centroid elution volume of the leading boundary which is normalized by $V_t = V_p + V_0$, since several columns had been used. V_p is the pore volume of the gel, V_0 the void volume. From curves 1 and 2 the dimerization constants $K_{1,2}$ (4.5 ± 0.2 ml/g) and $K_{1,2}$ (1.6 ± 0.1 ml/g) have been calculated for the systems Tris–HCl–KCl and Tris–HCl–KCl–$BaCl_2$; Tris–HCl–KCl–$BaCl_2$–monomer substrate, respectively. This result has been confirmed by sedimentation equilibrium data on the weight-average molecular mass. Thus it was found that inhibitor (Ba^{2+}) shifted slightly the equilibrium of dimerization to monomer, whereas the substrate in its molecular form had no influence on dimerization. The effect of micellar substrate is quite different (curve 3 in Fig. 7). In this case there is no $V_w(C)$ dependence, which is typical for reversible association, and the weight-average elution volume corresponds to dimer (E_2) elution volume, which is significantly higher than the elution volume of the dimer–micelle complex E_2S_n. Two important conclusions may be drawn from this: (1) the dimer–micelle complex is not formed in the presence of Ba^{2+}; (2) in the presence of mi-

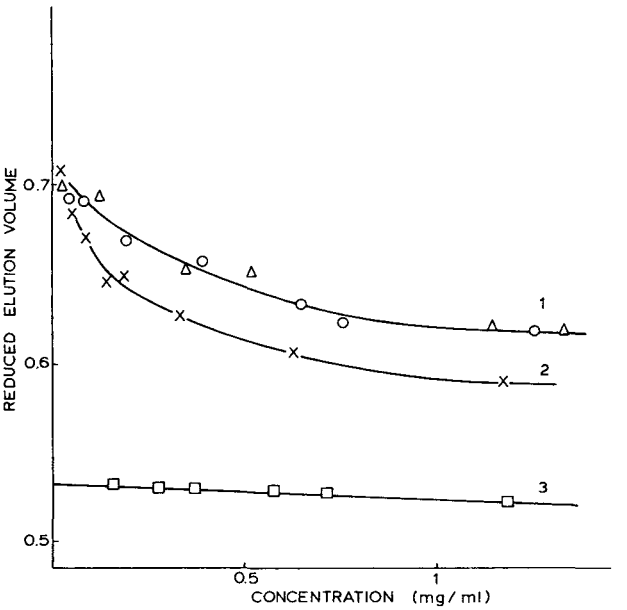

Fig. 7. Concentration dependence of the reduced weight-average elution volume V_w/V_t for phospholipase A_2 (Sephadex G-75 column) obtained in the systems: Tris—HCl—KCl (2, ×); Tris—HCl—KCl—$BaCl_2$ (1, ○); Tris—HCl—KCl—$BaCl_2$—S ([S] = 1 mg/ml < CMC) (1, △); and Tris—HCl—KCl—$BaCl_2$—S ([S] = 20 mg/ml ⩾ CMC) (3, □), S = substrate (dihexanoyl-lecithin); CMC = critical micelle concentration.

cellar substrate, the association equilibrium of phospholipase A_2 is entirely shifted towards active dimer.

The constancy of the dispersion coefficient in the examined range of concentration is also indicative of one-component behaviour of dimerized phospholipase A_2 in the system containing substrate micelles.

Thus we arrive at the conclusion that Ca^{2+} and Ba^{2+} promote, or respectively suppress, enzymatic activity at the stage of complex formation, but not at the stage of association. These results, obtained by means of MCSEC, have clarified to some extent the mechanism of catalytic action of phospholipase A_2. In conclusion, the specific character of the MCSEC experiment is worth noting. In our experiments as little as 1 mg of protein was sufficient for a complete study of association equilibrium and kinetics. For detection we used a spectrophotometer with a cell volume of ca. 1 μl. Due to low dead volumes of the cell and connection tubes the contribution of extra-column spreading to total dispersion did not exceed 5%.

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SEPARATION OF URINE PROTEINS ON THE ANION-EXCHANGE RESIN MONO Q™

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SUMMARY

Proteins excreted in urine due to renal failure were separated on Mono Q™, a new strong anion exchanger designed for fast high-resolution protein separations. The separation procedure was divided into two steps. The first step involved removal of low-molecular-weight substances by rapid desalting on a Sephadex G-25 Superfine column. In the second step, the total protein fraction (3—6 ml) was loaded onto the Mono Q column with the aid of a superloop. The proteins were adsorbed onto the top of the ion-exchanger column and gradually displaced by a combined pH and salt gradient in 40 min. The choice of ion exchanger and initial operating conditions were based on data obtained from electrophoretic titration curve experiments. Identification of separated proteins was achieved by fused rocket electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, respectively.

INTRODUCTION

The identification of serum proteins excreted into the urine in renal failure has traditionally been carried out by electrophoretic techniques such as immuno- or sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis [1]. The major drawback of all electrophoretic methods is their comparatively slow speed and results are generally not available until one day or more after sample delivery. This can be partly compensated by running several parallel samples on the same gel.

The major alternative to electrophoresis is chromatography. Protein chromatography has been used in clinical research for several decades, but has not been recognized as a substitute for electrophoresis in routine clinical chemistry, mainly due to the comparatively low resolution and slow speed

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of operation of conventional chromatography [2]. This situation has been dramatically changed by recent developments in gel materials suitable for high-performance liquid chromatography (HPLC) of proteins [3]. New media with an optimal combination of rigidity, porosity, hydrophilicity and inertness enable complex protein mixtures in urine to be separated in an hour or less. The patterns of the various absorbance peaks as they elute can provide important information on the likely pathophysiological state of the urinary tract. HPLC is not only faster than electrophoresis, but also allows fractions to be collected for subsequent multiparametric analysis of the separated proteins.

Recently Pharmacia Fine Chemicals has introduced a new hydrophilic, polyether resin with a narrower particle size distribution (9.8 ± 2%): Mono Q (containing $CH_2N^+(CH_3)_3$ groups) which appears suitable for solving the above problem.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Sephadex G-25 Superfine, Mono Q HR 5/5 prepacked columns, PD-10 columns, Pharmalyte 3—10, Agarose A, gradient gels PAA 4/30 and antibodies against κ-light chains, λ-light chains, IgG, transferrin, albumin, α_1-antitrypsin and whole blood serum from Pharmacia Fine Chemicals (Uppsala, Sweden); Bis Tris propane chloride (1,3-bis[tris(hydroxymethyl)-methylamino]-propane) from Sigma (St. Louis, MO, U.S.A.); antibodies against β_2-microglobulin, retinol-binding protein and α_1-acid glycoprotein from Dako (Copenhagen, Denmark).

Buffer solutions

Standard starting buffer for the ion-exchange chromatography step was Bis Tris propane chloride (6.25 m*M*) adjusted to pH 7.5 with hydrochloric acid. Standard limit buffer was Bis Tris propane chloride (6.25 m*M*) adjusted to pH 9.5 with hydrochloric acid with the addition of sodium chloride (0.35 *M*). In initial experiments the buffer salt, pH and ionic strength of the starting and limiting buffers were varied systematically to determine the optimum separation conditions.

Equipment

All chromatographic runs were performed using an FPLC System from Pharmacia Fine Chemicals. This system avoids contact of the sample and eluent with stainless steel. The use of standard HPLC equipment had to be abandoned due to rapid corrosion of the pumps at high pH in the presence of salt and subsequent degradation of column performance.

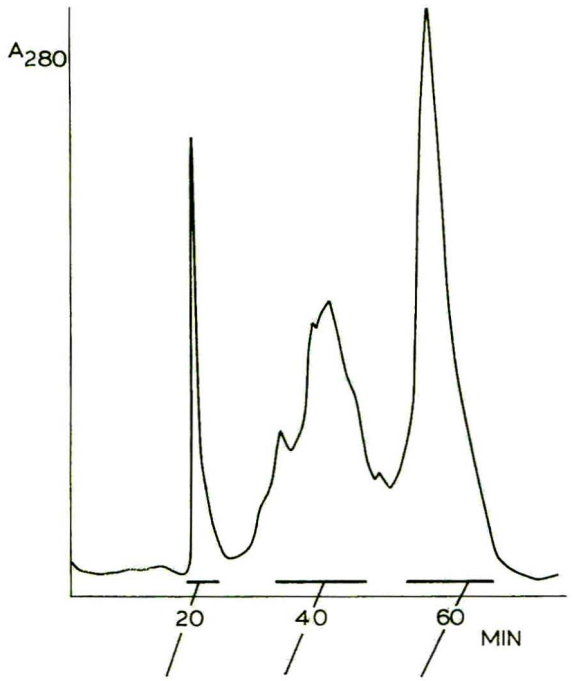
Electrophoretic techniques

SDS-polyacrylamide gradient electrophoresis and fused rocket immunoelectrophoresis were carried out by standard methods [4]. Electrophoretic titration curves were performed by the method of Rosengren et al. [5] using Pharmalyte 3—10 in place of Ampholine 3.5—10.

RESULTS AND DISCUSSION

Removal of low-molecular-weight components

To free the proteins from the low-molecular-weight salts and degradation products in urine, we used a rapid desalting step on Sephadex G-25 Superfine. The gel was swollen for 1 h in the starting buffer for the ion-exchange step



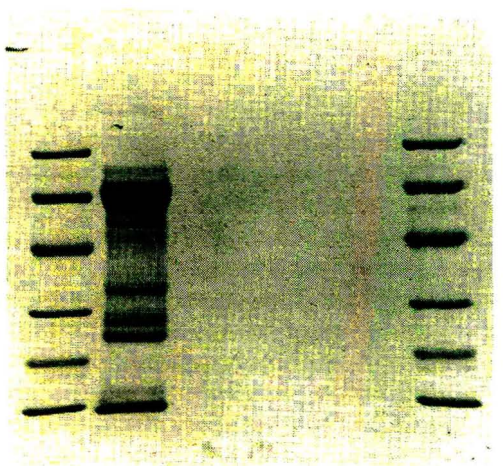


Fig. 1. Gel chromatography of urine (0.5 ml) on Sephadex G-25 Superfine. Column C 16/40, bed height 34 cm. Freeze-dried, pooled fractions were examined by SDS-electrophoresis. The total protein content elutes in the first peak. The outside lines are low molecular weight marker proteins.

and slurry-packed into a C 16/40 column at a flow-rate of 5 ml/min. Centrifuged urine (0.5 ml) was applied to the column and eluted at a flow-rate of 1.35 ml/min. The purified protein fraction eluted after 20 min in a volume of 3—6 ml depending on the amount of protein present (see Fig. 1). The recovery of proteins from this step was > 90%.

Prediction of the optimal separation conditions for ion-exchange chromatography

In ion-exchange chromatography of molecules with a very high net charge, e.g. proteins, there is a correlation between the ionic strength needed for elution and the net charge of the protein [6]. Since the net charge on a protein, and consequently the ionic strength at which it will be eluted, varies with pH as the ionogenic groups on the protein are titrated, titration curves are of great assistance in predicting the elution pattern over a range of pH. The titration curves of proteins in a complex mixture can be determined by an electrophoretic technique of Rosengren et al. [5].

Fig. 2 shows a schematic of electrophoretic titration curves for a mixture of urine proteins. To identify the curves for the individual proteins, immunoprints were obtained by placing acetate paper wetted with the corresponding antibody on the separation gel. The strips were then stained with Coomassie Blue-R. Fig. 3 shows examples of the immunoprints for β_2-microglobulin and albumin. As can be seen from the schematic titration curves in Fig. 2, the biggest differences in charge between the four main proteins in this pathological urine (β_2-microglobulin, retinol-binding protein, α_1-acid glycoprotein and albumin) are found in the basic region, pH 7—10. The optimum pH for separation of these major components should, therefore, be within this pH range.

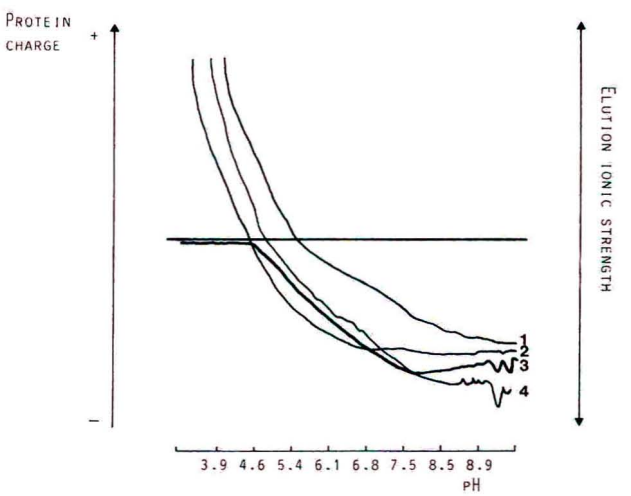
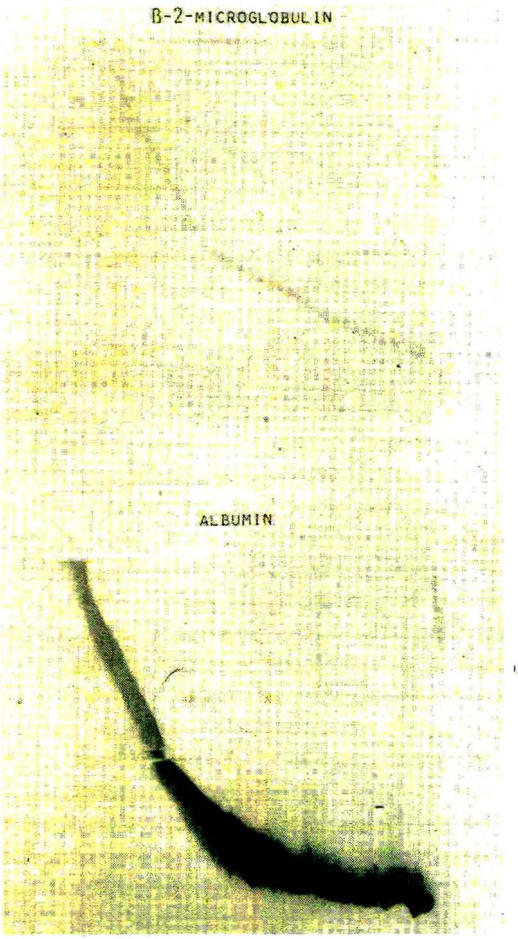


Fig. 2. Schematic electrophoretic titration curves. The numbered curves are (1) β_2-microglobulin, (2) retinol-binding protein, (3) α_1-acid glycoprotein, (4) albumin.



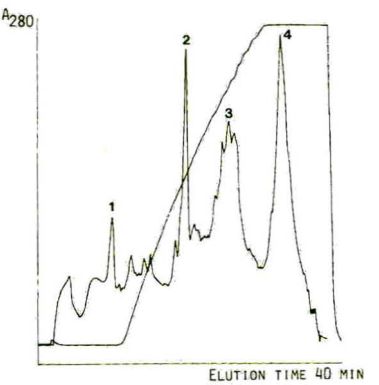


Fig. 3. Immunoprints from the electrophoretic titration curves.

Fig. 4. The elution of β_2-microglobulin (peak 1) at an ionic strength of 12.5 m*M* Bis Tris propane (pH gradient from 7.5 to 9.5, sodium chloride gradient to 0.35 *M*). Compare to Fig. 8.

Ionic strength and pH of the starting buffer

The low ionic strength of the starting buffer for the ion-exchange separation is necessitated by the elution characteristics of β_2-microglobulin. The elution of this protein is very sensitive to both the pH and the ionic strength if a sharp peak is required. At a lower pH than 7.5, it is not retarded on the column and elutes in the front. At higher pH values, β_2-microglobulin tends to elute as a broad peak with lower separation efficiency as a result. A higher starting ionic strength than 6.25 m*M* also results in a broader peak. Fig. 4 shows β_2-microglobulin eluting in 12.5 m*M* Bis Tris propane pH 7.5 as starting buffer.

Optimization of pH

Separations at three different pH values in the range 7—10 with the same gradient of sodium chloride concentration are shown in Figs. 5—7. At pH 7.5, there is optimal resolution between β_2-microglobulin and retinol-binding protein and no separation between α_1-acid glycoprotein and albumin. The optimal resolution for these two proteins is at pH 9.5. To obtain good resolu-

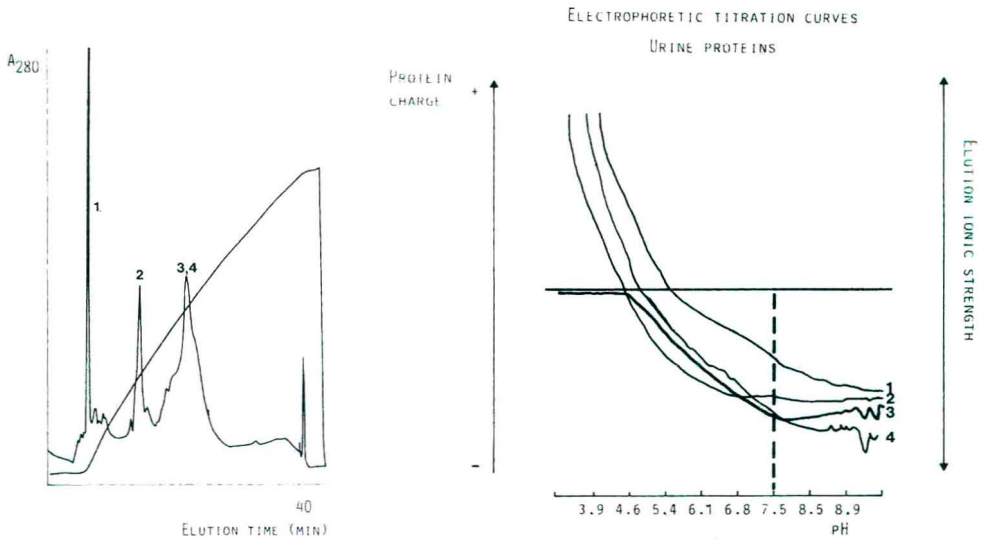


Fig. 5. Separation of urine proteins at pH 7.5 (6.5 m*M* Bis Tris propane, sodium chloride gradient to 0.35 *M*). Injection volume 3 ml. The dashed line on the titration curve indicates the charge differences between the four proteins at the pH of chromatography. Compare Figs. 6—8. Peak identification as in Fig. 2.

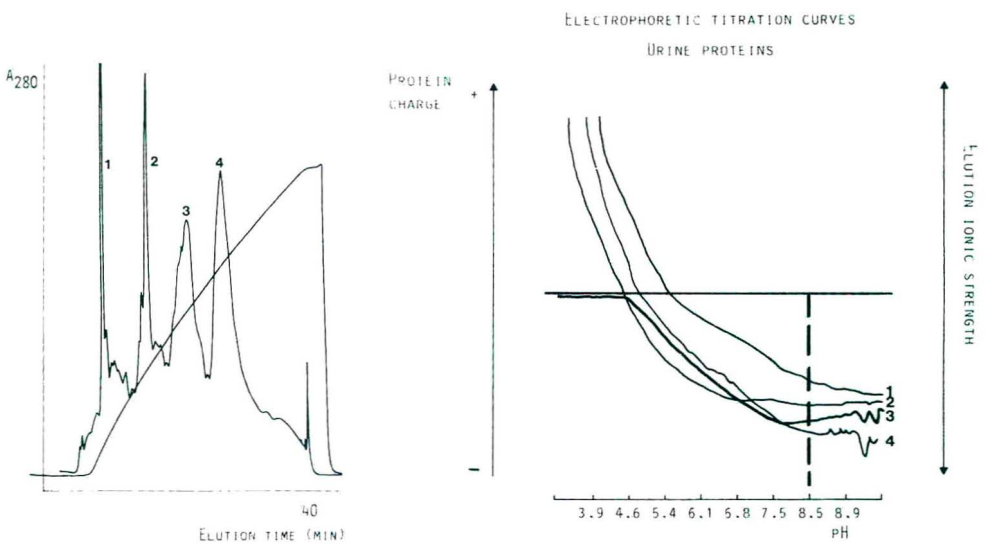


Fig. 6. Separation of urine proteins at pH 8.5 (gradient as in Fig. 5). Injection volume 3.5 ml. Peak identification as in Fig. 2.

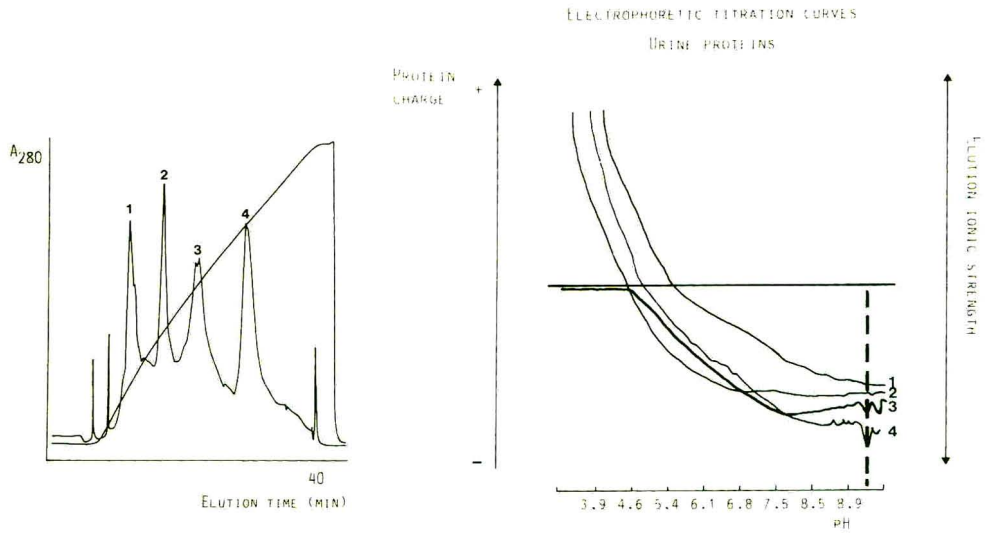


Fig. 7. Separation of urine proteins at pH 9.5 (gradient as in Fig. 5). Injection volume 3 ml. Peak identification as in Fig. 2.

tion between all four proteins, a combined pH (7.5—9.5) and salt gradient (0—0.35 *M* sodium chloride) was used in Fig. 8. Bis Tris propane with pK_a 6.8 and 9.0 gives a linear pH gradient between 7.5 and 9.5 [7]. It is important that the buffer has the same ionic strength at both the start pH and at the final pH in the absence of added salt otherwise a concave or convex pH gradient will form.

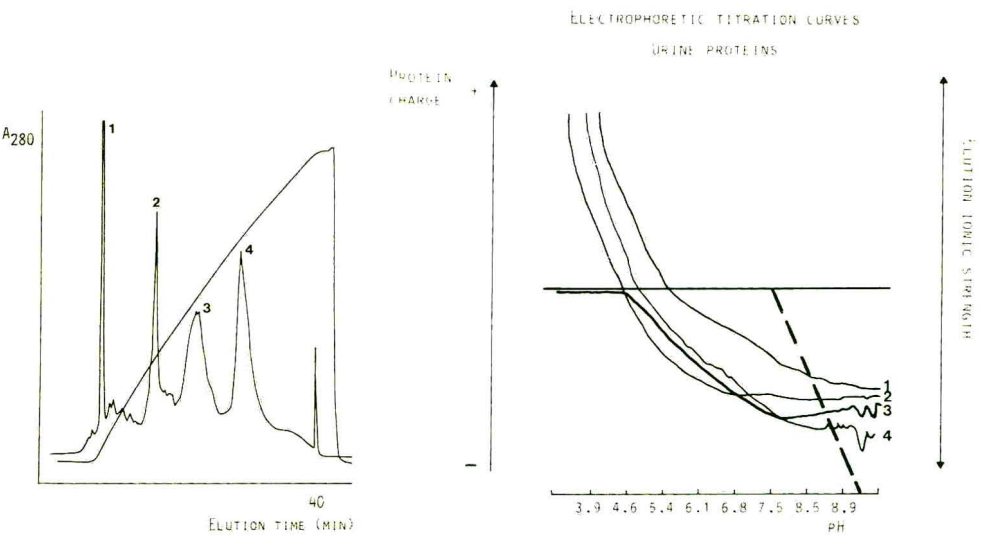
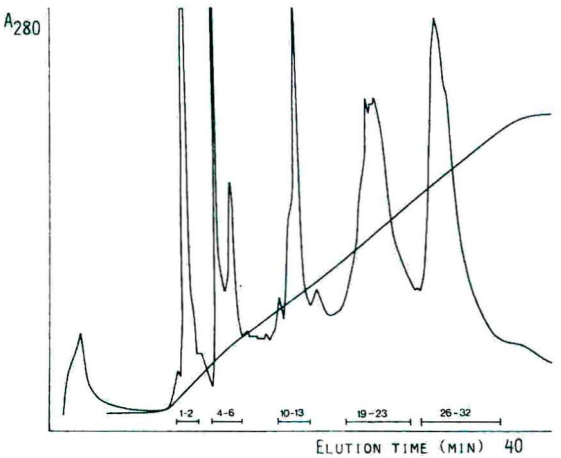


Fig. 8. Separation of urine proteins with a pH gradient 7.5—9.5. Injection volume 3 ml. Peak identification as in Fig. 2.

Identification of the individual proteins

Fig. 9 shows a typical chromatogram for urine excreted in renal disease. Fractions (1 ml) were collected, desalted on a PD-10 column, concentrated



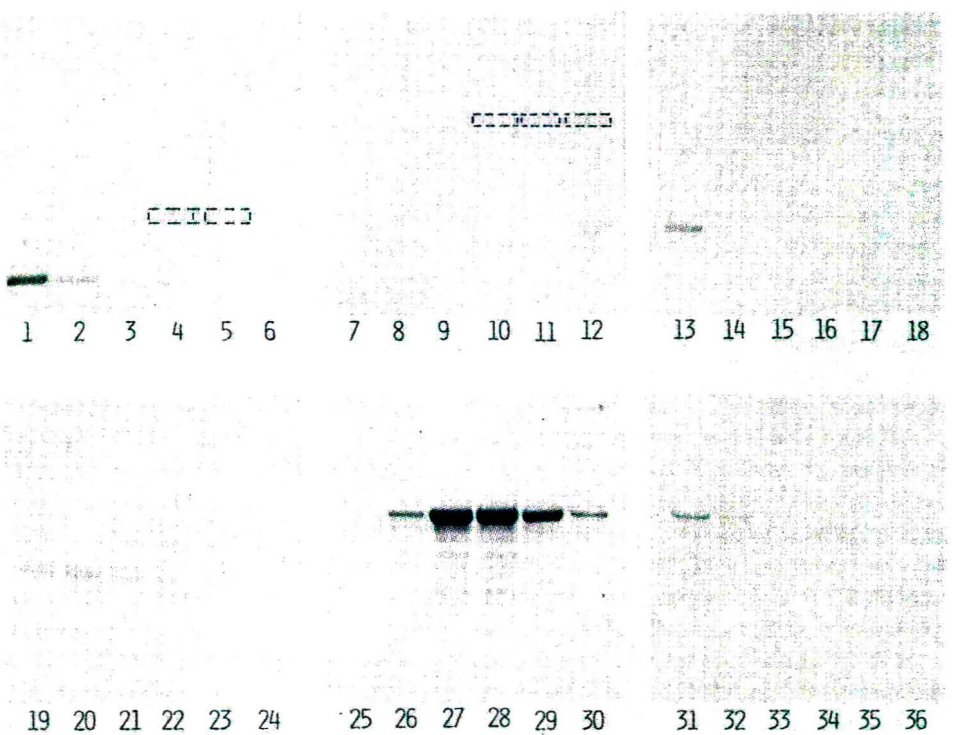


Fig. 9. Urine from chronic pyelonephritis on Mono Q column, 1-ml fractions analysed by SDS-gel electrophoresis. Lanes 1, 2, 4, 5: β_2-microglobulin. Lanes 4, 5: κ- and λ-chains. Lanes 10—12: transferrin. Lanes 12, 13: retinol-binding protein. Lanes 18—21: α_1-microglobulin. Lanes 19—23: α_1-acid glycoprotein. Lanes 20—24: α_1-antitrypsin. Lanes 26—32: albumin.

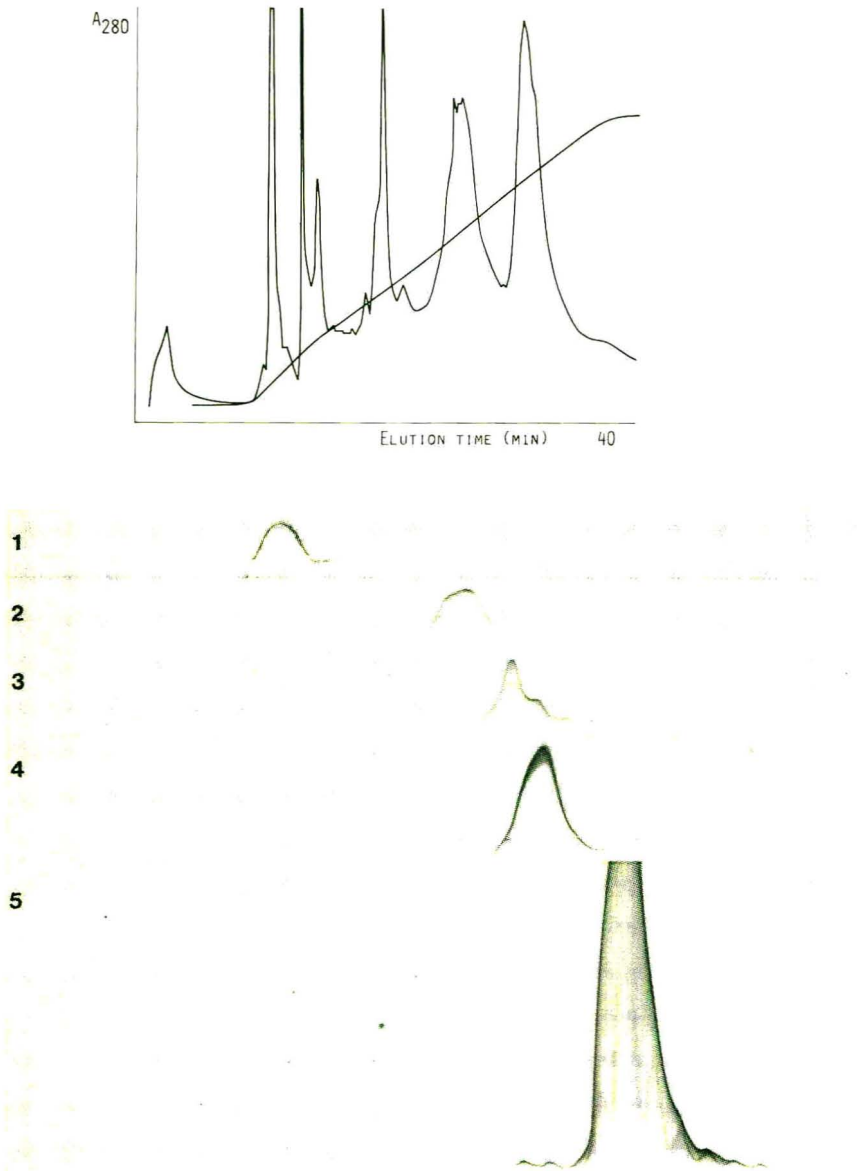


Fig. 10. Urine from chronic pyelonephritis on Mono Q column, 1-ml fractions analysed by fused rocket immunoelectrophoresis. 1 = β_2-microglobulin, 2 = transferrin, 3 = α_1-antitrypsin, 4 = α_1-acid glycoprotein, 5 = albumin.

by freeze-drying and dissolved in aliquots for analysis by SDS-polyacrylamide gel electrophoresis and fused rocket immunoelectrophoresis (Figs. 9 and 10).

Injection volume and lowest detectable limit

Since the efficiency of ion-exchange chromatography is limited by the total protein load and not by the injection volume, large volumes of dilute

protein solution can be concentrated on the column. The way to increase the sensitivity of detection for this application is thus to pool the protein fractions from a number of gel filtration runs and inject the pooled fractions directly onto the Mono Q column via a superloop.

CONCLUSIONS

Several major protein peaks can be resolved by two-step chromatography of urine. Immunochemical analysis and SDS-polyacrylamide gel electrophoresis have shown that each peak contains several proteins usually with one predominant or characteristic single protein.

Clinical investigations by one of us (E.H.C.) indicate that the peak profiles can be correlated to certain pathophysiological changes in renal function. The optimization technique is being applied to obtain maximum separation between protein fractions that are of major interest in different forms of proteinuria. The system can provide a basis for the separation of individual proteins as well as reveal information about the relative concentration of a wide variety of plasma proteins in the urine.

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ISOTACHOPHORETIC ELECTRODESORPTION OF PROTEINS FROM AN AFFINITY ADSORBENT ON A MICROSCALE

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SUMMARY

Cationic isotachophoresis was used for the desorption of mouse monoclonal antibody to transferrin strongly affinity-bonded to transferrin immobilized on polyethyleneglycol terephthalate powder. The electrodesorption under nondestructive conditions was effected in the capillary isotachophoresis apparatus of our own construction which was equipped with an adsorption element. The electrodesorption is on-line connected with quantitative isotachophoretic analysis of the antibody desorbed. Only a few tens of microliters of the affinity adsorbent and several nanomoles of the antibody are needed for the characterization of the capacity of the affinity adsorbent and the conditions of adsorption and desorption.

INTRODUCTION

One of the key problems in affinity chromatography is the release of the bio-affinity-adsorbed, soluble, complementary component or ligate [1] from the bond to the affinity ligand immobilized on a solid support. In some cases of very strong complexes, such as, for example, antigen—antibody complexes, the ligate cannot be set free from the bond to the affinity ligand by changes in the composition of the solution, pH, ionic strength, temperature or by the addition of chaotropic reagents or detergents without damage or even complete loss of the biological activity of the protein ligate. The nondestructive release of the ligate by an excess of the low molecular weight ligands, if available at all, leads to undesirable contamination and often requires an additional separation step. If the ligand—ligate complex is in equilibrium with its components [2] and if the ligate is of an ionogenic character, the ligate can be liberated by electrophoretic desorption according to Dean et al. [3].

The electrophoretic desorption (electrodesorption), also called electrophoretic elution by Grenot and Cuilleron [4], can be effected by all the three

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basic electromigration methods, i.e. by electrophoresis in a homogeneous electrolyte, by isotachophoresis in a discontinuous system of two electrolytes and, with amphoteric ligates, also by isoelectric focusing in a pH gradient of mixed ampholytic electrolytes. As yet the desorption of protein ligates, especially of antibodies, has been mostly carried out by electrodesorption in a homogeneous electrolyte [2—11] which represents a special mode of affinity electrophoresis (the affinity adsorbent occupies only a part of the separation chamber). A disadvantage of electrodesorption in a homogeneous electrolyte is not only a slow release of the antibodies and of other ligates in a dilute state but also the elaborate detection or isolation of the ligate released.

The focusing electromigration methods of electrodesorption of proteins were employed in the form of isoelectric focusing by Vesterberg and Hansén [12] and by Haff et al. [13]. In such a case the protein ligate is not only to be exposed to an unfavourable effect of the pH of the medium during the focusing but also to the risk of precipitation after the quasistationary state of the isoelectric point has been achieved.

The self-focusing and concentrating effect of isotachophoresis enables us to use for electrodesorption a properly selected isotachophoretic system of electrolytes where the pH of both the leading and the terminating electrolyte does not exceed values at which the protein desorbed is stable and at the same time shows an intermediary mobility with respect to both the leading and the terminating ion of the system. In the process of desorption the ligate released migrates between the leading and the terminating ion thus forming a zone of a relatively high steady-state concentration [14, 15] which can, moreover, be regulated by the concentration of the leading ion. The pH of this zone is always different from the isoelectric point of the protein desorbed and the ionic strength is higher than in the case of isoelectric focusing; the risk of precipitation of the protein desorbed is generally lower compared to isoelectric focusing. This study was undertaken in an effort to develop the conditions of the use of capillary analytic isotachophoresis for the release of the ligate and its on-line analysis and conditions of the characterization of the properties of the affinity adsorbent on a microscale; the affinity pair antibody—antigen served as a model.

MATERIALS AND METHODS

Chemicals

Acetic acid, A.G., hydrochloric acid, A.G. (Normanal), boric acid, A.G., potassium hydroxide, A.G., and sodium azide pure were purchased from Lachema, Brno, Czechoslovakia. Sodium borohydride (98%) was made in the Institute of Inorganic Chemistry, Czechoslovak Academy of Sciences, Rež, Czechoslovakia. β-Alanine (BALA) puriss., and ϵ-aminocaproic acid (EACA) puriss., were from Koch-Light, Colnbrook, Great Britain. 2-(N-Morpholino)-ethanesulfonic acid (MES) A-grade, was purchased from Calbiochem, San Diego, CA, U.S.A. Polyvinyl alcohol (PVA) Mowiol was a product of Hoechst, Frankfurt, G.F.R. The solution of mouse monoclonal antibody to porcine transferrin (MMAbT), IgG1 subclass [16], in phosphate-buffered saline (PBS, Serva PM 16, Heidelberg, G.F.R.) with the addition of 0.1% sodium azide, and

polyethyleneglycol terephthalate microgranular carrier (Sorsilen) from the Insolmer kit (a product of the Institute of Sera and Vaccines, Prague, Czechoslovakia), coated with porcine transferrin immobilized by glutaraldehyde (Insol-transferrin) were kindly supplied by Dr. F. Franěk, Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. A part of the Insol-transferrin was treated with sodium borohydride according to the method of Royer et al. [17].

Instrumentation

Electrodesorption was carried out in the apparatus of our construction, made in the workshops of the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. The core of the instrument is the analytical capillary isotachophoresis apparatus. The separation takes place in a PTFE capillary of 0.45 mm I.D. and 0.7 mm O.D., 230 mm long. The capillary is accommodated in a thermostated bath filled up with the Savant EC 123 coolant and temperature-controlled over the range 7—25° C by Peltier thermocouples. The apparatus is provided with two universal potential gradient detectors connected in series and with a photometric detector of ultraviolet absorption at 254 nm. The adsorption element containing the affinity adsorbent is placed between the six-way application PTFE valve and the block of the terminating electrolyte; the element is connected to the apparatus by a PTFE capillary of 1 mm I.D. and 1.4 mm O.D. of variable length. The arrangement can be seen in Figs. 1 and 2.

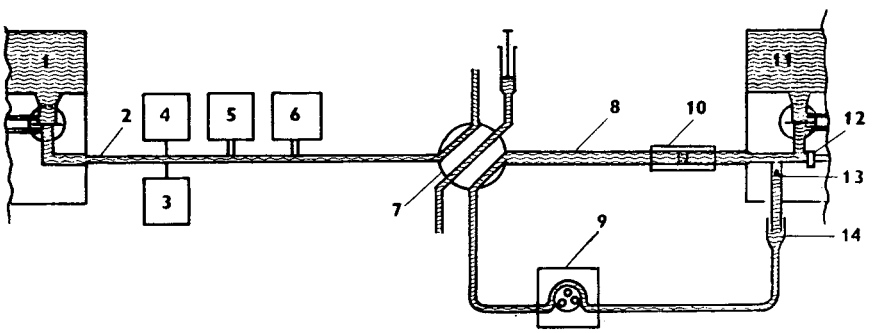

Fig. 1. Scheme of isotachophoretic apparatus for analytic desorption during sample application. 1 = Leading electrolyte compartment; 2 = PTFE capillary of 0.45 mm I.D.; 3 = UV source, 254 nm (low-pressure mercury lamp); 4 = photometric detector (photomultiplier); 5, 6 = potential gradient detectors; 7 = six-way valve for application of sample (the middle bore connected to a syringe containing the leading electrolyte); 8 = PTFE capillary of 1 mm I.D.; 9 = peristaltic micropump; 10 = adsorption element; 11 = terminating electrolyte compartment; 12 = PTFE-coated silicon rubber septum; 13 = outlet valve (open); 14 = solution reservoir.

The driving current is supplied by a high-voltage source of current stabilized over the range of 2—500 μA and 0.2—30 kV, manufactured in the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. The source is equipped with a low-voltage outlet and with a recorder of output voltage and current.

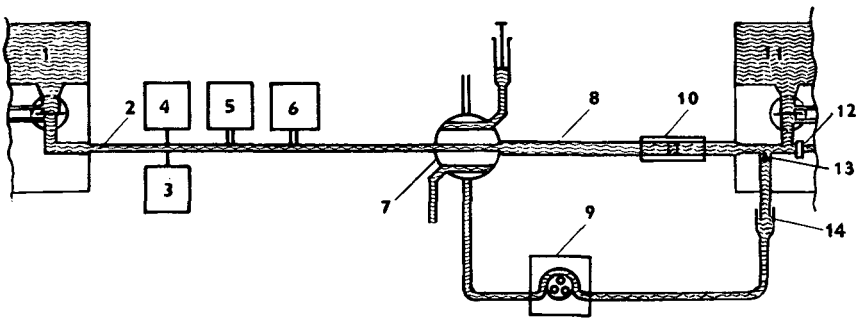

Fig. 2. Scheme of isotachophoretic apparatus for analytical desorption during separation; the designation of the functional elements is the same as in Fig. 1. The leading electrolyte compartment 1 represents the cathodic part of the system under conditions of cationic isotachophoretic separation; the peristaltic pump circuit 9 is disconnected and the outlet valve 13 is closed during isotachophoresis.

Adsorption elements

Four different models of adsorption elements shown in Fig. 3A—D were manufactured. The simplest element A represents a segment of a PTFE capillary of 1 mm I.D. and 1.4 mm O.D. with a porous polypropylene disc (Vyon, Porvair Ltd., Norfolk, Great Britain), 1 mm thick. Th suspension of the affinity adsorbent is placed on the disc, allowed to sediment, and subsequently the capillary is closed up also at the other side by another porous polypropylene disc. In the apparatus the extending terminal parts of the PTFE capillary are inserted into two PTFE connectors (not shown in the figure) and the position of the element is thus fixed.

Element B (Fig. 3B), more suitable for larger adsorbent volumes, is composed of a polyethylene tube of 3 mm I.D. and 5 mm O.D., provided with a porous polypropylene disc; the suspension of the adsorbent is placed on the latter, allowed to sediment and another disc is then used as a partition. The outside space beyond the disc is closed up by conically tapered tips limiting the effect of electric shading. If the particle size of the microgranular adsorbent is too small and the adsorbent particles are likely to penetrate through the porous discs, the adsorption element (model C, Fig. 3C) is covered with a microporous membrane of cellulose acetate (such as, for example, Membranfolien zur Elektrophorese, Membranfiltergesellschaft GmbH, Göttingen, G.F.R.), fixed in place by another polypropylene disc. The membrane will allow for the flow during the adsorption, but will not, however, let through the fine adsorbent of particle size around 3 μm. If it is necessary to change the volume of the affinity adsorbent, model D (cf. Fig. 3D) with removable PTFE tips is used to advantage. These tips serve as grooves for polypropylene porous discs. The disc diameter is in this case 1 mm smaller than the I.D. of the polyethylene tube. Table I shows the characteristics of the individual variants of the adsorption elements.

Procedure

The procedure of adsorption, electrodesorption, and isotachophoretic

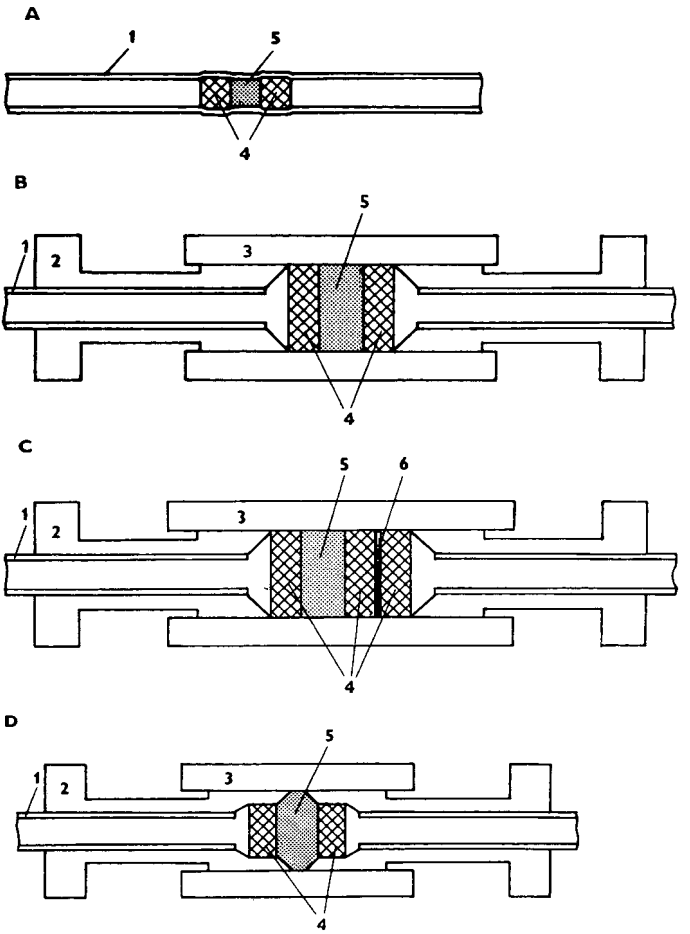

Fig. 3A—D. Scheme of adsorption element, models A—D: 1 = PTFE capillary of 1.0 mm I.D.; 2 = PTFE connector outlet; 3 = polyethylene tube of 3 mm I.D.; 4 = porous polypropylene disc; 5 = microgranular affinity adsorbent; 6 = cellulose acetate microporous membrane for electrophoresis (placed in the system closer to the outlet valve).

analysis of desorbed ligate consists of the following operations:

(1) Washing of affinity adsorbent in the adsorption element in the apparatus by the leading electrolyte (a volume of ca. 1 ml is used for model A whereas ca. 2 ml are necessary for models B, C, and D). Establishment of the boundary between the leading and the terminating electrolyte at the site of the outlet valve of the terminating electrolyte; closing of the valve.

(2) Blank electrodesorption—isotachophoretic analysis of the leading and terminating electrolyte with the adsorption element containing the void adsorbent to check on the possible leakage of the ligand from the adsorbent in the electric field.

(3) Filling. The application valve is turned to the filling position (cf. Fig. 1), the outlet valve is opened and the solution of the ligate is pumped by a peristaltic pump through the application valve to the adsorption element and back to the pump.

TABLE I

CHARACTERISTICS OF ADSORPTION ELEMENTS

The alphabetic designation corresponds to the models shown in Fig. 3A—D.

Type	Size of porous polypropylene discs (mm)		Volume of sorbent (mm^3)	Width of sorbent layer	Electrical screen
	Diameter	Distance			
A	1.0	1.0	0.8	Constant	None
		1.5	1.2		
		2.0	1.6		
B, C	3.0	1.0	7.1	Constant	Small
		1.5	10.6		
		2.0	14.1		
D	2.0	1.0	6.1	Variable	Medium
		1.5	9.6		
		2.0	13.2		

(4) The application stopcock is turned to "separation", the adsorption element is washed by the leading electrolyte and the boundary between the leading and the terminating electrolyte is established at the site of the outlet valve.

(5) Connection of the apparatus to the current source and electrodesorption

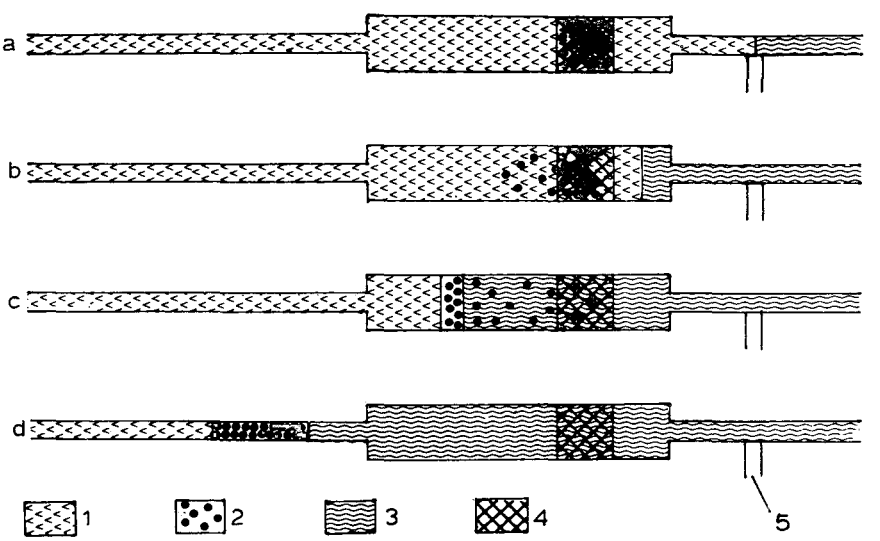

Fig. 4. Scheme of electrodesorption under conditions of isotachophoresis: a = before the start of isotachophoretic electrodesorption; b = initial stage — electrodesorption analogous to electrodesorption in a homogeneous electrolyte; c = formation of zone of desorbed ligate after passage of the terminating electrolyte boundary through the adsorption element; d = final stage — the ligate desorbed migrates in steady-state through a thin capillary to the detection system. 1 = Leading electrolyte; 2 = ligate; 3 = terminating electrolyte; 4 = adsorption element; 5 = outlet valve (closed).

of ligate under isotachophoretic conditions. The individual stages of the desorption are shown in Fig. 4. The course of the desorption was checked by recording the voltage of the stabilized current source (cf. Fig. 5).

(6) Isotachophoretic analysis of desorbed antibody; record of the passage of the individual zone boundaries through the detection system at decreased values of current passing through the apparatus.

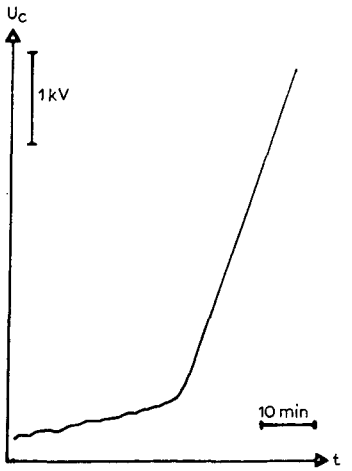

Fig. 5. Time profile of voltage of the high-voltage source of stabilized current. Inflection of the slope of the recorded curve indicates the passage of the boundary of terminating electrolyte or ligate from the 1-mm capillary to the application stopcock.

RESULTS AND DISCUSSION

The capillary isotachophoretic analysis of the MMAbT, carried out in electrolyte system I (see Table II), is documented by the record in Fig. 6 showing additional experimental data. Fig. 7 documents the result of isotachophoretic analysis of the desorbed MMAbT by electrodesorption in electrolyte system I. The quantity of the desorbed antibody, i.e. the ratio of MMAbT

TABLE II

COMPOSITION OF ELECTROLYTE SYSTEMS I AND II FOR ISOTACHOPHORETIC DESORPTION OF MMAbT FROM INSOL-TRANSFERRIN

BALA = β-alanine, EACA = ε-aminocaproic acid, MES = 2-(N-morpholino)ethanesulfonic acid, PVA = polyvinyl alcohol (Mowiol).

System number	Leading electrolyte				Terminating electrolyte		
	Leading ion (mol l^{-1})	Counterion	pH	Additive	Terminating constitutent (mol l^{-1})	Counterion	pH
I	K^+ 0.01	CH_3COO^-	5.2	PVA 0.02%	BALA 0.01	CH_3COO^-	5.0
II	K^+ 0.01	MES^-	5.9	—	EACA 0.01	Cl^-	5.2

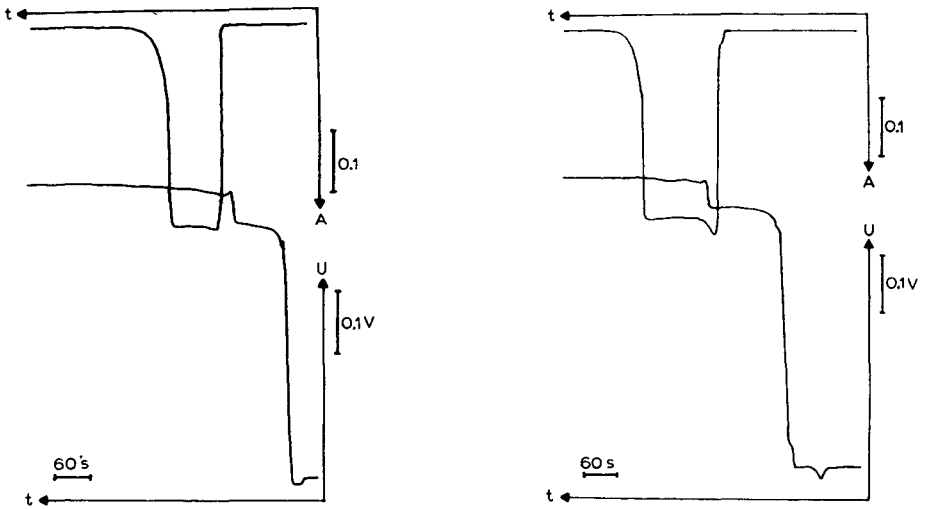

Fig. 6. Control analysis of ligate (isotachophoretic standard of MMAbT) in the absence of the adsorption element in electrolyte system I (see Table II). Sample, 5 μl of 1% solution of MMAbT in phosphate-buffered saline containing 0.1% sodium azide applied via a septum; current intensity 50 μA, during detection 20 μA; temperature 20°C.

Fig. 7. Isotachophoretic analysis of on-line desorbed fraction of MMAbT with binding affinity in electrolyte system I. Sample: 20 μl of 1% MMAbT in phosphate-buffered saline containing 0.1% sodium azide, made up to 1 ml with the leading electrolyte of system I, adsorbed at pump flow-rate of 2 ml h^{-1} for 2700 sec; adsorption element B; volume of affinity adsorbent 10 μl; length of 1-mm capillary 100 mm; time of electrodesorption 1 h at current intensity of 50 μA; detection at current intensity of 20 μA; capillary temperature in thermostated bath 20°C.

showing binding affinity for immobilized transferrin, was determined as follows. The length of the zone of a known quantity of antibody analyzed in the absence of the adsorption element (cf. Fig. 6) was compared with the length of the zone of the desorbed antibody (see Fig. 7), shorter by 10% because of decreased rate of zone migration due to changes in the electro-osmotic flow through the capillary in the presence of the adsorption element. The migration rate of the zone was determined as the ratio of the known distance between the potential gradient detector and the UV detector (15 mm in our apparatus) and the time during which the front boundary of the zone traveled over this distance at a current of 20 μA.

The quantity of the antibody desorbed was determined in this manner in two electrolyte systems (see Table III), in the B, C, and D adsorption elements (see Table IV), and also at various flow-rates during adsorption in element B (Table V).

The quantity of the unadsorbed fraction MMAbT in the sample solution after its passage through the adsorption element was determined colorimetrically by the method of Lowry et al. [18] and was a few per cent lower than the quantity of unadsorbed MMAbT determined as the difference between the quantity of MMAbT applied and desorbed. The low result can be accounted for both by the experimental error of the colorimetric determination of the un-

TABLE III

QUANTITY OF MMAbT WITH BINDING ACTIVITY IN ELECTROLYTE SYSTEMS I AND II

Conditions: pump flow-rate 2 ml h^{-1} during adsorption; time of circulation of 1-ml sample 2700 sec; for composition of sample see legend to Fig. 7; adsorption element B.

Electrolyte system	Applied amount of protein (μg MMAbT)	Desorbed amount of protein (μg MMAbT)
I	200	64—70
II	200	60—64

TABLE IV

QUANTITY OF DESORBED MMAbT IN VARIOUS MODELS OF ADSORPTION ELEMENTS IN ELECTROLYTE SYSTEM I

Conditions: flow-rate 2 ml h^{-1} during adsorption; adsorbent volume 10 ± 0.5 mm^3; adsorption time 2700 sec; for composition of sample see legend to Fig. 7.

Adsorption element type	Applied amount of protein (μg MMAbT)	Desorbed amount of protein (μg MMAbT)
B	200	64—70
C	200	66—68
D	200	62—64

TABLE V

QUANTITY OF DESORBED PROTEIN OF MMAbT (FRACTION WITH BINDING ACTIVITY) AS A FUNCTION OF FLOW-RATE UNDER CONDITIONS OF ADSORPTION IN ELECTROLYTE SYSTEM I

Adsorption element B; for composition of sample see legend to Fig. 7.

Peristaltic pump volumetric flow (ml h^{-1})	Adsorption element linear flow (μl mm^{-2})	Adsorption time (sec)	Applied amount of protein (μg MMAbT)	Desorbed amount of protein (μg MMAbT)
2	278	2700	200	70.0
8	1110	675	200	65.0
16	2220	338	200	27.6

adsorbed fraction of MMAbT and by irreversible adsorption which can never be eliminated completely.

The capacity of Insol-transferrin (reduced by $NaBH_4$) was determined as the quantity of antibody desorbed from a fully saturated adsorption element and represents 8.0 μg of MMAbT per μl of adsorbent.

The electrodesorption of MMAbT from Insol-transferrin in the isotachophoretic system of buffers, followed by isotachophoretic analysis of desorbed MMAbT, showed that the method of isotachophoretic desorption can be used for the desorption of ligates forming strong complexes with immobilized affinity ligands. Compared to the remaining electromigration methods which have been used for electrodesorption there are numerous advantages to the isotachophoretic procedure.

The experimentally determined steady-state concentration of electrophoretically desorbed MMAbT (3.2% in electrolyte system I and 4% w/v in electrolyte system II) is in good agreement with our earlier data [14] on the average steady-state concentration of polyclonal porcine immunoglobulin G and is, for example, ten times higher than the concentration of the sex hormone binding protein [10] obtained by electrodesorption in a homogeneous electrolyte. The concentration factor was 160 or 200 in our case if the original MMAbT concentration was 0.02%. Far more concentrated protein solutions can be obtained by isotachophoresis provided that there are no solubility problems; this factor is important from the viewpoint of future applications of the method. Another fact advantageous as regards these applications is that the antibody desorbed contains the counterion of the leading electrolyte only and there is no contamination with the carrier electrolyte or mixed ampholytes, a contamination which is observed when the electrodesorption is carried out by electrophoresis in a homogeneous electrolyte or by isoelectric focusing.

The conditions of isotachophoretic desorption (pH, ionic strength) must be chosen with respect to the stability of ligate and, if necessary, of ligand, and with respect to the solubility of the ligate. The deviation in pH of the terminating electrolyte zone in steady-state from the pH of the leading electrolyte can be calculated [19] and from these calculations the composition of the electrolyte system adjusted so that the values critical both from the viewpoint of ligate solubility and from the viewpoint of ligate and/or ligand stability may not be exceeded. The pH of the zone of desorbed ligate lies within the interval represented by the pH value of the leading electrolyte and the pH zone of the terminating electrolyte in steady-state. The calculated steady-state pH value of the leading electrolyte zone was 4.0 in system I and 5.0 in system II. System II [20] is more convenient as regards the stability of MMAbT whereas electrolyte system I is more advantageous from the viewpoint of the desorption rate. The electrodesorption process is most effective if the probability of the resorption of ligate, resulting from the decomposition of the complex, has been limited to a minimum. The probability of resorption can be decreased by decreasing the path of ligate ions in the adsorbent layer [6] and by increasing the migration rate of ligate ions in this layer [2]. The reduction of the path can be effected by a geometric arrangement of the adsorbent layer; a larger diameter and a thinner layer are more convenient. The increase of migration rate can be achieved by a proper choice of pH and ionic strength of the leading electrolyte and of the intensity of the electric field. As a rule, in common practice it is necessary to compromise and to choose the current density and the adsorbent cross-section to prevent thermal denaturation of ligate and/or ligand since the removal of Joule's heat is less efficient if the

cross-section is larger. Joule's heat was the critical factor when adsorption element A was used and the current density was 64 μA mm^{-2} : because of the small diameter of the adsorbent layer the thickness of the adsorbent had to be expanded to 10 mm in order that the properties of the individual adsorption element models could be compared. The isotachophoretic concentration of the antibody desorbed took place in the adsorbent layer. Hence, the specific conductivity in the adsorbent layer significantly decreased and thermal denaturation of the antibody occurred.

Our arrangement of the apparatus where the adsorption element of models B, C and D is connected in series to a capillary of 0.45 mm I.D. and 0.70 mm O.D. was used with relatively low current densities in the adsorbent layer, i.e. at 7 μA mm^{-2}. The maximal current densities in the adsorbent layer used by other authors are 100—250 μA mm^{-2} in adsorption elements without cooling and up to 1060 μA mm^{-2} in a cooled adsorption element [4]; the intensity of the electric field in this element did not exceed the value of 1.7 V mm^{-1}, whereas in our arrangement [21] these values go up to 5 V mm^{-1} similarly to the experiments of Haff [2] who showed that the intensity of the electric field is the decisive factor from the viewpoint of the efficiency of electrodesorption.

The time necessary for the desorption of MMAbT including its isotachophoretic analysis is 70—105 min. This period was controlled by the length of the capillary of 1 mm I.D. (see Figs. 1 and 2). The decrease in the desorption time results in a decrease in the quantity of desorbed ligate. More than 95% of MMAbT was liberated in 90 min.

The dependence of the quantity of MMAbT desorbed on the flow-rate of the sample solution during the adsorption (Table V) shows that the transferrin—MMAbT complex is kinetically very labile since a four-fold increase in the relatively high linear flow-rate resulted in a decrease of complex formation by only 7%.

If the quantity of the antibody is determined, it should be kept in mind that the size of the electroosmotic flow in the presence of the adsorption element is other than if this analysis is carried out in the absence of the adsorption element. The rate of zone migration is thus also different and the same real zone length may manifest itself in a different manner in the isotachopherogram. The rate of zone migration was in the individual cases 10—30% lower than the rate in the absence of the adsorption element. The zone length under the conditions of electrodesorption must therefore be corrected for zone length corresponding to the rate of zone migration in the absence of the adsorption element. If l_n is the uncorrected zone length, v_d the rate of zone migration under the conditions of electrodesorption, and v_a the rate of zone migration in the absence of the adsorption element, then the corrected zone length l_c can be expressed by the relation

$$l_c = \frac{v_d}{v_a} \cdot l_n$$

We used isotachophoretic desorption to check the quality of the Insol-transferrin adsorbent, i.e. its binding activity in the sense of its complex for-

mation with MMAbT. The determined capacity of this affinity adsorbent (8 $\mu g/\mu l$) is merely 2—3 times lower than the capacity of affinity adsorbent gels (mostly Sepharose derivatives) used by other authors for electrodesorption [2, 7]. The relatively high capacity of Insol-transferrin can be explained both by the high unit surface [22] of the polyethyleneglycol terephthalate carrier of the ligand [80—100 $m^2\ g^{-1}$) and also by the fact that the entire surface of the carrier particles is coated by the cross-linked antigen which, however, shows binding activity. The pellicular character of Insol-transferrin obviously contributes to the enhancement of the electrodesorption process since the electrodesorption rate is not limited by the ligate diffusion from the interior of adsorbent particles.

It appears that our on-line arrangement of qualitative isotachophoretic analysis may yield data on the specificity of sorbents of similar type and on the homogeneity of the ligate desorbed in the near future.

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GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION OF METOPROLOL FROM HUMAN PLASMA AFTER REACTION WITH PHOSGENE

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SUMMARY

A method for the determination of therapeutic levels of metoprolol in human plasma is presented. Metoprolol and the internal standard are extracted from the buffered plasma sample to an organic phase containing 4×10^{-3} *M* phosgene. After 10 min the organic phase is taken to dryness. The residue is dissolved in ethyl acetate and the formed oxazolidine derivatives are analyzed by gas chromatography with nitrogen-selective detection.

With packed columns, rectilinear standard curves through the origin were obtained down to 80 nmoles/l of plasma. The precision of the method at 200 nmoles/l was 1.5% ($n = 8$).

The sensitivity of the method was improved by using capillary column gas chromatography. Linear standard curves were obtained down to 10 nmoles/l of metoprolol in plasma. The precision of the method at the 50 nmoles/l level was 2.2% ($n = 7$).

With this simple and straightforward method using extractive derivatization 30 samples can be handled in a day.

INTRODUCTION

In addition to pharmacokinetic studies, the monitoring of plasma levels of β-blocking drugs is of a certain importance in drug compliance and toxicological cases. The most common method used is gas chromatography with electron-capture detection of perfluoroacyl derivatives [1, 2]. In most instances it is possible to perform the derivatization after a simple extraction without any extra purification steps. However, due care has to be taken to ensure reliable derivatization. The quality of the perfluoroacyl reagents may vary from batch to batch, sometimes resulting in low yield or extraneous peaks. Besides, the risk of hydrolysis can never be ruled out completely. Recently, it has been demonstrated that this type of derivative exhibits instability on certain capillary columns [3].

Phosgene is widely used in industry for the production of a great number of

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chemical compounds in large quantities. It is commonly used in organic synthesis, normally under anhydrous conditions [4—6]. The use of phosgene as a derivatizing agent in gas chromatographic analysis is rare [7]. A reverse procedure exists [8], however, for the trapping and determination of phosgene formed metabolically from chloroform [9] and carbon tetrachloride [10] with cysteine. The formed oxathiazolidine carboxylic acid is isolated and methylated with diazomethane prior to quantitation by gas chromatography—mass spectrometry.

Our interest in phosgene as a potential derivatizing agent for amino alcohols originated from the use of chloroformates in aqueous media [11, 12] and the use of boronates as cyclization reagents [13]. As phosgene is slowly hydrolyzed by water [14, 15] it looked promising as a derivatizing agent in an aqueous environment.

In the present method, for the β-blocking drug metoprolol in plasma, extraction and derivatization with phosgene are performed simultaneously. After evaporation of the organic phase the residue is analyzed by gas chromatography with nitrogen-selective detection.

EXPERIMENTAL

Gas chromatography

A Varian 3700 gas chromatograph with flame ionization and thermionic (nitrogen—phosphorus-selective) detectors was used equipped with 120 × 0.2 cm I.D. glass columns. These were filled with 3% OV-17 on 100—120 mesh Gas-Chrom Q or 3% Hi-EFF-8BP (= cyclohexanedimethanol succinate) on the same support. Gas flow-rates of nitrogen carrier gas were 30 or 45 ml/min. The thermionic detector was supplied with 5 ml of hydrogen and 175 ml of air per min. The detector bead current setting was in the range 400—500. The injector, oven and detector were maintained at 250°C, 240°C and 300°C, respectively.

Capillary column gas chromatography was performed in the same instrument by the aid of stainless-steel adaptors for split effluent and make-up gas [16]. A soda glass column (7 m × 0.26 mm I.D.) coated dynamically with Carbowax 20M was used [17]. The inlet pressure of helium carrier gas was 150 kPa. The carrier gas flow-rate was 7 ml/min and that of the split effluent 20 ml/min (both measured at 150°C). The flow-rate of helium make-up gas was 20 ml/min. The temperature of the injector and the detector were as above. Samples were injected with the split valve closed at a column temperature of 150°C. After 0.8 min the split valve was opened and after 1 min the column temperature was increased to 240°C at 30°C/min.

Mass spectrometry

A Varian MAT-112 mass spectrometer coupled to a Varian 1400 gas chromatograph was used. The glass column was filled with the same OV-17 packing as above. Helium was used as carrier gas at a flow-rate of 20 ml/min. After injection and venting of the solvent, the slit valve to the ion source was opened manually to a pressure of 2×10^{-5} Torr. The temperatures were 260°C (oven) and 250°C (injector, transfer line and ion source). The electron energy was

70 eV and the emission current 1.5 mA.

Liquid chromatography

The system consisted of a pump (Altex 110A), an injection valve (Rheodyne 7010) fitted with a sample loop (250 μl), a 150 × 4.5 mm stainless-steel column filled with 5 μm LiChrosorb RP-8 and a Cecil 212 variable-wavelength ultraviolet (UV) monitor. The mobile phase was 0.01 *M* phosphoric acid in 60% methanol (metoprolol oxazolidine) and 0.01 *M* of tetrabutylammonium hydrogen sulphate in aqueous buffer pH 2 (μ = 0.1) with 9% of acetonitrile (metoprolol). The flow-rate was 1 ml/min and the UV absorption of the eluate was measured at 276 nm.

Liquid scintillation

The radioactivity measurements of the fractions containing radioactive metoprolol and derivative from the liquid chromatography eluate were performed by liquid scintillation counting. Insta-Gel^R (Packard) was added to the samples before counting on a Mark III spectrometer (Searle Analytical Instruments). The quenching was corrected for by external standardization.

Reagents and chemicals

Phosgene 2 *M* in toluene, purum, was from Fluka (Buchs, Switzerland), methylene chloride and ethyl acetate p.a. from Merck (Darmstadt, G.F.R.), acetonitrile and hexane HPLC grades were from Rathburn Chemicals (Walkerburn, Great Britain).

Metoprolol, (1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy-2-propanol]-tartrate), H 87/31 (internal standard, 2-ethoxymethyl), H 93/47 (internal standard for electron-capture gas chromatography, ethoxyethyl) and tetrabutylammonium hydrogen sulphate were synthesized at AB Hässle (Department of Organic Chemistry).

3-Isopropyl-5-[4-(2-methoxyethyl)phenoxymethyl]oxazolidine-2-one, the oxazolidine derivative of metoprolol, was synthesized from metoprolol base dissolved in pyridine and a one-fold excess of phosgene in toluene. After standing overnight, excess phosgene was destroyed by the addition of water. The toluene phase was washed twice with 1 *M* hydrochloric acid before evaporation. The crystalline residue was recrystallized with boiling isopropyl ether. The m.p. was 81—82°C. Büchi 2°C/min.

Tritiated metoprolol (AB Hässle) was at a concentration of 2.5 μM in distilled water (250,000 dpm/ml).

n-Octacosane from Fluka and trichloroethyl carbamate of dibenzylamine [11] were used as non-reacting internal standards.

Sodium phosphates and carbonates were used for the preparation of aqueous buffers (μ = 1).

METHODS

Determination of metoprolol in plasma

Plasma (2 ml or 2 g) and 1 ml of buffer pH 8.1 with 900 n*M* of the internal standard H 87/31, 5 ml of the extraction solvent (hexane—methylene chloride,

4:1) and 10 μl of 2 *M* phosgene in toluene were shaken horizontally for 10 min (160 rpm). After centrifugation the organic phase was transferred to a 5-ml centrifuge tube, either after freezing of the aqueous phase (dry ice and acetone) or by using a pipette. The organic solvent was removed by evaporation with a stream of nitrogen. The residue was dissolved in 25—50 μl of ethyl acetate and 1—2 μl was taken for analysis by gas chromatography on 3% Hi-EFF-8BP with nitrogen-selective detection.

Samples for analysis with capillary column gas chromatography were prepared by the same procedure except that 1 ml of plasma was taken and that the concentration of the internal standard was 225 n*M*. Of the final 25-μl solution 1 μl was injected into the gas chromatograph equipped with a Carbowax 20M capillary column and nitrogen-selective detection.

Electron-capture gas chromatography of metoprolol from plasma

Plasma (2 g), 0.3 ml of 1 *M* sodium hydroxide and 100 μl of the internal standard, 7.29 μ*M* (H 93/47), were extracted for 10 min with 5 ml of hexane—methylene chloride (4:1). After centrifugation and freezing of the aqueous phase the organic phase was decanted to a new tube and mixed with 200 μl of trifluoroacetic anhydride. The reaction was allowed to proceed for at least 45 min at 40°C before evaporation with nitrogen. The residue was dissolved in 200 μl of toluene and a 3-μl aliquot was analyzed by gas chromatography on 3% OV-17.

RESULTS AND DISCUSSION

Identity of derivative formed

The derivative formed by the reaction of metoprolol with phosgene was identified by gas chromatography—mass spectrometry. The structure and the mass spectrum are presented in Fig. 1. Prominent ions are *m/z* 100 and 56. The former is formed by the loss of isopropyl from *m/z* 142 which is followed by the elimination of carbon dioxide to give *m/z* 56. The latter ion is also dominant in the fragmentation of cyclic boronates of alprenolol [18]. The ions

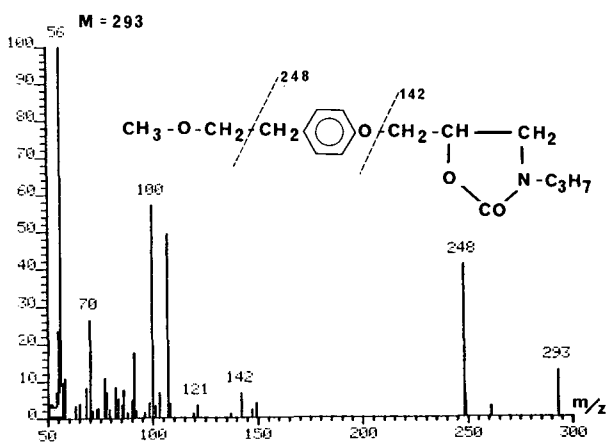

Fig. 1. Mass spectrum of the phosgene derivative of metoprolol at 70 eV.

in the higher mass range have sufficient relative intensity to be promising for mass fragmentography.

Reaction conditions

pH of the aqueous phase. Initially the derivatization reaction with phosgene was carried out in aqueous buffer without plasma or organic phase present. A constant yield of the derivative was obtained in the pH range 7.5—12.1 as shown in Table I. A buffer of pH 8.1 was selected as a suitable medium for the reaction.

TABLE I

INFLUENCE OF pH ON THE DERIVATIZATION OF METOPROLOL WITH PHOSGENE IN AQUEOUS MEDIA

Method: 1 ml of 1 mg/ml metoprolol in 0.01 *M* hydrochloric acid was mixed with 1 ml of buffer (μ = 1) and the pH was measured. Then 50 μl of 2 *M* phosgene in toluene were added and the mixture was vibrated for 30 sec and allowed to stand for 5 min before extraction with 5 ml of methylene chloride containing 1 mg of *n*-octacosane. Then 0.5 ml of the organic phase was taken to dryness and dissolved in 50 μl of ethyl acetate before analysis by gas chromatography with flame ionization detection.

Starting pH	Relative yield (%)	Final pH
6.2	28	4.9
6.6	69	6.0
7.0	87	6.2
7.5	99	6.5
8.1	103	6.6
8.3	101	6.7
9.7	93	7.7
10.4	98	9.5
12.1	99	11.1

Presence of plasma. When plasma was present in the reaction mixture the yield of the derivative was reduced relative to that obtained when buffer only was the reaction medium. This may be due to the inclusion of metoprolol or the derivative in the protein precipitate formed by the rather vigorous shaking conditions used at this stage. The problem could be circumvented if the extraction solvent was present before the addition of phosgene. The yield of the derivative was then the same as the aqueous reference. An advantage with this procedure is also that less handling of the sample is required when derivatization and extraction are performed simultaneously, so called extractive derivatization (cf. extractive alkylation, acylation or dealkylation).

Partition properties of the metoprolol derivative. The distribution ratio of the oxazolidine derivative of metoprolol is independent of pH. It was found to be 2.3 in the system hexane—buffer pH 8.1 (μ = 1) with equal phase volumes. For the intact compound this ratio is 1.3 at pH 11 (pK_a + 1.3, negligible amounts of ionized metoprolol present). This means that only a slight increase in the lipophilic character is obtained by derivatization with phosgene. The

practical implication of this is that only 92% of the derivative will be recovered in the hexane phase if a phase ratio of 5:1 is used. By including 20% of methylene chloride in the hexane phase less than 1% of the derivative could be retrieved from the aqueous phase when this was subjected to a new extraction.

Amount of phosgene. The amount of phosgene required for a constant yield in the two-phase system at pH 8.1 is illustrated in Table II. The yield of derivative is constant from 5 to 20 μl of 2 *M* phosgene added; 10 μl was selected as a suitable amount. The molar excess of phosgene was approximately 10 in this study (C_0 metoprolol 8×10^{-4} *M*). At lower concentrations of the substrate the excess of the reagent will be considerable. Plasma constituted 25% of the aqueous phase in these experiments and appears to have no detrimental effect on the yield when compared with the aqueous reference samples (Table II). It was later found that plasma up to 67% of the aqueous phase had no harmful effect on the yield (0.4 μmoles/l of metoprolol in plasma).

Internal standard. An analogue of metoprolol, H 87/31, was selected as a suitable internal standard. The only structural difference is that of the position of the ether oxygen in the short side-chain. With 3% Hi-EFF-8BP as stationary phase it elutes just before metoprolol (Fig. 2).

Reaction time. When the time course for the reaction of phosgene with metoprolol and the internal standard was followed in the two-phase system with plasma present, a constant peak height ratio was obtained from 5 min up to 2 h. A shaking time of 10 min was used hence-forward.

TABLE II

AMOUNT OF PHOSGENE REQUIRED FOR THE DERIVATIZATION OF METOPROLOL IN A TWO-PHASE SYSTEM WITH PLASMA PRESENT

Method: 0.5 ml of buffer pH 8.1, 1 ml of metoprolol 0.46 mg/ml in 0.01 *M* hydrochloric acid and 0.5 ml of plasma were mixed and shaken together with 5 ml of hexane—methylene chloride (4:1) and phosgene as indicated in the table. The organic phase also contained 106 μg/ml of the trichloroethyl carbamate of dibenzylamine as marker. After 5 min shaking a 0.5-ml aliquot was withdrawn and evaporated. The residue was dissolved in 50 μl of ethyl acetate and analysed by gas chromatography with flame ionization detection.

Amount of 2 *M* phosgene in toluene added (μl)	Relative yield (%)
20	100 ± 4*
20	98
20	99
15	97
15	98
10	97
10	98
5	97
5	100
2	88
2	82
1	78
1	72

*Aqueous reference, $n = 3$.

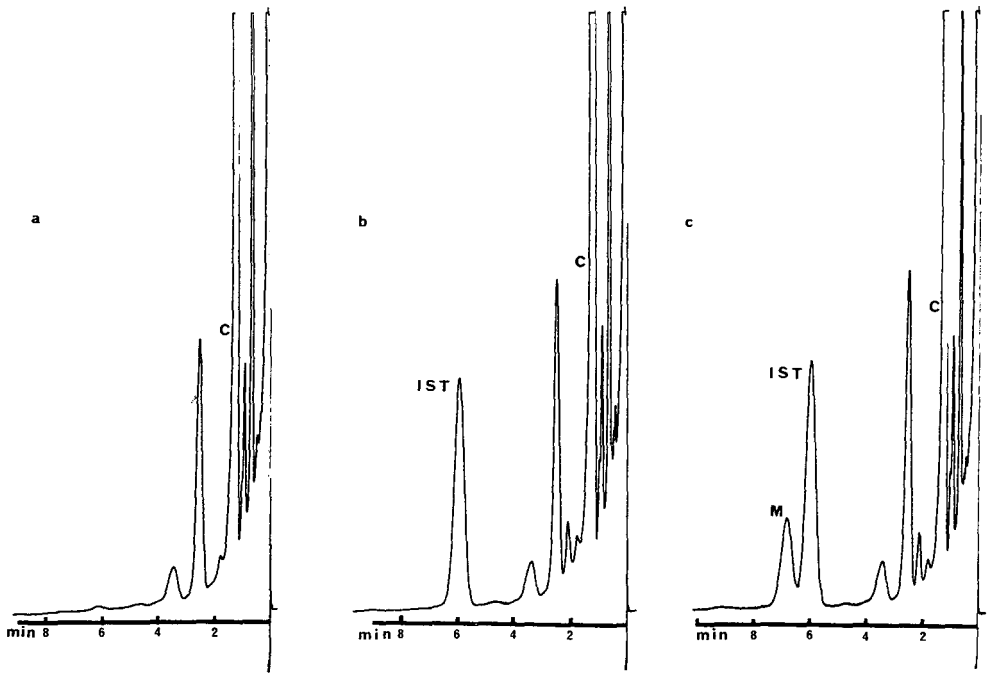

Fig. 2. Packed column gas chromatograms from the analysis of metoprolol in plasma. (a) Blank plasma. (b) Blank plasma and 900 pmoles of the internal standard. (c) As in (b) plus 200 nmoles/l of metoprolol. Column: 3% cyclohexanedimethanol succinate at 240°C; 2 μl injected (of 25 μl final volume) at attenuation 8. M = metoprolol, IST = internal standard, C = caffeine.

Stability of phosgene and the metoprolol derivative. In the presence of water phosgene is hydrolyzed to hydrogen chloride and carbon dioxide. The hydrolysis of phosgene in the two-phase system used was studied in the absence of metoprolol. At various time intervals aliquots of the organic phase were withdrawn and mixed with an excess of metoprolol [19] and a known amount of the trichloroethyl carbamate of dibenzylamine as marker. The amount of derivative formed was taken as a measure of the amount of phosgene present. Without plasma present the half-life of phosgene was 4 min and with plasma present ca. 2 min. As the extrapolated initial concentration of phosgene from the plot coincided with that of the initial concentration before the aqueous phase was added, the conclusion is that phosgene is mainly present in the organic phase. From the calculated half-life with plasma present, 2 min, phosgene can be expected to be present in ca. 3% of the initial concentration after 10 min. The corresponding amount is equal to the threshold limit value for phosgene if the organic phase is completely evaporated in 100 l of air. This fact emphasizes that the sample tubes should be handled in a vented hood, and that the reaction time should not be reduced, in order to prevent the risk of exposure to phosgene.

The stability of the oxazolidine derivatives was investigated shaking equal phase volumes of organic phase and 0.1 *M* of sulphuric acid or sodium hydroxide as the aqueous phase. The initial concentration of the derivatives

was 1 μM and a suitable amount of the trichloroethyl carbamate of dibenzylamine was added as marker. At various time intervals aliquots were withdrawn and analysed by gas chromatography after evaporation and reconstitution in a small volume of ethyl acetate. No degradation of the derivatives could be observed for up to 8 h.

Standard curves, precision and absolute recovery

The reaction conditions were optimized using milligram amounts of metoprolol and gas chromatographic analysis with flame ionization detection and OV-17 as stationary phase. When the amounts injected were reduced a hundredfold, and the nitrogen-selective detector was used, severe peak broadening was noted. Some more polar silicone stationary phases were tested but without success (QF-1, OV-225 and Silar 5 CP). The peak symmetry on the cyanosilicone phases was adequate but the bleeding excluded the use of these phases in combination with the nitrogen-selective detector. The polyester phase cyclohexanedimethanol succinate (Hi-EFF-8BP) proved to be suitable for the analysis of nanogram amounts of the oxazolidine derivatives on column. Symmetrical peaks (Fig. 2) and acceptable precision was obtained when 5-ng amounts were injected repeatedly and quantitated with an inert internal standard (S.D. $< 3\%$, $n = 6$).

Linear standard curves obtained from 800 to 80 nmoles/l of plasma (213—21 ng/ml) passed through the origin. The precision at the 200 nmoles/l level was 1.5% ($n = 8$).

The absolute recovery of metoprolol as oxazolidine derivative in the organic phase was 85% as determined with the synthesized material as reference. This value was supported by liquid chromatography and liquid scintillation counting.

Selectivity of the present method

Actual plasma samples from patients on metoprolol therapy were analysed by the present method and the electron capture gas chromatography method [2] used for routine determinations of metoprolol in our laboratories. The results of the comparison are shown in Fig. 3. The latter method tends to give somewhat higher values.

Interferene from metoprolol metabolites is not likely. The major metabolites have alcoholic hydroxyl groups beside the intact β-sidechain [20] or the β-sidechain oxidized to a carboxylic group [20]. The alcoholic metabolites are less probable to interfere as they require further derivatization prior to gas chromatography, e.g. trimethylsilylation of the alcohol group [21]. The carboxylic acid metabolite is not likely to cyclize with phosgene under the present conditions.

The secondary amine 1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-propane (metoprolol devoid of the alcohol group) does also react with phosgene. The formed chlorocarbonyl derivative (identified by mass spectrometry) could only be chromatographed at low levels after treatment with methanol, that is after conversion to its methyl carbamate derivative. Thus, interference from moderate levels of amines is not likely either. This shows clearly the advantage of selective cyclic derivative formation [7].

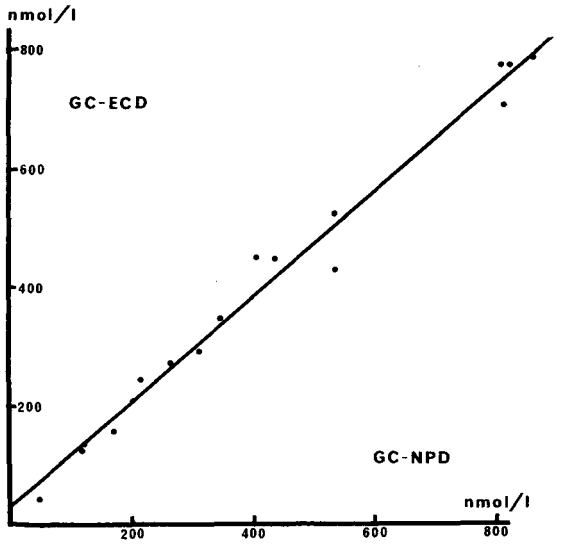

Fig. 3. Comparison between the trifluoroacetylation method and the phosgene derivatization method for metoprolol. GC—ECD = gas chromatography with electron-capture detection after trifluoroacetylation; GC—NPD = gas chromatography with nitrogen-selective detection after reaction with phosgene. Linear regression = 0.991, slope = 0.877, intercept = 31 nmoles/l, number of samples = 17.

The present derivative of metoprolol has a five-membered ring structure. Also, six- and seven-membered rings can be formed and chromatographed as was demonstrated with two metoprolol homologues with one or two extra methylene groups inserted between the amino and the hydroxy group [3], identified by mass spectrometry.

Derivatization of some other β-blocking drugs

The relative retention of some other β-blocking drugs after phosgene derivatization is shown in Table III. The table shows the potential of this method for the determination of other drugs of this type. Some of the more structurally complex β-blocking drugs gave two peaks in the chromatograms, e.g. pamatolol and timolol. The poor symmetry of the atenolol peak could be improved by derivatization of the amide moiety to the corresponding nitrile by trifluoroacetic anhydride [22]. The derivatization and extraction conditions were not optimized in any of these experiments. Gas chromatography with mass spectrometry was used to confirm the structures of the formed derivatives.

Interferene from caffeine

Some plasma samples from patients contained high concentrations of caffeine (> 1 μg/ml). This resulted in an overload of the chromatographic system, as confirmed by injecting corresponding high amounts of pure caffeine. In the present chromatographic system this interference did not affect the derivative peaks. The position of caffeine in a normal chromatogram is indicated in Fig. 2. With the actual organic phase less than 10% of the caffeine present in the aqueous phase is extracted to the organic phase.

TABLE III

RELATIVE RETENTION OF SOME β-BLOCKING DRUGS AS OXAZOLIDINE DERIVATIVES

Compound	Relative retention time on	
	3% OV-17	3% Hi-EFF-8BP
Alprenolol	0.47	0.40
Oxprenolol	0.64	0.56
H 87/31 (internal standard)	—	0.87
Metoprolol	1.00*	1.00**
Propranolol	1.67	2.12
Pindolol	2.83	7.42

*Absolute retention time: 1.8 min at 240°C.
**Absolute retention time: 6.8 min at 240°C.

Application to capillary column gas chromatography

The sensitivity of the present method was improved by using capillary column gas chromatography. The oxazolidine derivatives chromatographed without any sign of decomposition or adsorption. A chromatogram with 9 ng of the metoprolol derivative is presented in Fig. 4a. The Carbowax 20M column

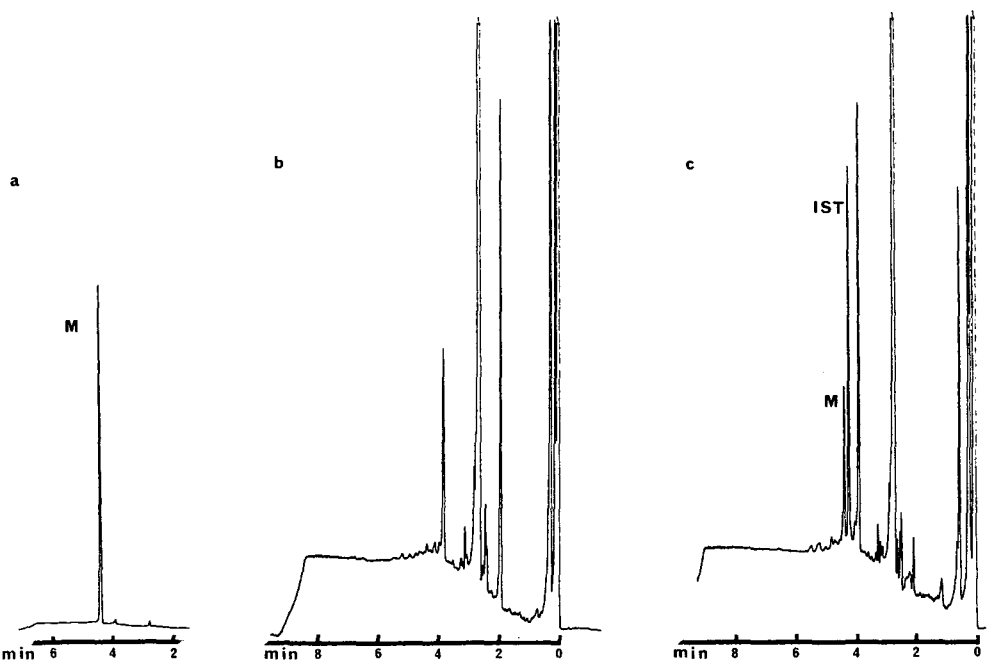

Fig. 4. Capillary column chromatograms from the analysis of metoprolol in plasma. (a) 9 ng of metoprolol derivative, attenuation 64. (b) Blank plasma, attenuation 8. (c) as (b) with 225 pmoles of the internal standard and 100 nmoles/l metoprolol, attenuation 8. Column: Carbowax 20M, 150°C for 1 min then 30°C/min to 240°C; 1 μl injected of the 25 μl final solution (b and c). M = metoprolol, IST = internal standard.

used in this study was not the only column capable of chromatographing these derivatives. A 30 m × 0.25 mm SE-30 fused silica column (DB-1, J and W Scientific) was also used without problem. This indicates an improved stability of these derivatives as compared with perfluoroacylated derivatives on capillary columns [3].

Linear standard curves for metoprolol were obtained down to 10 nmoles/l of plasma. The precision at the 50 nmoles/l level in plasma was 2.2% (n = 7). The presence of minute interfering peaks near that of metoprolol, and not the nitrogen-selective detector, limit the detection of lower levels of metoprolol. Chromatograms from blank plasma and plasma spiked with 100 pmoles of metoprolol and 225 pmoles of the internal standard per 1 ml of plasma are shown in Fig. 4b and c, respectively.

ACKNOWLEDGEMENTS

We are grateful to Dr. Anders Arfwidsson for synthetic work, to Mr. Lars Johansson for the capillary columns and the analysis of plasma metoprolol by electron-capture gas chromatography, and to Dr. Kurt-Jürgen Hoffmann for recording the mass spectra.

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CHROMBIO. 1543

PENTAFLUOROBENZOYL DERIVATIVES OF DOPING AGENTS

I. EXTRACTIVE BENZOYLATION AND GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION OF PRIMARY AND SECONDARY AMINES*

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SUMMARY

A sensitive and rapid method for the gas chromatographic (with electron-capture detection) confirmation of derivatizable sympathomimetic amines is described. Extractive derivatization with pentafluorobenzoyl chloride is performed on 2-ml urine or plasma samples. Especially for primary amines, the method appears to be very sensitive. Mass spectral data allowed confirmation of the monobenzoylation of all congeners.

INTRODUCTION

Various methods for the identification of doping agents in biological material have been presented during recent years [1—6]. Although it is well known that gas chromatography combined with mass spectrometry (GC—MS) is superior for the identification of drugs, it is relatively simple to prove or disprove the identity of a drug by cumulative analytical data. Therefore positive or suspected cases need to be confirmed on columns of different polarities after derivatization of the analyte. The present work reports on the derivatization

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with pentafluorobenzoyl chloride (PFBCl) of primary and secondary sympathomimetic amines by extractive benzoylation.

EXPERIMENTAL

Materials

The following amines were studied: *l*-amphetamine sulphate (SKF, Philadelphia, PA, U.S.A.), chlorphentermine hydrochloride (Tropon Werke, Köln, G.F.R.), cyclopentamine hydrochloride, dioxadrol (Cutter, Berkeley, CA, U.S.A.), ethylamphetamine hydrochloride, fencamfamine hydrochloride and its metabolite 2-amino-3-phenylnorbornane hydrochloride (Merck, Darmstadt, G.F.R.), fenfluramine hydrochloride (Servier, Orleans, France), mephentermine sulphate (Wyeth, Philadelphia, PA, U.S.A.), methylamphetamine hydrochloride (Merck), methylphenidate hydrochloride (Ciba, Basle, Switzerland), pentorexum (Nordmark Werke, Hamburg, G.F.R.), phacetoporane hydrochloride (Specia, Paris, France), phenmetrazine hydrochloride (Boehringer, Ingelheim, G.F.R.), phentermine hydrochloride and tranylcypromine sulphate (SKF). Stock solutions of these drugs were freshly prepared with double-distilled water.

Pentafluorobenzoyl chloride (PFBCl) was obtained from Aldrich Europe (Beerse, Belgium).

The triethanolamine—cyclohexane (CH—TEA) extraction solvent was prepared by briefly refluxing cyclohexane with small amounts of triethanolamine, cooling and separating the two phases.

The ammonium buffer was a saturated ammonium chloride solution adjusted to pH 9.4 with undiluted ammonia.

All glassware was silanized and the organic solvents (analytical grade) were freshly distilled before use.

Dilutions were made with a Hamilton diluter/dispenser.

Gas chromatography

A Varian 3700 gas chromatograph equipped with a ^{63}Ni detector and connected to a Varian CDS 111 integrator was used. The glass column (200 × 0.25 cm I.D.) was packed with 2% OV-225 on Chromosorb W AW DMCS, 80—100 mesh. Nitrogen was used as carrier gas at a flow-rate of 25 ml min^{-1}. Temperature was programmed from 165°C, 5 min hold-time, to 225°C at a rate of 5°C min^{-1}. The injector and detector temperatures were kept at 240° and 320°C, respectively. Chart speed was 20 cm/h.

Gas chromatography—mass spectrometry

Spectra were obtained with a Hewlett-Packard 5985B quadrupole instrument, equipped with a directly coupled fused-silica open tubular capillary column (OV-101, 10 m × 0.22 mm I.D.). Helium (70 cm sec^{-1}) was used as carrier gas. Injections were made via an all-glass "moving needle" system. Temperature settings were: injection port 290°C; oven temperature programmed from 140° to 240°C at 16°C min^{-1}; transfer line 250°C; source temperature 200°C. Spectra were taken under electron-impact conditions (70 eV).

Methods

Two millilitres of urine (or plasma) were made alkaline with 0.2 ml of NH_4^+/NH_4OH buffer and 6 ml of CH—TEA were added followed by 0.01 ml of PFBCl (0.5% in cyclohexane). Extractive benzoylation was performed during 5 min. The organic phase was evaporated under nitrogen (40°C) and the residue redissolved in 500 or 100 μl of ethyl acetate depending on the starting material (urine or plasma). One microlitre was injected into the gas chromatograph.

The lowest concentration which allowed comfortable detection ("routine detection limit") was determined by adding different volumes (0—100 μl) of several aqueous amine solutions to 2 ml of horse urine or plasma. The extraction was done as described and a 5-ml aliquot of the organic phase was used for evaporation.

The effect of the reaction time and the stability of some PFB derivatives was measured using tetrachlorodiphenylethane (TDE) as internal standard.

RESULTS AND DISCUSSION

After extraction, PFB derivatives of amines are generally made with trimethylamine as catalyst [7] or at elevated temperatures [8—10] during periods varying from 20 min to 2 h [7]. Moreover, after derivatization it is often necessary to remove the strongly interfering hydrolysis product (pentafluorobenzoic acid) of PFBCl by supplementary washings with alkali. We found that most of this reagent interference could be diminished by using minute amounts of PFBCl.

The extractive benzoylation with PFBCl as presented here combines extraction, derivatization and consumption of the reagent.

The addition of triethanolamine to the extraction solvent results in higher recoveries in contrast with cyclohexane alone.

Identity of the postulated derivatives was investigated by GC—MS under electron-impact (70 eV) conditions. Although only for some components was a weak molecular ion observed, the presence of diagnostic ions allowed confirmation of the monobenzoylation of all congeners.

The stability of the pentafluorobenzyloxonium ion (*m/z* 195), through electron sharing with the non-bonding orbital of the oxygen atom, was characteristic for all derivatives. This ion was either the base peak or very abundant for all derivatives. The ion at *m/z* 167 originated from further fragmentation with loss of neutral CO molecule.

The strong electron-donating ability of the nitrogen atom also provided abundant ions by α-cleavage with loss of the benzyl moiety, $[M - 91]^+$. For the piperidyl congeners this reaction was even more important; for example, phacetoperane gave a base peak at *m/z* 278.

Another typical fragment included McLafferty rearrangement with charge migration to the alkyl side-chain, the intensity reflecting the stability of the formed ion. For tranylcypromine this ion was the base peak (*m/z* 116), while for pentorexum this ion yielded only a relative intensity of 1.3% (*m/z* 146). Also for fencamfamine this type of fragmentation gave rise to an abundant ion (*m/z* 170, 86.1%).

Other ions were indicative of the cyclic structure of the congeners, e.g. *m/z* 91, *m/z* 77 for benzyl and *m/z* 115 for bicyclic congeners. Mass spectra and the fragmentation of tranylcypromine and chlorphentermine are given as illustrative examples in Figs. 1—4. Mass spectral data of all congeners are summarized in Table I, according to the major fragmentation pathways discussed.

The effect of the reaction time on the formation and stability of the PFB

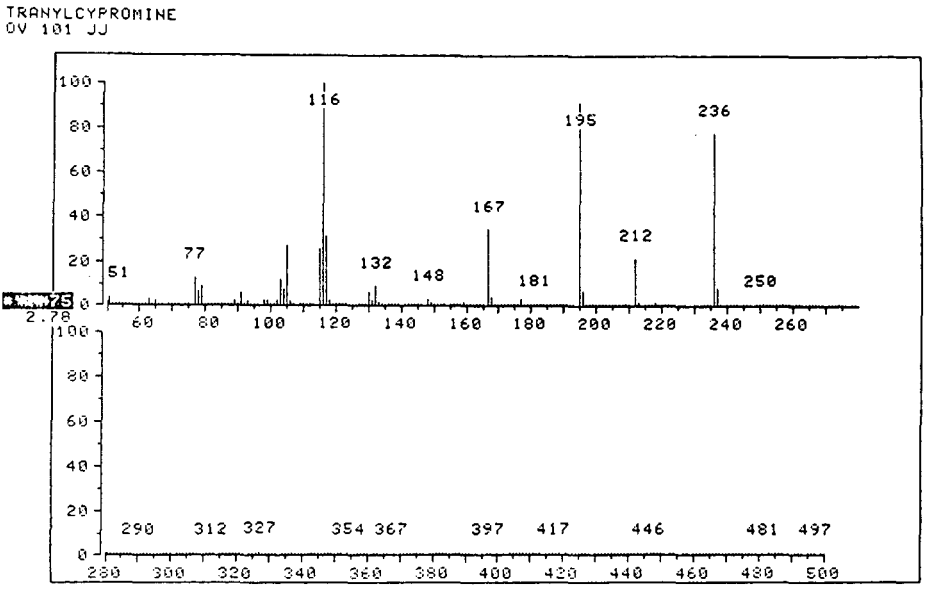

Fig. 1. Electron-impact mass spectrum of N-pentafluorobenzamide of tranylcypromine.

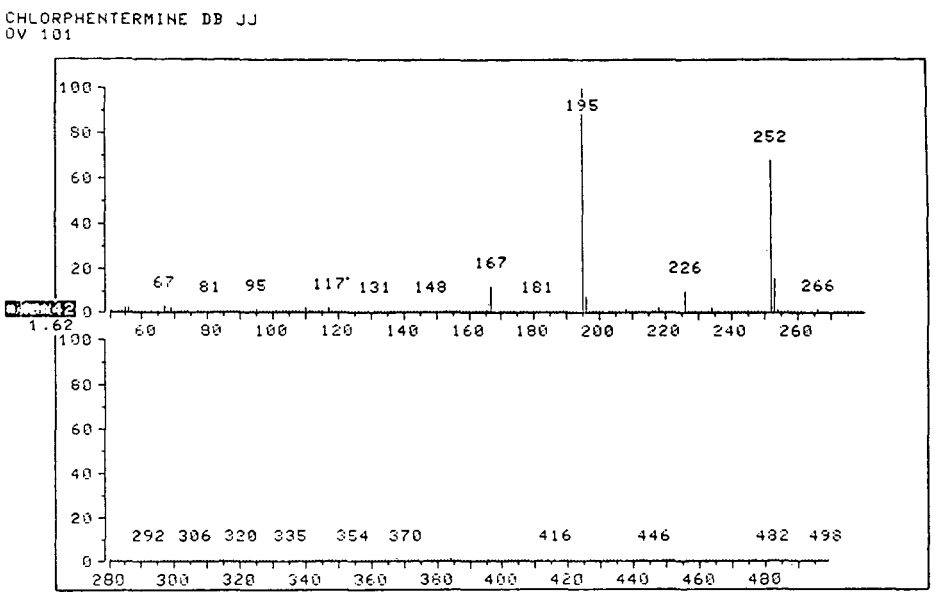

Fig. 2. Electron-impact mass spectrum of N-pentafluorobenzamide of chlorphentermine.

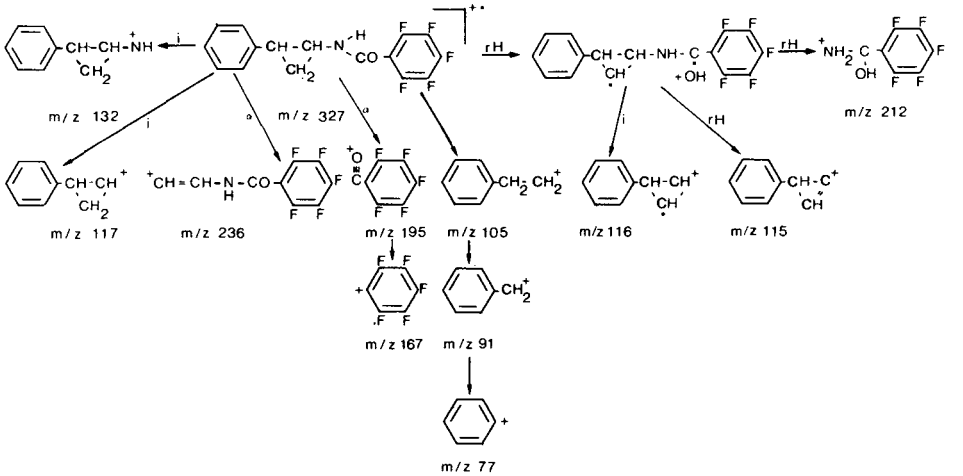

Fig. 3. Fragmentation scheme of N-pentafluorobenzamide of tranylcypromine.

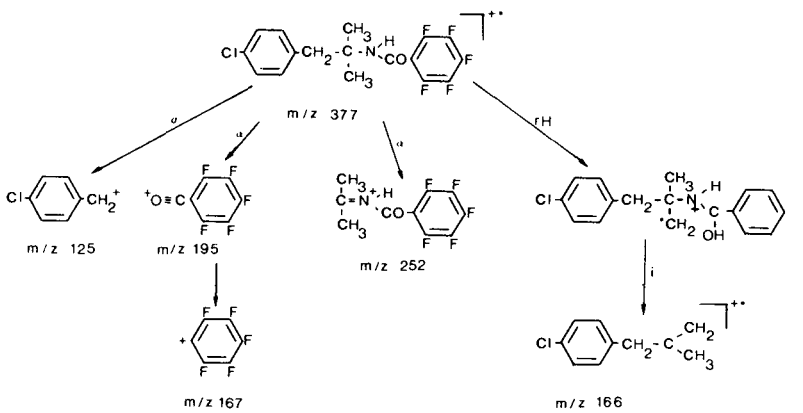

Fig. 4. Fragmentation scheme of N-pentafluorobenzamide of chlorphentermine.

derivatives of the primary amine amphetamine and the secondary amine phenmetrazine was measured in quadruplicate for each time period. As shown in Fig. 5, the amount of both derivatives did not increase after 1 min of extractive benzoylation. However, cleaner chromatograms were obtained after 5 min reaction time, probably due to the complete removal of the unreacted PFBCl.

For PFB-amphetamine no decrease was found after 1-h period, contrary to PFB-phenmetrazine which slowly decomposed after 30 min in the two-phase system.

Moreover, a solution of PFB-amphetamine in ethyl acetate was stable for at least two weeks (room temperature) while the PFB derivative of phenmetrazine was already decomposed after 48 h.

A reference chromatogram of N-pentafluorobenzamides of doping agents is given in Fig. 6.

The detection limits for some derivatives starting with 2 ml of urine or plasma are given in Table II and illustrated in Figs. 7 and 8.

TABLE I

TABULATION OF PRINCIPAL FRAGMENTATION PATHWAYS OF SOME PFB DERIVATIVES

Tranylcypromine	$M^{+\cdot}$ 327 (0.9)	$M-91^+$ 236 (40.8)	$M-115^+$ 212 (14.5)	$M-182^+$ 195 (60.9)	$M-120^+$ 160 (26.5)
Phentermine	$M^{+\cdot}$ 343 (—)	$M-91^+$ 252 (61.1)		$M-148^+$ 195 (100.0)	$M-176^+$ 167 (15.1)
Chlorphentermine	$M^{+\cdot}$ 377 (—)	$M-125^+$ 252 (56.0)		$M-182^+$ 195 (100.0)	$M-210^+$ 167 (15.9)
Pentorexum	$M^{+\cdot}$ 357 (—)	$M-105^+$ 252 (69.2)		$M-162^+$ 195 (100.0)	$M-190^+$ 167 (13.5)
Amphetamine	$M^{+\cdot}$ 329 (—)	$M-91^+$ 238 (32.8)		$M-134^+$ 195 (100.0)	$M-162^+$ 167 (14.5)
Mephentermine	$M^{+\cdot}$ 357 (—)	$M-91^+$ 266 (52.6)		$M-162^+$ 195 (100.0)	$M-190^+$ 167 (14.3)
Methylamphetamine	$M^{+\cdot}$ 343 (0.1)	$M-91^+$ 252 (36.9)		$M-148^+$ 195 (100.0)	$M-176^+$ 167 (18.4)
Ethylamphetamine	$M^{+\cdot}$ 357 (0.2)	$M-91^+$ 266 (32.7)		$M-162^+$ 195 (100.0)	$M-210^+$ 167 (16.2)
Fenfluramine	$M^{+\cdot}$ 425 (—)	$M-159^+$ 266 (41.5)		$M-230^+$ 195 (100.0)	$M-258^+$ 167 (21.0)
Cyclopentamine	$M^{+\cdot}$ 335 (1.2)	$M-83^+$ 252 (57.6)		$M-140^+$ 195 (100.0)	$M-168^+$ 167 (12.6)
Methylphenidate	$M^{+\cdot}$ 427 (—)	$M-149^+$ 278 (67.2)		$M-132^+$ 195 (100.0)	$M-260^+$ 167 (13.9)
Phacetoperane	$M^{+\cdot}$ 427 (—)	$M-149^+$ 278 (100.0)		$M-132^+$ 195 (95.2)	$M-260^+$ 167 (12.5)
Dioxadrol	$M^{+\cdot}$ 503 (0.4)	$M-125^+$ 278 (85.5)	$M-298^+$ 225 (47.7)	$M-308^+$ 195 (100.0)	$M-336^+$ 167 (35.5)
Fencamfamine	$M^{+\cdot}$ 409 (—)	$M-214^+$ 195 (63.8)	$M-239^{+\cdot}$ 170 (86.1)	$M-342^+$ 167 (19.6)	$M-367^+$ 142 (100.0)
Fencamfamine metabolite	$M^{+\cdot}$ 381 (—)	$M-186^+$ 195 (65.2)	$M-211^{+c}$ 170 (100.0)	$M-214^+$ 167 (13.2)	$M-239^+$ 142 (82.5)
Phenmetrazine	$M^{+\cdot}$ 371 (0.8)	$M-106^+$ 265 (29.6)	$M-125^+$ 246 (29.6)	$M-133^+$ 238 (10.3)	$M-176^+$ 195 (100)

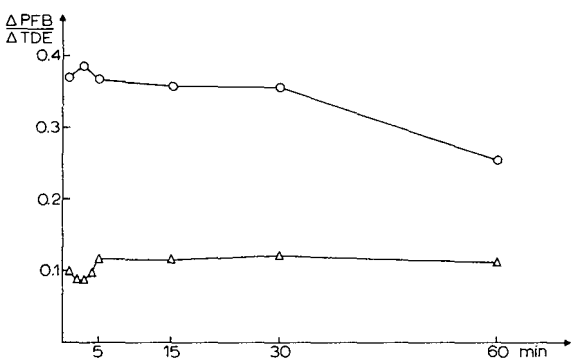

Fig. 5. Effect of reaction time on the pentafluorobenzoylation of amphetamine (△) and phenmetrazine (○).

Although the pentafluorobenzoyl group seems to be the one that confers the greatest sensitivity for electron-capture detection of amines [11], one can see from Table II that the response for derivatives of primary amines is much higher than for secondary amines.

$M-195^+$	$M-120^+$	$M-211^{+\circ}$	$M-212^+$	$M-222^+$	$M-236^+$
132 (9.5)	117 (28.3)	116 (100)	115 (28.9)	105 (27.3)	91 (3.9)
		$M-211^{+\circ}$			$M-252^+$
		132 (7.2)			91 (11.0)
		$M-211^{+\bullet}$			$M-252^+$
		166 (11.9)			125 (9.1)
		$M-211^{+\bullet}$			$M-252^+$
		146 (1.3)			105 (10.7)
		$M-211^{+\bullet}$			$M-238^+$
		118 (32.8)			91 (12.5)
		$M-225^{+\bullet}$			$M-266^+$
		132 (10.8)			91 (16.8)
	$M-224^+$	$M-225^{+\bullet}$	$M-226^+$		$M-252^+$
	119 (1.0)	118 (9.0)	117 (9.6)		91 (16.3)
	$M-238^+$	$M-239^{+\bullet}$	$M-240^+$		$M-266^+$
	119 (1.6)	118 (9.6)	117 (11.0)		91 (19.0)
		$M-239^{+\bullet}$			$M-266^+$
		186 (5.4)			159 (4.9)
		$M-225^{+\bullet}$			—
		110 (2.8)			
$M-398^+$	$M-426^+$				
105 (46.7)	77 (23.0)				
$M-392^+$	$M-394^+$	$M-318^+$			
117 (17.1)	115 (15.8)	91 (30.8)			
$M-264^+$	$M-266^+$	$M-290^+$			
117 (13.3)	115 (11.8)	91 (25.6)			
$M-204^+$	$M-254^+$	$M-266^+$			
167 (18.2)	117 (12.3)	105 (8.5)	70 (19.7)	56 (19.6)	

Taking into account that the doses used in doping practices are still higher than for therapeutic purposes, extractive benzoylation of sympathomimetic amines, especially those with a primary amino group, is a sensitive and rapid method for confirmation of suspected or positive cases.

Further studies with respect to selectivity (fused-silica capillary gas chromatography) and sensitivity (negative chemical ionization mass spectrometry) are currently being investigated.

TABLE II

DETECTION LIMITS (ng/ml) OF PFB DERIVATIVES OF SOME DOPING AGENTS STARTING WITH 2 ml OF BIOLOGICAL FLUID

Compound	Urine	Plasma	Compound	Urine	Plasma
Amphetamine	2.5	0.35	Phenmetrazine	50	25
Chlorphentermine	2.5	1	Fenfluramine	125	20
Phentermine	5	2.5	Methylphenidate	250	25
Fencamfamine metabolite	2.5	0.5	Mephentermine	500	200
Tranylcypromine	1	0.125	Ethylamphetamine	750	125

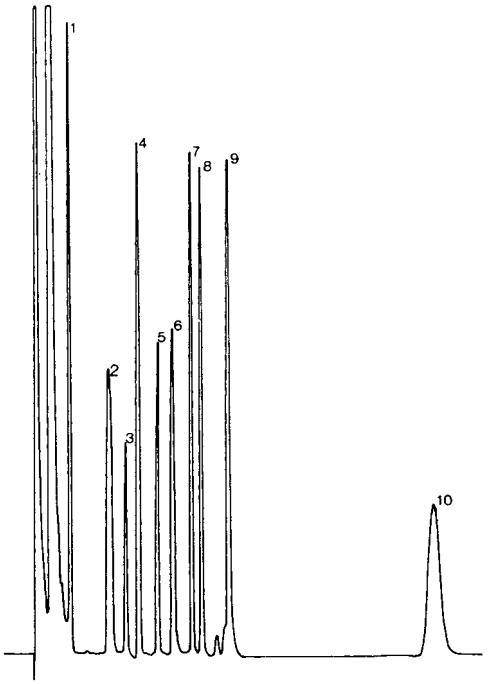

Fig. 6. Gas chromatography of N-pentafluorobenzoyl derivatives of doping agents. For GC conditions see section on gas chromatography. Peaks: 1 = cyclopentamine; 2 = ethylamphetamine, fenfluramine, mephentermine and methylamphetamine; 3 = phentermine; 4 = amphetamine; 5 = phenmetrazine; 6 = chlorphentermine, tranylcypromine, fencamfamine; 7 = phacetoperane; 8 = methylphenidate; 9 = fencamfamine metabolite; 10 = dioxadiol.

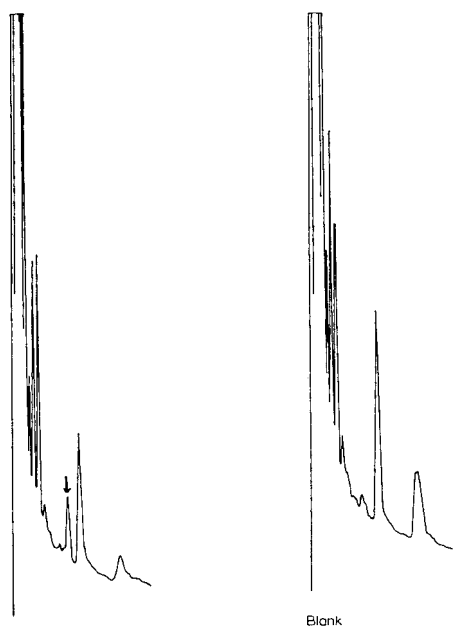

Fig. 7. Detection limit (2.5 ng/l) for the N-pentafluorobenzamide of amphetamine (arrow) in urine (2 ml).

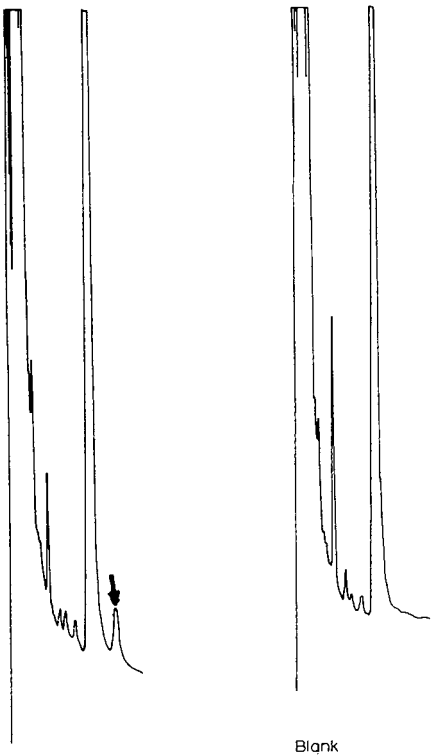

Fig. 8. Detection limit (50 ng/ml) for the N-pentafluorobenzamide of phenmetrazine (arrow) in plasma (2 ml).

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CHROMBIO. 1534

SOLVENT EXTRACTION OF TRICYCLIC AMINES FROM BLOOD PLASMA AND LIQUID CHROMATOGRAPHIC DETERMINATION

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SUMMARY

The extraction of seven tricyclic antidepressant amines from human plasma at different pH values was investigated for dichloromethane, diethyl ether and hexane—1-pentanol (95:5). The amines were extracted as bases and back-extracted to sulphuric acid, 0.10 mol/l, prior to the separation by bonded-phase liquid chromatography. Ether and hexane—1-pentanol (95:5) were most suitable, tertiary amines being best extracted at pH 8, and secondary amines at pH 10. Using ether, both tertiary and secondary amines required 30 min extraction time for a quantitative yield while 15 min was sufficient for hexane—1-pentanol (95:5). UV detection allowed concentrations down to 10 ng in 1 ml of plasma to be determined.

Three ammonium ions — octylammonium, dimethylammonium, and trimethylammonium — were added as modifiers to the mobile phase containing acetonitrile in phosphoric acid, 0.10 mol/l. In the concentration interval 0.010—0.030 mol/l all of the amine modifiers gave on Polygosil C_8 peak asymmetry factors of sufficiently low magnitude, while on LiChrosorb RP-18 this was so only for di- and trimethylammonium in a concentration of 0.030 mol/l.

INTRODUCTION

Tricyclic amines used as drugs are in many instances subject to plasma level monitoring as a support to rational drug therapy. In the last few years a great number of chromatographic methods for tricyclic antidepressants have been published in particular by liquid chromatography (e.g. refs. 1—7).

For liquid chromatography of tricyclic and other hydrophobic amines on chemically bonded stationary phases the use of an amine modifier in the aqueous mobile phase has been adapted to improve the chromatographic performance [8]. In addition these substances are of analytical interest since published methods often exhibit much lower recoveries in the work-up procedure, down to 65%, than would be expected from their distribution coefficients [9—11]. This effect may to some extent be due to adsorption losses not being prevented but the major reason must be inconsistency in the ex-

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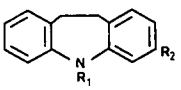
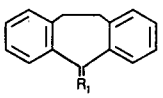
traction procedure used. In the present study three common organic solvents were used in the extraction from blood plasma of a number of tricyclic amines, and secondary and tertiary aliphatic amines (Table I). The extraction as a function of time was determined for each solvent at three different pH values.

TABLE I

LIST OF TRICYCLIC ANTIDEPRESSANT AMINES

Formula	Name	R_1	R_2
	Imipramine	$-(CH_2)_3N(CH_3)_2$	-H
	Desipramine	$-(CH_2)_3NHCH_3$	-H
	Trimipramine	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	-H
	Clomipramine	$-(CH_2)_3N(CH_3)_2$	-Cl
	Desmethylclomipramine	$-(CH_2)_3NHCH_3$	-Cl
	Amitriptyline	$=CH(CH_2)_2N(CH_3)_2$	
	Nortriptyline	$=CH(CH_2)_2NHCH_3$	

The liquid chromatographic studies comprised two bonded phases — LiChrosorb RP-18 and Polygosil C_8 — and amine modifiers of different structures to elucidate conditions for a satisfactory chromatographic behaviour of the tricyclic amines.

EXPERIMENTAL

Apparatus

The liquid chromatograph was composed of an Altex solvent metering pump Model 110 A (Berkeley, CA, U.S.A.) and a Waters 440 (Waters Assoc., Milford, MA, U.S.A.) UV detector operated at 254 nm. The injector was from Rheodyne (Berkeley, CA, U.S.A.) with 20-, 100-, and 150-μl loops. The separation column of stainless steel (length 150 mm, O.D. 6.35 mm, I.D. 4.5 mm) had end fittings of modified Swagelok connections. Room temperature was used.

The spectrophotometer used in the determination of molar absorptivities was a Beckman DU®-8 UV—Vis spectrophotometer (Fullerton, CA, U.S.A.).

The pH meter used in the extraction experiments was a PHM 64 research pH meter (Radiometer, Copenhagen, Denmark). The mixer used was a table universal shaker TU-1 (B. Braun Melsungen AG, Melsungen, G.F.R.) which operated in an orbital mode at a frequency of 200 rpm with the tubes being placed horizontally.

Chemicals and packing material

Octylamine (puriss, Fluka, Buchs, Switzerland), N,N-dimethyloctylamine (DMOA) (ICN Pharmaceuticals, Plainview, NY, U.S.A.) and N,N,N-trimethyloctylammonium (TMOA) bromide (Department of Organic Chemistry, Hässle, Mölndal, Sweden) were used.

The drug compounds were supplied as hydrochlorides except for trimipramine (maleate). They fulfilled the quality requirements of the Pharmacopoeia Nordica.

All other chemicals used were of analytical grade. The column packing material was Polygosil C_8, average diameter 5 μm (Macherey-Nagel & Co., Düren, G.F.R.) and LiChrosorb RP-18, 5 μm (E. Merck, Darmstadt, G.F.R.).

Column packing procedure

Microporous silica particles (1.8 g) were suspended in 12 ml of isopropanol—dichloromethane (1:1). The slurry was filled in a reservoir connected to the separation column which was packed downwards with methanol—water (60:40) at 400 bar.

Chromatographic systems

(A) Stationary phase: LiChrosorb RP-18, 5 μm. Mobile phases: 30% of acetonitrile in phosphoric acid, 0.10 mol/l, containing octylamine (OA), dimethyloctylamine (DMOA) and trimethyloctylammonium (TMOA) bromide in concentrations of 0.005, 0.010 and 0.030 mol/l. Flow-rate: 1 ml/min.

(B) Stationary phase: Polygosil C-8, 5 μm. Mobile phases: 25—30% of acetonitrile in phosphoric acid, 0.10 mol/l, containing OA, DMOA and TMOABr in concentrations of 0.005, 0.010 and 0.030 mol/l. Flow-rate: 1 ml/min.

Determination of distribution constants

The distribution constant (K_D) for each amine between diethyl ether or hexane—1-pentanol (95:5) and water was determined with equal volumes of organic and aqueous phases in centrifuge tubes at room temperature. As the aqueous phase phosphate buffer solutions (ionic strength = 0.10) of three different pH values around log D = 0 ($D = C_{org.}/C_{aq.}$) were used. The total concentration of each amine in the two phases was $2.5 \cdot 10^{-4}$ mol/l. The tubes were mechanically shaken for 30 min. After centrifugation the organic phase was transferred to another tube and the pH was measured in the aqueous phase. The concentrations of the amines in the aqueous phase were determined chromatographically after washing the phase with hexane and centrifugation.

The concentrations of amines in the organic phase were determined by back-extraction to sulphuric acid, 0.10 mol/l, for 10 min. After centrifugation, washing with hexane and centrifugation again, the sample was injected in the chromatograph; 20-μl samples of the aqueous phases were injected.

The molar absorptivities of the amines at 254 nm were determined to be around $8 \cdot 10^3$.

Determination of the extraction yield of amines from human blood plasma

Volumes of 2.00 ml of blank plasma and of buffer solution (Table II) were mixed with 200 μl of 0.001 *M* hydrochloric acid containing 100 nmol of each amine in centrifuge tubes. Then 4.00 ml of the organic solvent, dichloromethane, diethyl ether or hexane—1-pentanol (95:5), were added for each pH and amine solution. The plasma phases for each pH and organic solvent were then extracted for 2, 5, 10, 15, 30, 45 and 60 min.

TABLE II

BUFFER SOLUTIONS USED IN EXTRACTION AND BLANK PLASMA STUDIES

pH	Buffer	Volume (μl)
8	Tris 1 mol/l, pH 8.0	50
10	Na_2CO_3 0.5 mol/l	140—180*
11**	NaOH 1 mol/l	110
12**	NaOH 1 mol/l	180—200*

*The volume was determined by the buffering capacity of the plasma used.
**pH 11 was used for CH_2Cl_2 and pH 12 for $(C_2H_5)_2O$ and C_6H_{14}—n-$C_5H_{11}OH$ (95:5).

After centrifugation for 10 min, 3.00 ml of organic phase were transferred to a centrifuge tube containing 3.00 ml of sulphuric acid, 0.10 mol/l, and the amines were back-extracted to the aqueous phase by shaking for 10 min.

After centrifugation, the organic phase was removed by aspiration and 1 volume of hexane was added to remove dissolved organic solvent. After shaking for approx. 1 min and centrifugation for 3 min the hexane was removed by aspiration.

A 100-μl aliquot of the aqueous phase was injected onto the chromatographic column. The absorbance of the eluate was measured at 254 nm.

Quantitative evaluation

The extraction yield of the amines was estimated by peak height or area measurement and comparison with direct injection of a reference sample in sulphuric acid, 0.10 mol/l, which prior to injection was shaken with the relevant organic solvent followed by hexane as described above.

Determination of the sensitivity of the analytical method

The determination of the sensitivity was performed according to the analytical method above, except for some minor modifications. Half the volume of blank plasma and buffer solution was used; 100 μl of the 0.001 mol/l hydrochloric acid containing 0.1 nmol of each of desipramine, trimipramine and clomipramine were added to blank plasma. The extraction was performed with diethyl ether and hexane—1-pentanol (95:5), respectively, for 45 min at pH 8 and 10. The organic phase was transferred to a conical centrifuge tube containing 300 μl of sulphuric acid, 0.10 mol/l, which after aspiration of the organic phase was purified by shaking twice with hexane. Finally, 150 μl of the aqueous phase were injected.

RESULTS AND DISCUSSION

Extraction studies

In methods to determine tricyclic antidepressant amines in biological material, samples buffered to pH 12 and 10 are mostly used. The extraction times vary between 1 and 60 min and the volume ratio organic/aqueous phase

between 1 and 8. At pH 12 imipramine and desipramine in human plasma have been reported to be extracted in 90% yield after 10 min extraction time with hexane—1-pentanol (99:1) [1], in 68.5% and 65.5% yield, respectively, by 15 min with hexane—1-butanol (80:20) [2], > 80% yield by 60 min with hexane—isopentanol (99:1) [3], and 88.2% and 103.2% yield by 5 min with butyl chloride [4]. At pH 10 imipramine and desipramine have been extracted in 65.5% and 67.5% yield, respectively, in 10 min with hexane [5], 86.4 ± 4.3% and 100 ± 3.2% yield in 15 min with methanol—ethyl acetate—hexane (0.4:20:79.6) [6], and 89% and 84% yield by 15 min shaking with ether [7].

The extraction yield depends on the pH of the plasma phase and on the extraction time as is illustrated in Figs. 1—3 for ether, hexane—1-pentanol (95:5) and dichloromethane.

The distribution ratio, D, at pH 8, 10 and 12 (11 for dichloromethane) and the theoretical extraction yield from pure aqueous solutions can be estimated from the distribution constant, log K_D (Table III). More than 99% of the amines should be extracted, with the exception of the secondary amines at pH 8 where ether and hexane—1-pentanol (95:5) give between 90 and 99% yield.

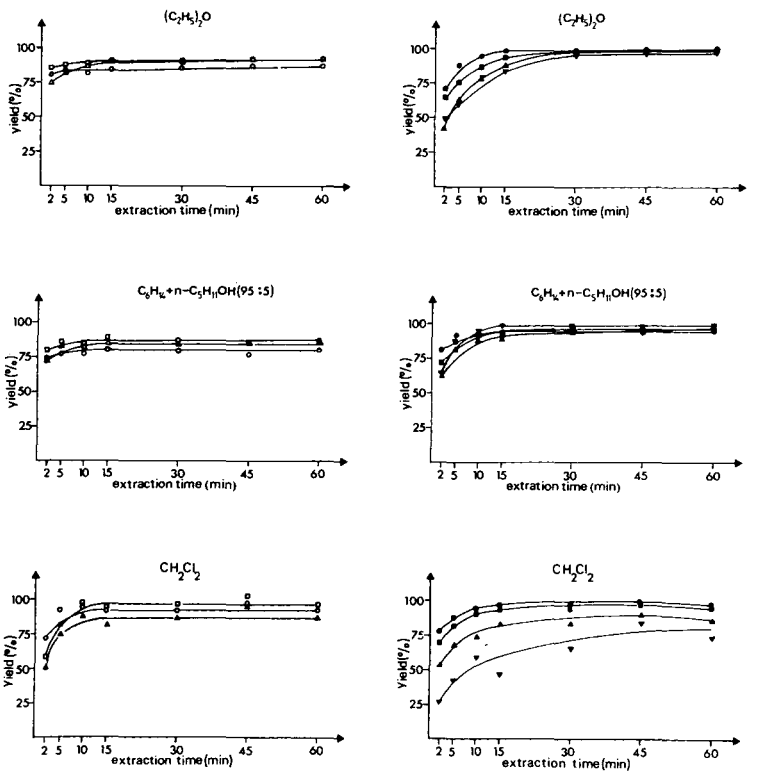


Fig. 1. Extraction yield of seven tricyclic antidepressant amines from plasma at pH 8 for three organic solvents. (○) Desipramine, (□) nortriptyline, (△) desmethylclomipramine, (●) imipramine, (■) amitriptyline, (▲) clomipramine, (▼) trimipramine.

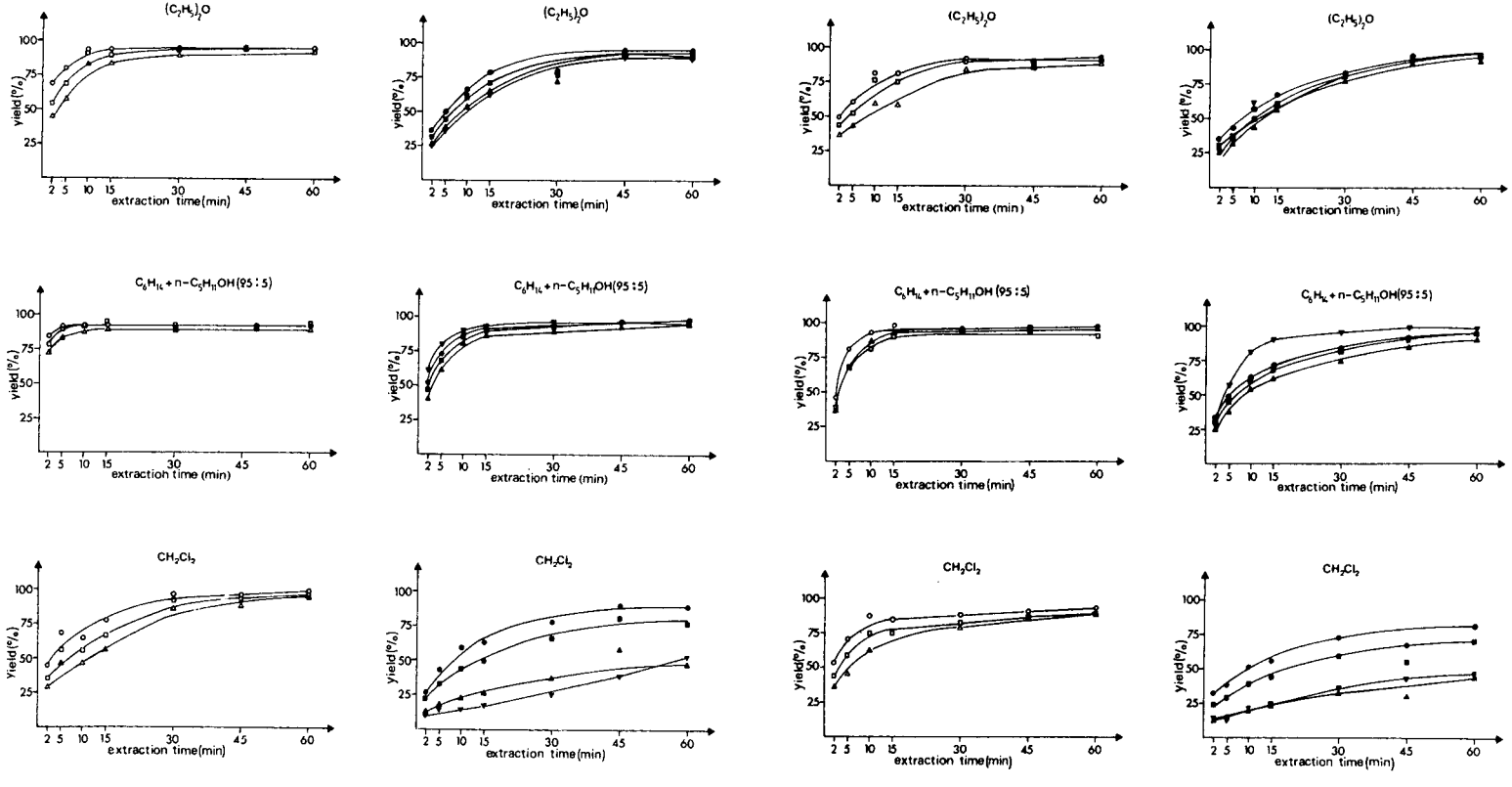


Fig. 2. Extraction yield of seven tricyclic antidepressant amines from plasma at pH 10 for three organic solvents. Symbols as in Fig. 1.

Fig. 3. Extraction yield of seven tricyclic antidepressant amines from plasma at pH 12 (pH 11 for CH_2Cl_2). Symbols as in Fig. 1.

TABLE III

DISTRIBUTION COEFFICIENTS

Ionic strength = 0.10.

Amine	log $K_{D(A)}$*			pK'_{HA}***
	CH_2Cl_2**	$(C_2H_5)_2O$	C_6H_{14}—n-$C_5H_{11}OH$ (95:5)	
Imipramine	5.8	4.27	4.33	9.4
Desipramine	5.1	3.38	3.17	10.2
Amitriptyline	6.2	4.72	4.82	9.4
Nortriptyline	5.6	3.87	3.78	10.2
Clomipramine	6.6	5.14	5.15	9.4
Desmethylclomipramine	5.8	4.26	4.11	10.2
Trimipramine	6.6	5.18	5.17	9.4

*Calculated from log ($K_{D(A)} \times K'_{HA}$).
**Values of imipramine and desipramine were taken from ref. 9. All other values in CH_2Cl_2 estimated from data for $CHCl_3$ from refs. 9 and 10.
***Taken from ref. 11.

The extraction yields were much lower than predicted for some of the extraction systems (Figs. 1—3). With dichloromethane as organic solvent, a very low extraction from plasma was achieved for the tertiary amines at pH 10 and 11, especially for clomipramine and trimipramine which also at pH 8 gave a much too low yield. Dichloromethane gave an extensive protein denaturation in the plasma which increased with increasing pH. This precipitation may cause inocclusion of the strongly protein-bound amines and insufficient access of the organic solvent. Hexane—1-pentanol (95:5) gave only a moderate protein denaturation, which also increased with increasing pH, while ether gave only a slight protein denaturation. In spite of the low protein denaturation of hexane—pentanol and ether, maximum extraction was achieved more rapidly at pH 8 than at pH 10 and 12. This may indicate that the binding of tricyclic antidepressant amines to plasma components are pH dependent and increase on going from pH 8 to pH 10 and 12.

As shown in Figs. 1—3, the secondary amines required shorter extraction times than the tertiary amines to give maximal extraction yield, which may reflect a weaker protein binding. However, dichloromethane should be avoided as it generally gave a lower yield and large deviation between the duplicate samples.

The secondary amines have to be extracted at pH 10 (Fig. 2) if a quantitative recovery is to be obtained when ether or hexane—1-pentanol (95:5) constitutes the organic phase. For ether, 30 min extraction time gave a maximum yield for nortriptyline and desipramine of 94% and 95%, respectively, but only 89% of N-desmethylclomipramine. Using hexane—1-pentanol (95:5) a 15 min extraction time was sufficient to obtain maximum yield, 89% for N-desmethylclomipramine and 92% for the other two amines.

The highest yields for the tertiary amines were obtained at pH 8 (Fig. 1)

using ether or hexane—1-pentanol (95:5) as organic solvent. Maximum extraction in ether was achieved after 30 min while 15 min extraction time was sufficient with hexane—1-pentanol (95:5) to obtain yields of about 95%.

Chromatographic studies

The tricyclic amines were separated as ion-pairs with $H_2PO_4^-$ at pH 3.5 on two types of reversed-phase columns — LiChrosorb RP-18, 5 μm, and Polygosil C_8, 5 μm. The asymmetry factor, Asf, and the capacity factor, k', were investigated without any alkylamine modifier present in the mobile phase and when OA, DMOA and TMOA were added to the mobile phase in the concentrations 0.005, 0.010 and 0.030 mol/l. The tricyclic amines were injected in sulphuric acid, 0.10 mol/l, as in the extraction studies. The results for trimipramine are presented in Fig. 4 and are representative of the other amines studied. In Table IV, Asf and k' values for the tricyclic amines on Polygosil C_8 and LiChrosorb RP-18 are presented using the same mobile phase containing OA as modifier.

The mobile phase content of acetonitrile had to be diminished when OA, DMOA and TMOA were added, because the alkylammonium ions reduce the capacity factors by competing with the tricyclic ammonium ions for the adsorption sites [8, 12].

Acceptable peak symmetry (Asf < 2) was obtained on Polygosil C_8 for all tricyclic ammonium ions at all three concentrations of alkylammonium ions

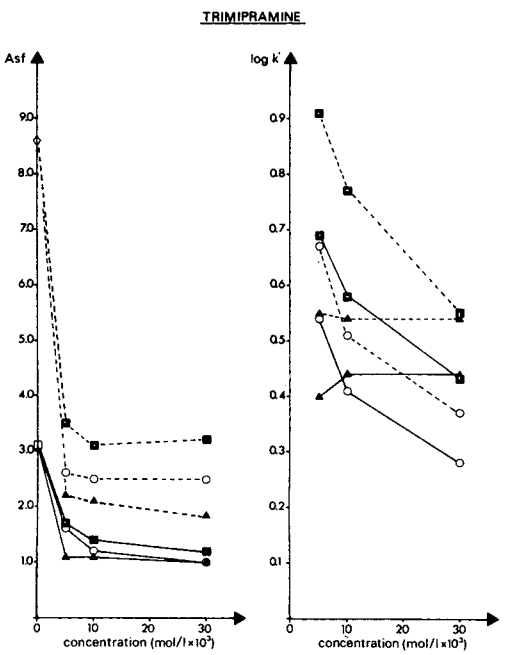


Fig. 4. Peak asymmetry factor (Asf) and capacity factor for trimipramine in the presence of different amine modifiers in the eluent: (■) OA, (○) DMOA, (▲) TMOA. Stationary phases: (— — —) LiChrosorb RP-18, (———) Polygosil C_8.

TABLE IV

COMPARISON OF ASYMMETRY FACTORS (Asf) AND CAPACITY FACTORS (k') OF TRICYCLIC AMINES SEPARATED ON TWO STATIONARY PHASES

Mobile phase: 30% of acetonitrile and OA 0.005 mol/l in H_3PO_4 0.10 mol/l.

Amine	LiChrosorb RP-18, 5 μm		Polygosil C_8, 5 μm	
	Asf	k'	Asf	k'
Desipramine	3.5	4.5	2.3	1.8
Trimipramine	3.5	8.1	2.3	2.8
Desmethylclomipramine	3.0	10.2	2.4	3.3
Clomipramine	3.5	12.3	2.2	3.8

(Fig. 4). At these concentrations TMOA gave approximately constant Asf and k' values.

On LiChrosorb RP-18, TMOA gave acceptable peak symmetry (Asf < 2) at higher concentrations of the modifier, while OA and DMOA improved the chromatographic performance less efficiently.

Sensitivity

The sensitivity of the determination for the tricyclic antidepressant amines in plasma was evaluated by extraction with ether and hexane—1-pentanol (95:5) at pH 8 and 10. A 1-ml volume of plasma was used and 150 μl of aque-

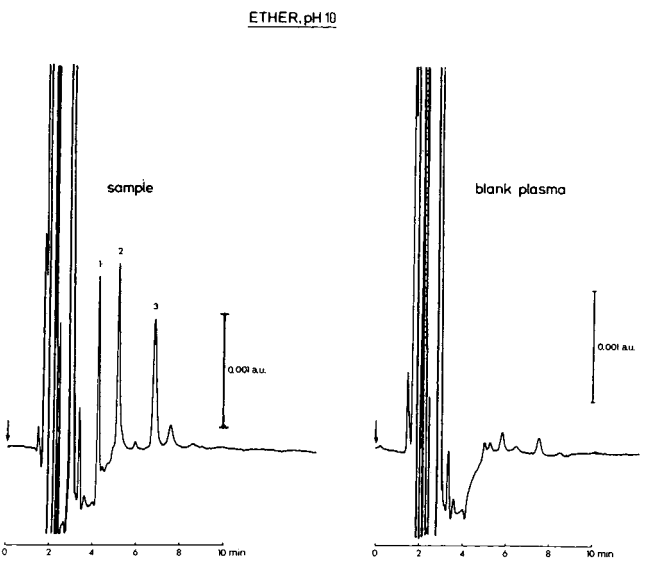


Fig. 5. Chromatograms of extract from 1 ml plasma spiked with 28.2 ng of desipramine (1), 40.1 ng of trimipramine (2), and 41.3 ng of clomipramine (3). Stationary phase: Polygosil C_8, 5 μm. Mobile phase: 25% acetonitrile and 0.015 mol/l DMOA in 0.1 mol/l H_3PO_4.

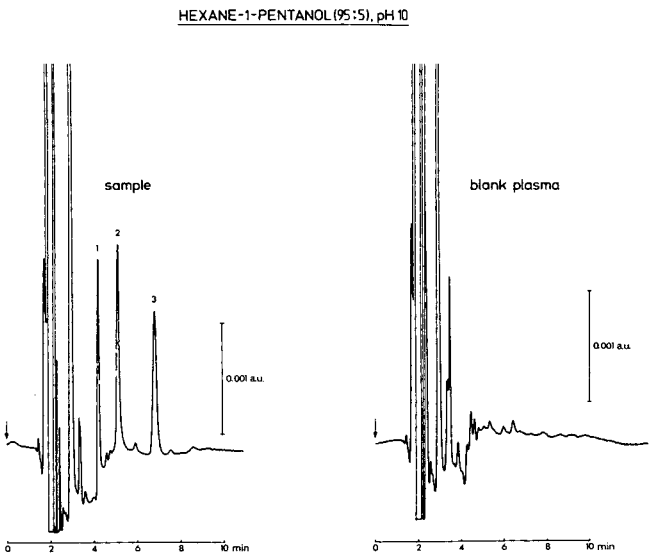


Fig. 6. Same conditions as in Fig. 5.

ous phase were injected onto the column, as these volumes were adequate. This analytical method allows determinations down to 10 ng/ml of plasma sample. The sensitivity limit is set by the background in the blank plasma at the place of the tricyclic amines (Figs. 5 and 6).

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Note

Determination of prostaglandin $F_{2\alpha}$ and 6-oxo-prostaglandin $F_{1\alpha}$ in urine by gas chromatography—positive chemical ionisation-mass spectrometry using stable isotope dilutions with selected ion monitoring

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Isotopically labelled substances are biochemically identical to non-labelled ones, but can be identified separately from naturally occurring ones by means of physical techniques. Isotopically labelled material permits the study of metabolic pathways of naturally occurring substances in vivo and in vitro, aids detection of metabolic disorders and enables the processes of resorption, distribution, storage and excretion to be followed in animals and humans. The development of very sensitive and precise instruments allows the establishment of so-called "definitive methods" and the precise measurement of very low amounts of biological substances using stable isotopes as tracers.

Radioimmunoassay offers exceptional sensitivity and allows the measurement of a large number of samples in a relatively short time, but the method is inherently suspect in terms of specificity. Consider, for example, the influence of unknown cross-reacting substances in body fluids, or of multistage preseparation procedures with a high percentage of losses. Losses in multistage separation methods can be compensated by radioisotopic labelling, but destruction of the molecules during the separation procedure is sometimes disregarded and falsifies the results. For example, cortisol and prednisolone were separated on thin-layer chromatographic (TLC) sheets, the corresponding zones were detected under UV light, and the substances measured by radioimmunoassay. The UV light caused destruction of the cortisol and prednisolone molecules, and neither substance bound to the binding protein (transcortin), but the radioactivity was present in the eluate [1]. Chromatographic procedures tend to give variable results if labile substances are studied or if two sterically different substances result from derivatisation for gas chromatography (GC). Such faults are avoided if the isotopically labelled standard and the substance are both measured with the same detector as is possible with mass spectrometry (MS).

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A sensitive and precise method for measurement of urinary prostaglandins (PG) is presented. Tetradeuterated PGs are used as internal standards and as tracers.

EXPERIMENTAL

Method

The method [2] described for measurement of serum PGs using a negative chemical ionisation (NCI) MS device was adapted for our needs to measure $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ from urine or from cell incubations with a positive chemical ionisation (PCI) MS device. The method consists of: 20 ml of urine or diluted sample + 50 ng of both PGs in tetradeuterated form + 1.4 ml of 2 *N* HCl → centrifugation → supernatant → sorption of the PGs on CP^{tm} Elut C 18 (Chrompack Cat. No. 19016; Chrompack, Middelburg, The Netherlands) 200 mg of sorbent → washing with 100 ml of water → elution of the PGs with 7 ml of ethyl acetate → sorption of the PGs on normal phase CP^{tm} Elut Si (Chrompack Cat. No. 19010) 500 mg of sorbent → washing with 50 ml of ethyl acetate → extraction of the PGs with 5 ml of methanol → evaporation under nitrogen at room temperature → TLC (Merck Kieselgel 60; Merck, Darmstadt, G.F.R.) solvent system: organic layer of ethyl acetate—acetic acid—isooctane—water (11:2:5:10), standards can be made visible by spraying with phosphomolybdate, R_F values: $PGF_{2\alpha}$ 0.17, 6-oxo-$PGF_{1\alpha}$ 0.12; elution with 2 ml of methanol + 2% acetic acid → filtration → evaporation → redissolution in citric acid—phosphate buffer, pH 3.8 → extraction two times with ether → evaporation → + 100 μl of 10% methoxyamine · HCl in pyridine → incubation overnight at room temperature → evaporation → methylation in 100 μl of methanol + 500 μl of ethereal diazomethane [3], 5 min at room temperature → evaporation → redissolution in dichloromethane → chromatography through a column (I.D. 5 mm) filled with 1 g of Sephadex LH-20 in dichloromethane → elution with 2 ml of dichloromethane → evaporation → addition of 20 μl of a mixture of hexamethyldisilazane—trimethylchlorosilane—pyridine (3:1:9) → splitless injection of 5 μl.

Instrumental conditions

A Finnigan 9610 gas chromatograph coupled to a Finnigan 4000 mass spectrometer with a positive ion electron-impact (EI) PCI device and an Incos data system are used. The gas chromatograph is equipped with a fused silica column coated with the chemically bonded DB-1 (30 m × 0.25 mm I.D.). The injector and the transfer line are kept at 260°C. The column is kept at 100°C for 1 min after injection, then heated with an increase of 40°C/min to 200°C and then at 3°/min to 300°C. The retention time of $PGF_{2\alpha}$ is 24 min, that of 6-oxo-$PGF_{1\alpha}$ 27 min. A shorter program resulted in inferior accuracy. The MS conditions are: CI with ammonia, ion source at 280°C, electron energy 120 eV, emission current 0.1 A.

RESULTS

The most prominent fragments in the higher mass range were monitored;

they are 405 for $PGF_{2\alpha}$ and 409 for its tetradeuterated form. For 6-oxo-$PGF_{1\alpha}$ we measured the mass 540 and 544 for the tetradeuterated $PGF_{1\alpha}$ (Fig. 1). Calibration curves were established for 30—2400 pg of each PG injected together with 10 ng of each in the tetradeuterated form. For 6-oxo-$PGF_{1\alpha}$ a correlation coefficient of 0.9989, an intercept of 0.004 and a slope of 0.021 were found. The correlation coefficient for $PGF_{2\alpha}$ was 0.9987, the slope 0.038 and the intercept 0.005. The standard deviation calculated from five parallel assays of pure PGs is about 2.5% and lies within 5% for urine samples. Since fragmentation in the ion source is slightly different for natural and for

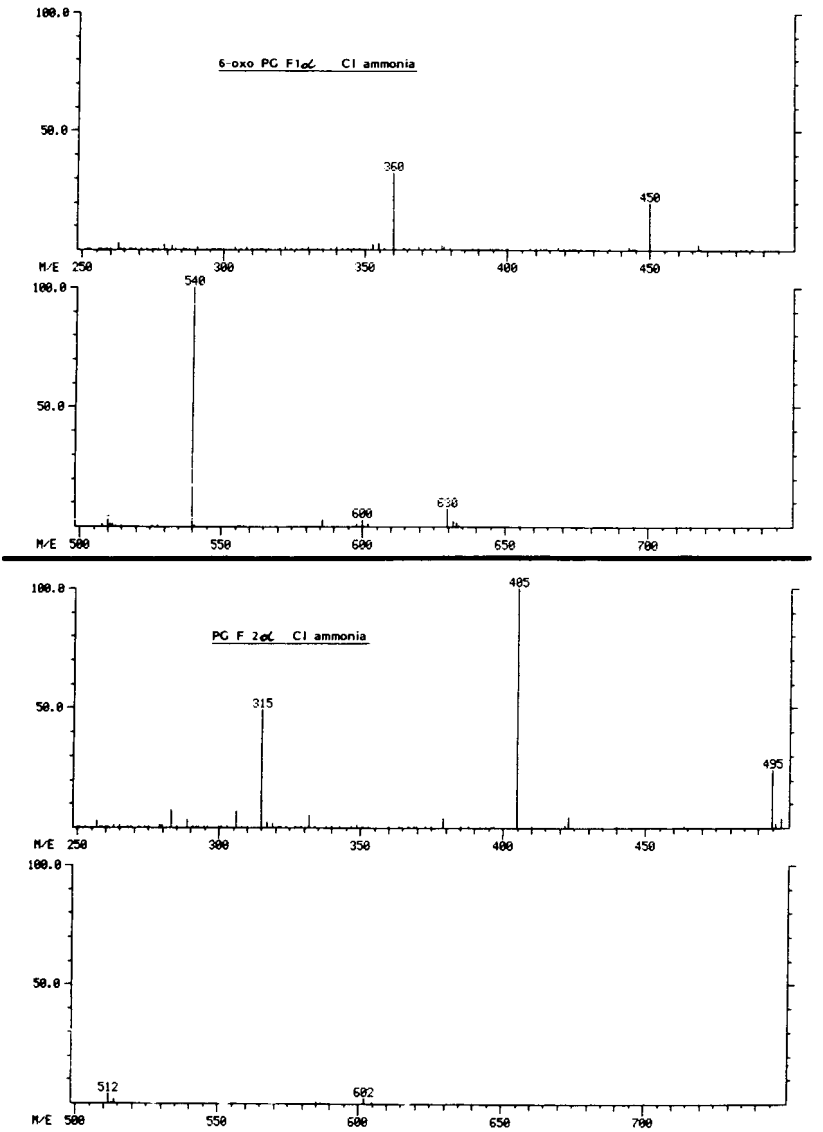

Fig. 1. Mass spectra of (bottom) $PGF_{2\alpha}$ as methyl ester—silyl ether and (top) of 6-oxo-$PGF_{1\alpha}$ as methyl ester—methyloxime—silyl ether obtained by chemical ionisation with ammonia as CI gas.

tetradeuterated PGs and because of the influences of the different source parameters on the fragmentation pattern, we establish a new calibration curve every day. The variations observed from day to day lie within 10%.

The detection limit for urine samples is 20 pg of each PG per ml urine (Fig. 2). For urine we detected 25—700 pg of 6-oxo-$PGF_{1\alpha}$ per mg creatinine. The values for $PGF_{2\alpha}$ are about ten times higher. The number of urines so far investigated, however, is too small to give reference values.

The specificity of the method is given by liquid—solid extraction, different chromatographic steps, formation of derivatives, GC on a 30-m fused silica column and selected ion monitoring (SIM) by MS.

The accuracy is ensured by addition of both PGs in tetradeuterated form as tracer and as internal standard. The overall recovery lies at 50% for urine samples. The losses, however, are compensated for by calculation via the internal standard.

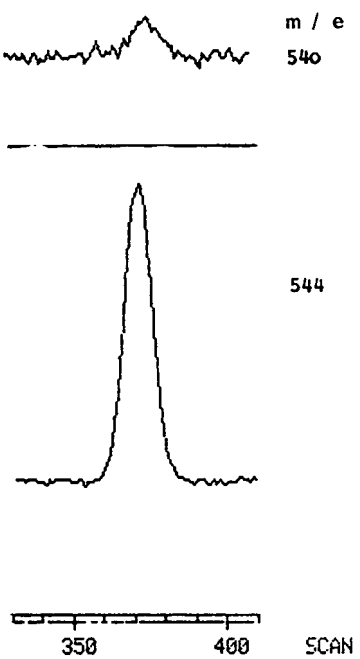

Fig. 2. Ion chromatogram obtained from urine showing the lower detection limit. The peak obtained for the mass 544 corresponds to 50 ng of tetradeuterated 6-oxo-$PGF_{1\alpha}$ added to 20 ml of urine. The amount of urinary 6-oxo-$PGF_{1\alpha}$ was calculated from the peak obtained for the mass 540 to be approximately 20 pg/ml urine.

DISCUSSION

The high purification of urine extracts combined with the addition of tetradeuterated PGs in great excess as tracers and as internal standards makes it possible to measure with PCI amounts as low as 20 pg PG per ml urine with high specificity and accuracy. A different behaviour of the tetradeuterated and

the natural PGs was not observed during the entire procedure, except during GC where the tetradeuterated PGs have slightly shorter retention times (the difference between labelled and unlabelled PG is about 4 sec at a retention time of 24 and 27 min). Further, we observed different fragmentation in the ion source in that the peak areas of identical amounts of labelled and unlabelled PG are not identical. These differences, however, are compensated for by the calibration curve. The results obtained for urinary $PGF_{2\alpha}$ correspond to those published by Seyberth et al. [4]. Those obtained for 6-oxo-$PGF_{1\alpha}$ can not be compared since we found only two methodological descriptions [2, 5] but no data.

Compared with the method for determinations from the serum [2] we use higher amounts for washing the columns to eliminate impurities from the urine. Further, we make the TLC purification step prior to derivatisation as the free PGs are better separated from interfering substances from the urine or from cholesterol if cell incubations are investigated. The difficulties reported [2] for methyloxime formation after TLC were overcome by our extraction procedure from the silica gel. Finally, methyl esters were used instead of pentafluorobenzyl esters since when working with PCI—MS these substances gave better results.

ACKNOWLEDGEMENTS

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Note

Direct coupling of fused silica columns to the ion source of a mass spectrometer applied to studies of arachidonic acid metabolism in human fibroblasts

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The metabolites of arachidonic acid are very labile polyunsaturated hydroxy fatty acids. When studying these compounds, problems of stability during gas chromatography—mass spectrometry (GC—MS) are to be expected. It is well known that the lower detection limit of prostaglandins is relatively high, owing not only to a disadvantageous fragmentation pattern, but also to losses during GC. Resorption and destruction phenomena have to be avoided to obtain reliable spectra. The interface and the transfer line between the GC column and the ion source of the mass spectrometer may be a source of leaks and also cause destruction of substances on active centers. Once precipitated, the residues will never be removed but influence the background. These reasons prompted us to introduce the thin, unbreakable and flexible, fused-silica column directly through the interface into the ion source of the mass spectrometer, thereby avoiding [1] the problems described above. Theoretical considerations, however, indicate that the separation quality of the GC column could be influenced by the high vacuum.

INSTRUMENTATION

A Finnigan GC 9610 coupled to a Finnigan 4000 mass spectrometer with a positive electron-impact ionisation (EI) and a positive chemical ionisation (CI) device combined with an Incos data system were used for the experiments. Fused silica columns, 30 m × 0.25 mm I.D., coated with the chemical bonded phase DB-1 (equivalent to the methyl-polysiloxane SE-30) were purchased from J & W Scientific Inc. (Rancho Cordova, CA, U.S.A.). The injector and the connection to the ion source were kept at a temperature of 260°C. The GC column was kept at 100°C for 1 min after injection, and then programmed to rise to 300°C. The EI mass spectra were obtained with an electron energy of 70 eV and an emission current of 0.2 A. The CI mass spectra

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were obtained with ammonia as CI gas, an electron energy of 120 eV, and an emission current of 0.1 A. The ionizer temperature was 280°C in both cases. The scan time was chosen to be 1 sec for a mass range of m/z 100—700 (mass per electron).

INSTRUMENTAL BEHAVIOUR

The high vacuum measured with the ion gauge in the mass spectrometer was about 1.6×10^{-4} Pa when the glass capillary column was connected in the usual manner to the mass spectrometer. If a fused-silica column was directly introduced, the pressure in the mass spectrometer remained unchanged or even decreased a bit. This can be explained by the smaller internal diameter of the quartz column as compared to the glass column. When the capillary column is disconnected from the interface, the small internal diameter of the glass interface causes the mass spectrometer pressure to be maintained at 6.66×10^{-3} Pa. A 50-cm long fused-silica column directly introduced results in the same pressure level. These results indicated the probability that the direct insertion of the fused silica column into the mass spectrometer would not pose greater problems or influence the separation quality of the GC column. To test this assumption, we connected the fused-silica column first through the normal interface, and then without interface. Retention times and theoretical plates of a column coated with DB-1 were measured under both conditions for the prostaglandin (PG) 6-oxo PG F1α. The temperature program was the following: 1 min at 100°C, then increasing at 40°C/min to 200°C, then at 3°C/min to 300°C. The retention times were calculated under both conditions to be 1522 sec, reproducible at ± 2 sec. The peak widths were also identical, with ten scans at half the peak height, the scan time being 858 msec. The number of theoretical plates was 170,600 under both conditions, if calculated according to the formula of Cremer

$$n = \left(\frac{t_R}{w}\right)^2 \times 5.54$$

where n = number of theoretical plates, t_R = retention time, and w = width at half the peak height. These measurements were made on the fragments 508 and 598 in the EI mode. We then measured the fragment 540 in the CI mode with ammonia as CI gas. Thereby the pressure in the ion source was increased to 46.7 Pa, from less than 1.33 Pa in the EI mode. The retention time was not affected by this pressure increase on the end of the capillary column. So far, no side-effects could be detected; however, we found that the ion source had to be cleaned more often when a new column was used, than when using an old one. This problem could be overcome by heating the new column overnight at 320°C in the GC oven before inserting it into the mass spectrometer for the first time.

EXPERIMENTS WITH LIPOXYGENASE PRODUCTS FROM CELL INCUBATIONS

To study the GC and the MS behaviour of the different metabolites of arachidonic acid we isolated these substances from platelet incubations ac-

cording to the methods of Hamberg and Samuelsson [2] and Nugteren [3], as well as from incubations with polymorphonuclear leucocytes (PMNL) following the procedure of Borgeat and Samuelsson [4]. Thereafter, we started our own experiments with fibroblast cultures.

EXPERIMENTS WITH FIBROBLAST CULTURES

Other groups [5—8] incubated fibroblast cultures with radioactively labelled arachidonic acid and measured the metabolites by radio-thin-layer chromatography (TLC) or they used cold incubations and detected the prostaglandins by radioimmunoassay. We used GC—MS for the detection of the hydroxy fatty acids.

Fibroblasts for culture were obtained from healthy humans. For the present study, two (500 cm^2) roller flasks, each containing about 2×10^7 fibroblasts in a monolayer, were used. After washing three times with physiological saline, the fibroblasts were incubated at 37°C and 3 rpm for 60 min in 15 ml of Dulbecco minimal essential medium (DMEM; Serva, Heidelberg, G.F.R.) containing 250 μg of arachidonic acid, 0.01% albumin, and 75 μg of ionophor A 23187 [8]. Thereafter, the metabolism was stopped with 10 ml of absolute ethanol, followed by extraction as described [9]. A preseparation was carried out by TLC on silica gel (Merck Kieselgel 60) plates (Merck, Darmstadt, G.F.R.), with the solvent system diethyl ether—hexane—acetic acid (60:40:1). Zones with R_F values of 0.06, 0.39 and 0.59 were scraped off and eluted. In order to increase the reliability of our results one part of the eluate was directly derivatised for GC—MS, the other part was hydrogenated prior to further derivatisation with platinum dioxide and hydrogen in methanol [10]. Thereafter, methylester-silylether or methylester-methyloxime-silylether derivatives were formed and analysed by GC—MS. Each substance was therefore detected twice, once in its originally present unsaturated form and further fully saturated, and each form was identified by retention time as well as by mass spectra in EI mode and in CI mode with ammonia as reactant gas. We found that the saturated hydroxy fatty acids gave a better separation on the GC column and further that identification is sometimes easier, as the saturated form gives more intense fragments in CI with ammonia than the unsaturated form. Fig. 1 shows the spectrum of 12-hydroxyeicosanoic acid and that of 12-hydroxyeicosatetraenoic acid in CI with ammonia.

RESULTS

Five to 60% of the arachidonic acid incubated with the fibroblasts are converted into prostaglandin E2 (PG E2). Small amounts of 12-hydroxyheptadecatrienoic acid (HHT) could be verified in the eluate from the TLC zone containing also the different eicosatetraenoic acids (HETE). Formation of HETE was presumed on the basis of radio-TLC investigations carried out by incubation of fibroblasts with radioactively labelled arachidonic acid. With our ongoing study we found that arachidonic acid is converted by photooxidation into various HETEs; the mass chromatogram (Fig. 2) shows the presence of 5-, 9-, 11-, 12-, 14-, and 15-HETE obtained from arachidonic acid incubations; the formation of 5,12-di-HETE is also presumed.

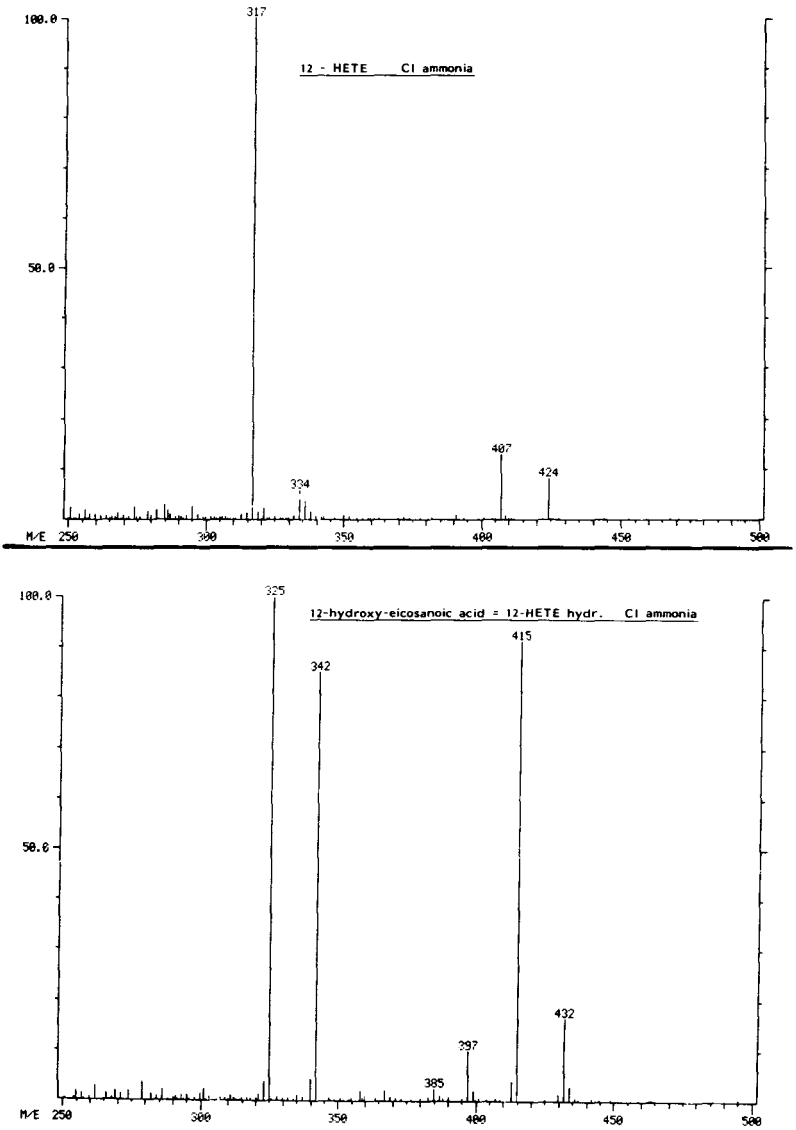

Fig. 1. Mass spectra of 12-HETE and its hydrogenated product (12-hydroxyeicosanoic acid) as methylester-silylether derivative, obtained with chemical ionisation using ammonia as CI gas.

DISCUSSION

The present study shows that the direct insertion of a fused-silica column into the ion source of a mass spectrometer is advantageous, the substances only being in contact with the coated wall of the GC column. With the present study we were able to prove that neither retention times nor number of theoretical plates are affected by this mode of coupling a GC column to the mass

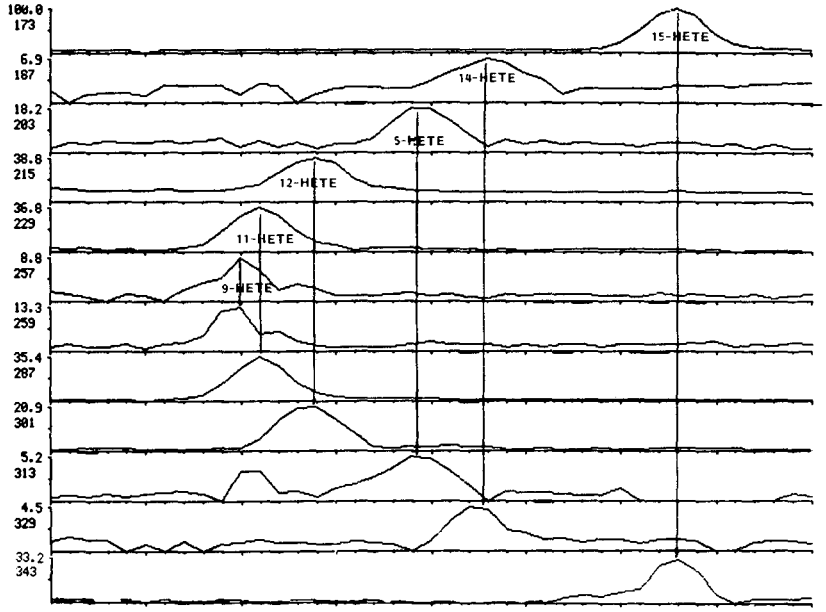

Fig. 2. Mass chromatogram of 5-HETE, 9-HETE, 11-HETE, 12-HETE, 14-HETE and 15-HETE in hydrogenated form as methylester-silylether derivatives in EI mode. The two main fragments of each substance, resulting from α-fragmentation, were monitored. The extract analysed was obtained from culture medium incubated with arachidonic acid and possibly demonstrates autooxidation.

spectrometer, which we therefore recommend. The validity of the method is demonstrated by analysing very labile arachidonic acid metabolites obtained from cell incubations. The identification of the different substances was carried out by comparison of the spectra with those obtained from substances isolated from incubations of arachidonic acid with platelets or with leucocytes according to the investigations of Hamberg and Samuelsson [2], Nugteren [3] and Borgeat and Samuelsson [4], as well as by comparison with published spectra. The two main fragments of the saturated hydroxy fatty acids result from *a*-fragmentation and help to differentiate the different HETEs since they are poorly separated by GC (Fig. 2).

The application of GC—MS enabled us to identify with high accuracy six different forms of HETE, namely 5-, 9-, 11-, 12-, 14-, and 15-HETE, by retention times of the natural product as well as of its saturated form and in both forms by MS in EI and CI modes. So far, identification of the different HETEs extracted from cell incubations would not pose further problems. The observation of a relatively rapid photo- or autooxidation of arachidonic acid into HETE but not into HHT suggests a very careful judgement of findings made on the enzymatic formation of HETE. We hope that we can precisely state if and which HETE is formed by fibroblast cultures. The widely varying amounts of PG E2 can probably be explained by the fact that prostaglandin formation is in close relation to the state of growth of the fibroblasts, as described by Taylor and Polgar [11].

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Note

Gas—liquid chromatographic analysis of intact long-chain triglycerides

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Direct gas—liquid chromatographic (GLC) analysis of triglycerides was first described more than 20 years ago [1—3]. Kuksis and co-workers [4—6] and Litchfield et al. [7] have made great advances in the theory and practical applications of this method. Triglycerides with carbon numbers of over 60 have been analysed by Litchfield et al. [8] and also by other authors [9, 10]. Quantitative analysis of low quantities of these compounds is very difficult because of their low recovery. In natural materials, triglycerides with higher carbon numbers appear primarily in the fat of sea animals. These triglycerides contain highly unsaturated long-chain fatty acids. This fact is the cause of their chemical heterogeneity resulting in poor GLC separation. Therefore, samples are often hydrogenated prior to analysis [8]. The hydrogenation is not difficult, but it represents several analytical operations that are not desirable in the analysis of large series of samples with low contents of triglycerides. The great number of analytical operations also has a negative effect on the reproducibility of the analysis. A further problem of the quantitative analysis of triglycerides with higher carbon numbers is commercial unavailability of these compounds in the pure form. Direct determination of the correction factors is not possible and the literature data cannot be generally used [11].

This paper describes optimization of the analytical conditions for the analysis of low amounts of long-chain triglycerides in blood plasma. Optimal conditions were defined by lowest possible values of f_w (weight correction factor; the ratio of weight percentage to area percentage for the individual compounds) and ΔC (peak resolution, which represents minimal carbon number difference between two compounds with baseline separation). Using the optimized method, which does not require sample hydrogenation, an effect of dietary mackerel fat on the plasma triglyceride composition was studied.

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MATERIALS AND METHODS

Materials

Triarachidin and tristearin were obtained from Supelco (Bellefonte, PA, U.S.A.). The Gas-Chrom Q support, 100—120 and 120—140 mesh, and the OV-1 stationary phase were supplied by Applied Science Labs. (State College, PA, U.S.A.) and Serva Feinbiochemica (Heidelberg, G.F.R.). The Supelcoport 100—120 mesh and the Chromosorb 750 100—120 mesh supports were supplied by Supelco. All solvents used were of analytical grade, supplied by Lachema (Brno, Czechoslovakia) and they were distilled before use.

Methods

The plot of f_w vs. the amount of injected compound was measured using the temperature programme, 5 or 8°C/min, and the optimized carrier-gas flow-rate (individually for each column). Before the measurement, recovery of the test compounds was stabilized by five injections of 5 μg of triarachidin. The plot of f_w vs. the carrier-gas flow-rate was measured using an injected amount of 300 ng of triarachidin and the temperature programme, 5 or 8°C/min. Analogously, the plot of f_w vs. the temperature programme rate was measured

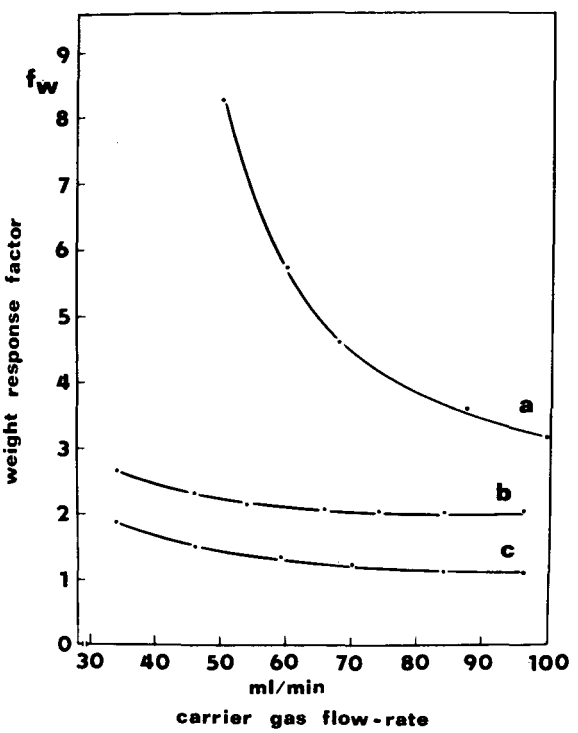

Fig. 1. Plot of f_w vs. the carrier-gas flow-rate for triarachidin measured with different columns at the same temperature programme rate (8°C/min) and the same analyzed amount of triarachidin (300 ng). (a) 3% OV-1 on Chromosorb 750, 100—120 mesh; (b) 1.5% OV-1 on Gas-Chrom Q, 120—140 mesh; (c) 1.5% OV-1 on Supelcoport, 100—120 mesh. For other analytical conditions see under Methods.

using the optimized carrier-gas flow-rate — concerning the peak resolution (ΔC) [7] and f_w values — for each column. Under the optimized conditions, the plots of ΔC vs. the carrier-gas flow-rate and the temperature programme rate were also measured. The samples contained the same amounts by weight of tristearin and triarachidin.

Apparatus and operating conditions

The analyses were performed on Perkin-Elmer gas chromatographs F-30, F-17 and 900. Peak areas were integrated using a Perkin-Elmer M-2 calculating integrator. In all cases, glass columns 0.5 m × 2.0 mm I.D. were used. The F-30 was equipped with on-column injection; the other gas chromatographs were equipped with the usual glass-lined injectors. Column packings were prepared in our laboratory by an evaporation technique. The columns were packed using the combined effect of suction and vibration.

Analytical conditions. Injector temperatures 300°C (F-30 and 900) and 320°C (F-17), detector temperatures 350°C (F-30 and 900) and 320°C (F-17), oven temperature programme 180—350°C, 2—10°C/min. Helium carrier gas was used at flow-rates of 30—180 ml/min. Quantitative analysis was performed using an internal standard method.

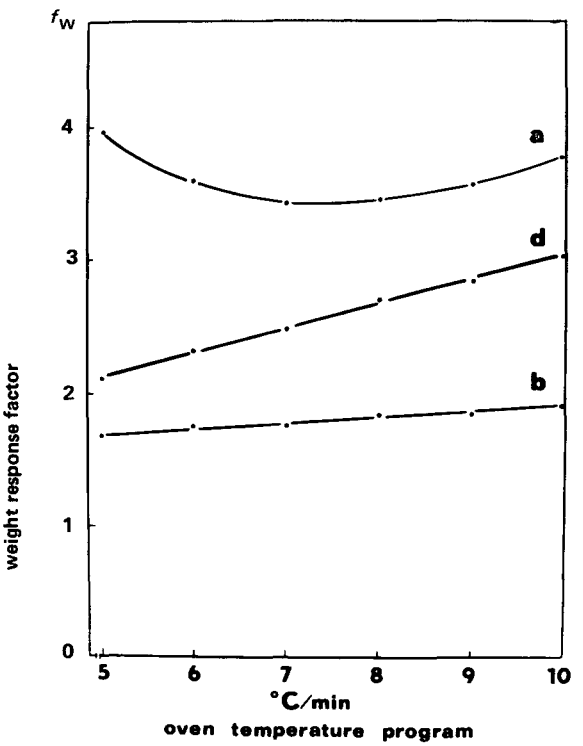

Fig. 2. Plot of f_w vs. the temperature programme rate for triarachidin measured with different columns at optimal carrier-gas flow-rates. (a) 3% OV-1 on Chromosorb 750, 100—120 mesh, carrier-gas flow-rate 90 ml/min; (b) 1.5% OV-1 on Gas-Chrom Q, 120—140 mesh, carrier-gas flow-rate 60 ml/min; (d) 1% OV-1 on Gas-Chrom Q, 100—120 mesh, carrier-gas flow-rate 44 ml/min. For other analytical conditions see under Methods.

RESULTS

First, the effect of different supports and loading with stationary phase on the recovery of the model compound, triarachidin, was studied. The f_w value was the criterion of the recovery of the compounds analysed; the values of the weight correction factors for 315, 100 and 56 ng of triarachidin are given in Table I. These values are also dependent on other parameters, especially on the carrier-gas flow-rate and the temperature programme rate. The course of these plots for some of the columns is given in Figs. 1 and 2. In addition to the recovery of triglycerides with high carbon numbers, the column should also attain the highest possible peak resolution. This value is again dependent on the above parameters. Our results obtained with the selected columns are shown in Figs. 3 and 4. Some practical applications of the results of the optimization in the analysis of neutral blood plasma lipids are shown in Fig. 5.

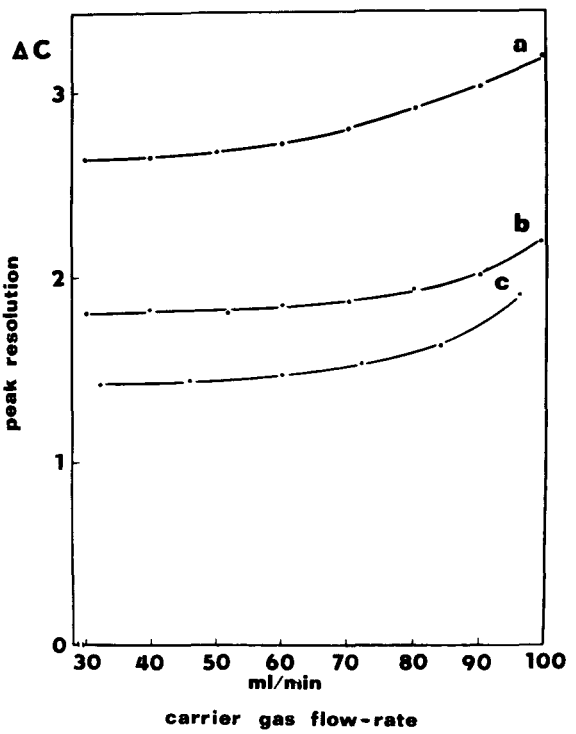

Fig. 3. Plot of ΔC vs. the carrier-gas flow-rate for triglycerides C_{54}—C_{60} using different columns. Analytical columns are the same as in Fig. 1.

DISCUSSION

The course of the plot of f_w vs. the injected amount of triarachidin is different for each individual instrument, even if the same column packing is used, as is shown in Table I. This observation is in good agreement with the results published by Litchfield et al. [7]. The course of the plot mentioned above and the f_w values are similar when they are measured with a series of

TABLE I

THE f_w VALUES FOR TRIARACHIDIN MEASURED WITH DIFFERENT COLUMNS UNDER OPTIMIZED CONDITIONS

Column	Instrument	Packing	Carrier-gas flow-rate (ml/min)	Oven temperature programme rate (°C/min)	f_w*		
					315 ng	100 ng	56 ng
A-33	F-30	1% OV-1 on Gas-Chrom Q, 100—120 mesh, batch No. 749	67	5	1.97	3.10	4.20
A-43	F-30	1% OV-1 on Gas-Chrom Q, 100—120 mesh, batch No. 749	75	5	2.02	3.10	4.10
A-51	F-17	1% OV-1 on Gas-Chrom Q, 100—120 mesh, batch No. 749	72	5	2.04	5.67	>10
B-79	F-30	1.5% OV-1 on Gas-Chrom Q, 120—140 mesh, batch No. 1422	60	8	2.74	6.77	9.38
A-74	F-30	3% OV-1 on Chromosorb 750, 100—120 mesh, batch No. 102	90	8	2.43	5.18	9.95
B-69	F-30	1% OV-1 on Gas-Chrom Q, 100—120 mesh, (Serva, batch No. 46559)	44	8	2.16	3.10	4.65
A-48	900	1% OV-1 on Gas-Chrom Q, 100—120 mesh, batch No. 749	77	5	3.16	5.94	9.43

*Each value represents the mean of two measurements. Values of f_w were measured with a C.V. better than 5% (calculated from 50 measurements).

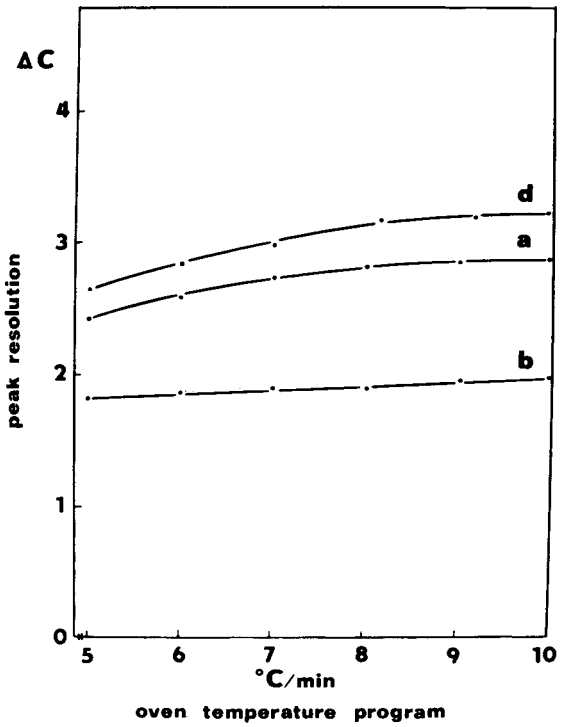

Fig. 4. Plot of ΔC vs. the temperature programme rate measured with the different columns. Analytical conditions are the same as in Fig. 2.

columns of the same length and packing, using the same instrument. Different f_w values were measured for the same analysed amount of triarachidin when different supports and different loading with stationary phase were used.

The f_w values for the given column and instrument are also dependent on the carrier-gas flow-rate and the temperature programme rate. These results are also in agreement with those published by Litchfield et al. [7] for the region of higher concentrations, where the f_w values are independent of the injected amount, and for triglycerides with lower carbon numbers.

The course of the plot of f_w vs. the carrier-gas flow-rate was the same for all the columns tested. The column packed with 3% OV-1 on Chromosorb 750 yielded high f_w values for low carrier-gas flow rates. On the other hand, the columns with lower loading of the support with the stationary phase yielded a sufficient recovery of triarachidin using relatively low carrier-gas flow rates.

The plot of f_w vs. the temperature programme rate was different for the different columns. The columns with the lower loading of the support with the stationary phase yielded a linear plot of f_w vs. programme rate. The same plot was non-linear using columns packed with 3% of OV-1 on Chromosorb 750. So far, we do not have a satisfactory explanation for this effect, which was observed repeatedly.

The higher values of the carrier-gas flow-rate, which are necessary for adequate f_w values (measured using the columns with 3% OV-1) result in decreased peak resolution. From this point of view, it is more advantageous to

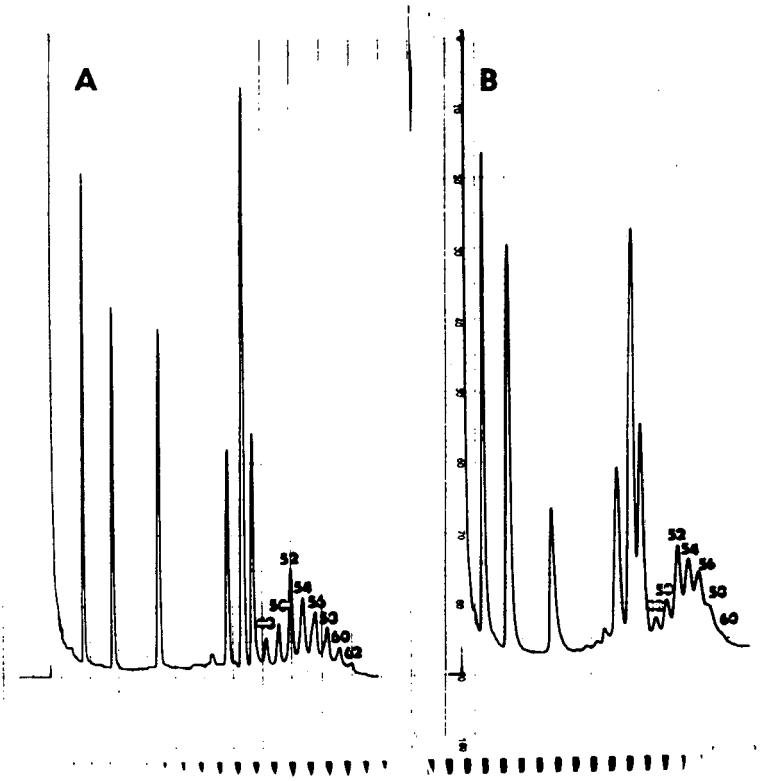

Fig. 5. (A) Gas chromatogram of plasma neutral lipids after the experimental diet containing the mackerel fat. Column was packed with 1.5% OV-1 on Gas-Chrom Q, 120—140 mesh. (B) Gas chromatogram of the same sample as in Fig. 5A. Column was packed with 3% OV-1 on Chromosorb 750, 100—120 mesh. For analytical conditions see under Methods and Table I. The figures 48—62 correspond to the carbon numbers of individual triglyceride fractions (carbon number is defined as the sum of the carbon atoms of the individual fatty acid moieties).

use the columns with a lower loading of the support with the stationary phase. Such columns yield markedly lower f_w values using 2—3 times lower flow-rates.

The peak resolution dependence on the temperature programme is similar for the different columns. It is probable that neither the ΔC value nor the course of the plot are basically different for the different loading of the support with the stationary phase. The high peak resolution of the columns with low loading can be illustrated by the results of the analysis of a sample of blood neutral lipids, which contains markedly heterogeneous triglycerides. The weight limit for the quantitative analysis of triglycerides with carbon number of over 60 is about 50 ng.

CONCLUSIONS

Quantitative analysis of higher molecular weight triglycerides in the concentration region where the f_w values are dependent on the amount

analyzed, requires individual optimization of the parameters for each column. Columns with a lower loading of support with the stationary phase yield more favourable values for the weight correction factors using relatively low carrier-gas flow-rates, and their peak resolution is good. The exact explanation of the role of the support under limit conditions of gas chromatography is very complicated and requires further experiments.

Comparing the supports under study, very good results were obtained using Gas-Chrom Q and Supelcoport, but significant differences were observed between individual batches of the same support. The quality of the column is also influenced by the coating technique of the support with the stationary phase, as well as by the technique of column packing and other factors already described [7, 9].

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Note

Gas—liquid chromatography profiling of intact lipids

Observation of differences between triglyceride structure of lipoproteins in type III and type IV hyperlipoproteinemia

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In recent years there has been great interest in the study of the metabolism of twenty-carbon polyunsaturated fatty acids which are precursors of prostaglandins and leukotrienes. For these studies efficient chromatographic techniques have been used. Kuksis et al. [1] introduced in 1967 the gas chromatographic analysis of intact lipids. Smith et al. [2] used high-performance liquid chromatography for the study of heterogeneous triglyceridemia in animal models. Mareš and co-workers [3, 4] modified the original method of Kuksis for gas chromatographic profiling of human plasma intact neutral lipids in the nanogram range; the method allows further study of subtle changes in triglyceride composition of individual plasma lipoprotein classes [3, 4].

In the present study we have monitored the triglycerides of total plasma and the triglycerides of individual plasma lipoprotein classes. The purpose of the study was to compare the molecular weight distribution of triglycerides with the corresponding fatty acid profile. Triglyceride composition was studied in subjects suffering from heterogeneous triglyceridemia. They were given an experimental diet supplemented with marine fish rich in very-long-chain fatty acids.

METHODS

Two female patients were given a diet supplemented with mackerel 300—400 g/day for a period of five days. Patient No. 1, 53 years old, had familial dyslipoproteinemia type III. She has had persistent heterogeneous triglyceridemia since our first contact with her [5]. Patient No. 2, 59 years old, was an insulin-dependent diabetic and her diet was adapted to this conditions. She had

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secondary hyperlipoproteinemia type IV and intermittent heterogeneous triglyceridemia.

Venous blood was drawn for analysis after an overnight fast before starting the experiment and after five days of mackerel-supplemented diet; EDTA was used as anticoagulating agent in a concentration of 1 mg/ml. For lipoprotein separation a sample of blood was drawn the fifth day of the experiment, 4 h after the midday meal. Individual plasma lipoprotein classes [chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL)] were isolated by preparative ultracentrifugation. The purity of the individual lipoprotein classes was checked by agarose gel electrophoresis.

Neutral lipid profile

Neutral lipids were extracted from 250 μl of plasma using 5 ml of extraction mixture composed of equal volume parts of chloroform, methanol and acetone. Polar lipids were removed by Florisil added to each plasma sample before the extraction. Lipids were analysed on a 0.5-m glass column packed with 3% OV-1 on Gas-Chrom Q 100—120 mesh under conditions already described [3].

Fatty acid analysis

Plasma lipids and lipoprotein fractions were extracted according to the procedure of Folch et al. [6]. Individual lipid classes (cholesteryl esters, triglycerides, and phosphatidylcholines) were separated by thin-layer chromatography on silica gel $HF_{254+366}$ plates (E. Merck, Darmstadt, G.F.R.). For the separation of cholesteryl ester and triglycerides, plates were developed in *n*-heptane—diethyl ether—acetic acid (85:15:1, v/v). Phosphatidylcholines were developed in chloroform—methanol—water (60:30:5, v/v). Separated lipid classes were detected under an UV lamp (366 nm) after the spraying with 0.01% 2,7-dichlorofluorescein in ethanol. Spots were scraped off and the lipids were isolated using the "dry column" technique. Cholesteryl esters and triglycerides were eluted three times with 2-ml portions of elution mixture composed of equal volume parts of chloroform and methanol. Phosphatidylcholines were eluted by the same technique using a mixture of chloroform—methanol—water—acetic acid (13:5:1:0.2, v/v). All lipid classes were transesterified using 0.5 *N* sodium methoxide and analyzed by gas—liquid chromatography (GLC) under the following conditions: gas chromatograph Perkin-Elmer Sigma 1, injector and detector temperature (250° C, oven programme from 120° C to 200° C (rate 2.5° C/min), flame ionization detector, attenuation 10 × 8, column 1.8 m × 2 mm I.D., glass, packed with 10% OV-275 (Supelco, Bellefonte, PA, U.S.A.) on Chromosorb W AW 80—100 mesh (Carlo Erba, Milan, Italy). For quantitative analysis an internal standard method was used and results were calculated by means of a Perkin-Elmer Sigma 10 Laboratory Data Station. The reproducibility of the results was checked using control samples prepared from pure compounds.

RESULTS

Fatty acid composition of plasma triglycerides

The fatty acid composition of whole plasma triglycerides after five days of mackerel-supplemented diet is shown in Table I. Twenty-carbon and 22 carbon polyenes and monoenes which are characteristic of mackerel oil were correspondingly higher after the diet. The effect of the diet was the same in both subject.

TABLE I

FATTY ACID COMPOSITION OF WHOLE PLASMA TRIGLYCERIDES BEFORE AND AFTER THE DIET

Results are expressed as mol%.

Fatty acid	Patient No. 1, dyslipoproteinemia type III		Patient No. 2, hyperlipoproteinemia type IV	
	Before	After	Before	After
14:0	1.8	2.5	1.5	1.6
16:0	27.7	17.8	31.2	24.9
16:1	5.0	3.6	5.6	4.3
18:0	2.1	2.6	2.2	2.9
18:1	46.1	29.0	48.2	35.2
18:2 (*n*—6)	13.6	9.6	8.8	8.2
18:3 (*n*—6)	0.4	0.2	0.2	0.3
18:3 (*n*—3)	0.7	1.2	0.6	0.9
20:0	0.2	0.1	0.3	0.1
20:1	0.3	3.3	0.3	1.4
20:3 (*n*—6)	0.2	0.1	0.2	0.1
20:4 (*n*—6)	1.0	2.1	0.5	1.2
20:5 (*n*—3)	0.2	11.9	<0.1	7.3
22:1	<0.1	1.6	<0.1	0.9
22:5 (*n*—3)	0.2	1.1	0.1	1.0
22:6 (*n*—3)	0.3	13.3	0.4	9.7
Totals of C_{20} fatty acids	1.9	17.5	1.3	10.2
Totals of C_{22} fatty acids	0.6	16.1	0.5	11.6

Fatty acid composition of lipoprotein triglycerides

Table II shows the fatty acid composition of plasma lipoprotein classes after the mackerel-supplemented diet. There is a considerable proportion of very-long-chain fatty acids in all lipoproteins with a decrease from low-density to high-density classes.

Molecular-weight profile of lipoprotein triglycerides

Fig. 1 demonstrates the molecular-weight profiles of neutral lipids isolated from individual lipoprotein classes. The patterns of triglyceride look more

TABLE II

FATTY ACID COMPOSITION OF PLASMA LIPOPROTEIN TRIGLYCERIDES AFTER THE DIET

Results are expressed as mol%.

Fatty acid	Patient No. 1, dyslipoproteinemia type III				Patient No. 2, hyperlipoproteinemia type IV			
	CM	VLDL	LDL	HDL	CM	VLDL	LDL	HDL
14:0	4.5	3.6	2.7	3.4	3.5	2.6	2.3	2.9
16:0	14.7	19.5	17.4	20.1	16.6	24.7	23.8	23.5
16:1	3.8	4.5	3.7	5.6	3.2	3.8	3.9	5.3
18:0	2.2	2.9	2.7	3.2	2.6	2.9	3.4	5.0
18:1	25.3	26.6	29.5	29.5	30.4	33.6	36.3	35.1
18:2 (*n*—6)	6.3	9.0	9.7	9.4	6.8	7.8	8.0	8.1
18:3 (*n*—6)	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3
18:3 (*n*—3)	2.3	1.5	1.2	1.2	2.0	1.4	1.3	1.5
20:0	tr.	<0.1	<0.1	<0.1	tr.	0.2	0.2	0.3
20:1	12.2	4.5	3.6	3.4	8.9	3.4	2.9	2.7
20:3 (*n*—6)	0.2	0.2	0.1	0.1	0.2	0.1	0.1	<0.1
20:4 (*n*—6)	0.9	1.8	2.1	2.0	0.8	0.8	1.2	1.3
20:5 (*n*—3)	6.9	9.5	11.4	8.5	7.5	5.3	5.0	4.3
22:1	11.1	3.6	1.7	1.8	8.9	2.8	2.1	1.6
22:5 (*n*—3)	1.3	1.4	1.1	1.3	1.2	2.2	1.4	2.1
22:6 (*n*—3)	8.2	11.3	12.8	10.0	7.4	8.3	7.6	5.9
Totals of C_{20} fatty acids	20.2	16.0	17.4	14.2	17.5	9.8	9.4	8.6
Totals of C_{22} fatty acids	20.5	16.2	15.6	13.2	17.5	13.3	11.1	9.6

informative than the corresponding fatty acid profiles. From the digital equivalents in Fig. 2 it is evident that the carbon-number* distribution of chylomicron triglycerides was rather symmetrical; it was very close to the random distribution calculated from the compositon of the fatty acids. The carbon-number profile of VLDL triglyceride reveals a tendency to non-randomness and this trend was again more expressed in LDL triglycerides. The composition of HDL triglycerides showed evidence of non-random selection of triglyceride fatty acids. Higher-molecular-weight species in the HDL triglyceride profile were only minimal.

DISCUSSION

Heterogeneous triglyceridemia in human subjects is characterized by a relative elevation of higher-molecular-weight triglyceride species containing

*For cholesteryl esters the carbon number corresponds to the total number of carbon atoms, whereas for triglycerides it corresponds to the sum of the carbon atoms in the fatty acid moieties.

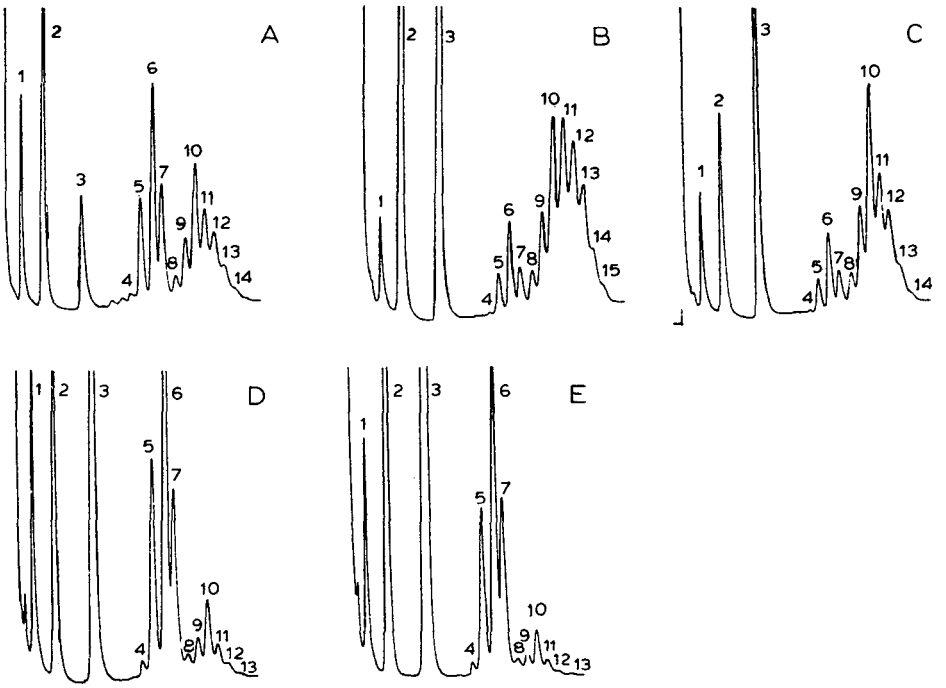

Fig. 1. Neutral lipid profiles of plasma and individual lipoprotein classes of the same subject. (A) Whole plasma; (B) chylomicrons; (C) VLDL; (D) LDL; (E) HDL. 1 = Cholesterol, 2, 3 = standards, 4—7 = cholesteryl esters with carbon numbers 41—47, 8—15 = triglycerides with carbon numbers 48—62. For analytical conditions see Methods section.

very-long-chain fatty acids. The anomaly is present in about 25% of hypertriglyceridemias but it can also be found in subjects with normal plasma triglyceride levels [5].

It is very easy to induce the condition with a diet rich in very-long-chain fatty acids as, for instance, contained in marine fish oils, but in some subjects the anomaly persists even if very-long-chain fatty acids are excluded from the diet as we have observed in our patient No. 1, who is an index member of a dyslipoproteinemia type III family referred to previously [5]. We have studied the effect of mackerel diet on the fatty acid composition and molecular-weight profile of triglycerides both in whole plasma and in isolated lipoprotein fractions.

Chylomicron triglycerides were characterized by a high proportion of very-long-chain triglyceride fractions with carbon numbers of 60, 62 and even 64. The triglyceride component of chylomicrons is formed in the intestinal mucosa, mostly from exogenous dietary fatty acids. Molecular-weight distribution is very close to the theoretical random distribution of fatty acids of triglyceride molecules. With increasing density of lipoprotein particles the effect of non-random selection of fatty acids for triglyceride molecule formation is more and more evident. In HDL triglycerides the proportion of high-molecular-weight species is minimal even if very-long-chain fatty acids are still present in high proportions.

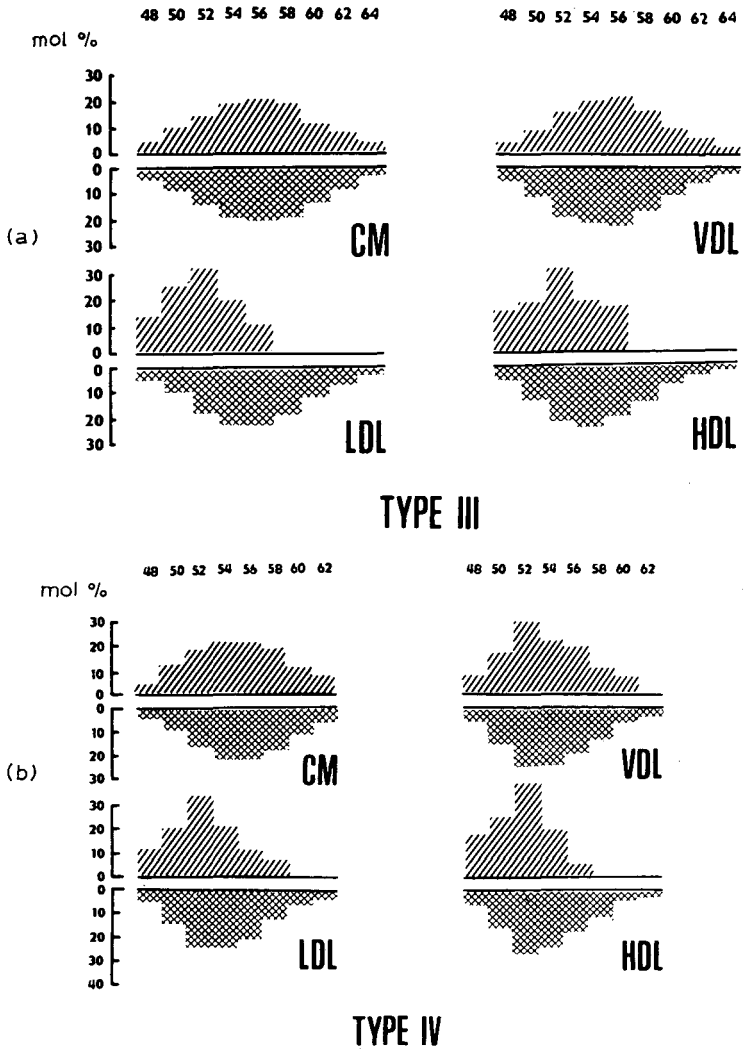

Fig. 2. Molecular-weight distribution of lipoprotein triglyceride components in type III and type IV hyperlipoproteinemia. Comparison of GLC-assessed values (above) with theoretical values calculated as random distribution of fatty acids in triglyceride molecules (below).

The essential divergence between fatty acid composition and molecular-weight profile of lipoprotein triglyceride species is most evident from geometrical representation in multidimensional euclidean space. Let us represent lipoprotein triglycerides as vectors calculated as molar per cent carbon number fractions in one case and as molar per cent fatty acids in the other case. Sets of angles among vectors in both systems were quite different. Projection of tips of vectors on the same plane generated a map of distances between lipoprotein triglyceride in both systems (Fig. 3). It is evident that the intact lipid profile was much more informative for discovering subtle differences among triglycerides composed of similar sets of fatty acids but in distinct non-random combinations.

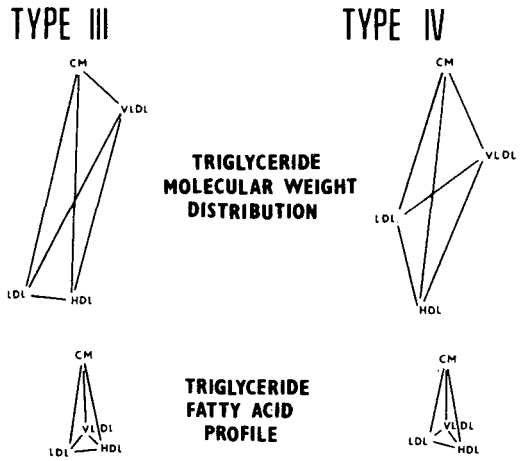

Fig. 3. Map of distances between lipoprotein triglyceride structure in type III and type IV hyperlipoproteinemias. Distances were calculated as angles between vectors whose elements are the molecular-weight fractions (mol %) of the lipoprotein triglyceride component (top), or fatty acids (mol %) of the same lipoprotein triglycerides (bottom). Tips of vectors are projected on the same plane and relative distances were calculated.

CONCLUSIONS

(1) GLC carried out on a column with a low coating of stationary phase is able to analyse quantitatively high-molecular-weight fractions of triglycerides with very good reproducibility and sensitivity.

(2) Heterogeneous triglyceridemia in man is characterized by a relative elevation of high-molecular-weight fractions of plasma triglycerides.

(3) Molecular-weight distribution in comparison with the corresponding fatty acid profile of triglyceride components in the lipoprotein metabolic cascade was studied in a patient with familial type III dyslipoproteinemia and in a patient with secondary type IV hyperlipoproteinemia after a diet rich in very-long-chain fatty acids.

(4) In both patients molecular-weight profiling of triglycerides revealed a trend from randomness to non-randomness in the selection of fatty acid triplets for triglyceride molecules.

(5) For the detection of heterogeneous triglyceridemia and to follow the metabolism of high-molecular-weight fractions, intact lipid GLC profiling is a more informative method than fatty acid analysis.

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Note

Influence of corticosteroid therapy on the fatty acid composition of serum lecithins monitored by means of thin-layer and gas—liquid chromatography

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One of the most provoking problems in clinical neurology is the elucidation and treatment of multiple sclerosis. According to some studies [1—6], symptoms of polyunsaturated fatty acid deficiency in multiple sclerosis patients were found.

In this preliminary trial we have studied the fatty acid pattern in serum choline phosphoglycerides (CPG) in patients other than multiple sclerosis patients. Comparison of untreated and prednisone-treated patients suffering from various diseases could elucidate whether corticosteroid therapy has any effect on the fatty acid composition of serum lecithins. CPG was studied as it is a principal phospholipid class of mammalian tissue [7] which is easy to isolate and transmethylate [8]. From the methodological point of view, the most suitable method in following the phospholipid fatty acid pattern is a combination of thin-layer chromatography (TLC) and gas—liquid chromatography (GLC). In this study a modified procedure of Karlsson et al. [8] was used.

MATERIAL

Group A: five women with progressive polyarthritis, mean age 56 years, not treated with corticosteroids. Group B: eleven patients (two men and nine women, mean age 61.3 years) treated for years with prednisone (10—20 mg/day). Group C: eight patients (four men and four women, mean age 58 years) with osteoarthrosis (coxarthrosis, gonarthrosis) treated with daily doses of 10—20 mg of prednisone for several months. Group D: eight asthmatics treated for several weeks by daily doses of 20—50 mg of prednisone. Group E: five patients of group D studied 1.5 h after parenteral application of 0.25 mg of adrenocorticotropin (ACTH). Group F: twelve healthy blood donors (men, mean age 28 ± 5 years, 178 cm, 81 kg).

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METHODS

For GLC a Perkin-Elmer F-30 gas chromatograph equipped with dual flame ionization detectors and an electronic integrator SIP 1 was used. A Chromaton N-AW DMCS, 0.125—1.16 mm (No. 751194, Lachema, Brno, Czechoslovakia) coated with 15% DEGS. The carrier gas was nitrogen at a flow-rate of 40 ml/min; injection and detection temperature was 250° C.

For TLC silica gel plates (Fertigplatten Merck No. 5721; Merck, Darmstadt, G.F.R.) were used in a saturated chamber. The mobile phase was choroform—methanol—water—acetic acid (65:35:5:1, v/v).

For sample preparation, venous blood was taken after 12 h of fasting and the serum stored at —20° C until analysis. Extraction of lipids was performed with chloroform—methanol (1:1, v/v) [8]. Total lipid extract corresponding to 0.1 ml of serum was applied on silica gel plates and separated in chloroform—methanol—water—acetic acid (65:35:5:1) at room temperature (Fig. 1). The

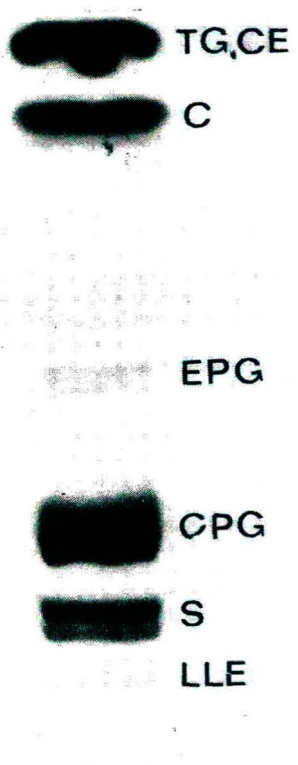

Fig. 1. TLC of total serum lipid extract (preparative, TLC, 0.1 ml of serum). C = Cholesterol, CE = cholesterol esters, TG = triglycerides, EPG = ethanolamine phosphoglycerides, CPG = choline phosphogylcerides, S = sphingomyelins, LLE = lysolecithins. Mobile phase: chloroform—methanol—water—acetic acid (65:35:5:1, v/v), silica gel G (Merck Fertigplatten No. 5721); detection copper acetate reagent.

CPG fraction after visualization with bromphenol blue were scraped off and dried in a dessicator over phosphorus pentoxide for 48 h. Transmethylation was done with 2 ml of 0.2 mol/l sodium methoxide in methanol at 37° C for 60 min. The fatty acid methyl esters (FAME) after acidification were extracted into petroleum ether, dried under N_2 and redissolved in 5 μl of petroleum ether, of which 2 μl were analysed by GLC (isothermal at 180° C). The peaks were identified by comparing their retention times with those of pure FAME (Applied Science Labs., State College, PA, U.S.A., and Serva, Heidelberg, G.F.R.). Values are given in molar per cent (18:1 = 1).

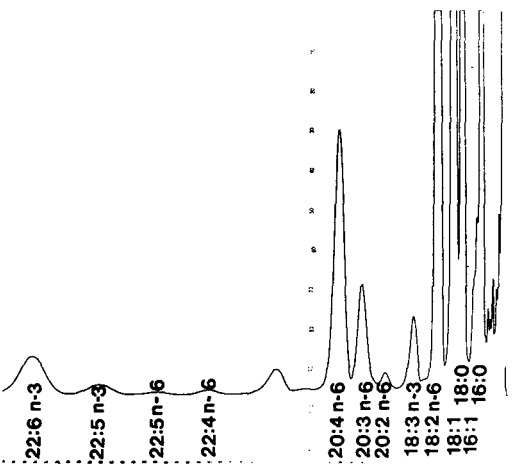

Fig. 2. GLC of serum lecithin FAME (see text for experimental details).

RESULTS

Mean values and standard deviations (S.D.) of FAME in serum CPG are presented in Table I.

Group A (non-prednisone-treated patients with progressive polyarhtritis) shows in comparison with healthy blood donors (group F) a significant decrease in linoleic acid (18:2, $n-6$) and docosahexaenoic acid (22:6, $n-3$), an increase in oleic acid (18:1) and surprisingly also arachidonic acid (20:4, $n-6$).

In all groups (B, C, D, E) treated with prednisone the levels of linoleic, arachidonic and docosahexaenoic fatty acids seem to be normalized. The amount of linoleic acid still remains lower than in healthy blood donors (group F).

There were no statistically significant differences in serum CPG FAME pattern in asthmatics under chronic treatment with prednisone (group D) and in those patients (group E) studied 1.5 h after ACTH application.

The level of polyunsaturated fatty acids in serum lecithins seems to be influenced by prednisone in chronic experiments. An acute bolus of ACTH (and endogenous steroids) evokes no apparent change in serum CPG FAME composition.

TABLE I

FATTY ACID METHYL ESTERS (FAME) OF SERUM LECITHINS IN NON-PREDNISONE-TREATED PROGRESSIVE POLYARTHRITIS (PAP) PATIENTS AND IN PREDNISONE-TREATED PATIENTS

FAME	PAP $\bar{x}$ ± S.D., $n = 5$		Treated with prednisone								Healthy blood donors $\bar{x}$ ± S.D., $n = 12$	
			PAP $\bar{x}$ ± S.D., $n = 11$		Osteoarthroses $\bar{x}$ ± S.D., $n = 8$		Asthmatics $\bar{x}$ ± S.D., $n = 8$		Asthmatics + ACTH $\bar{x}$ ± S.D., $n = 5$			
16:0	30.30	2.37	33.07*	1.86	31.11	1.66	32.60*	2.32	33.09*	1.77	31.63	1.34
16:1	2.20	1.03	1.13*	0.60	1.70	0.40	1.31	0.56	1.57	0.88	0.71**	0.28
18:0	15.28	0.96	16.81*	1.66	15.87	1.48	14.90	0.99	14.96	0.79	15.31	0.49
18:1	14.72	1.51	14.93	1.00	14.23	1.83	13.80	0.43	13.70	0.44	13.56**	0.43
18:2 (n—6)	18.90	1.21	20.79*	2.39	21.20*	1.43	21.23*	1.59	21.43*	1.32	23.42**	1.50
18:3 (n—3)	0.97	0.85	0.53	0.11	0.46	0.13	0.47	0.09	0.59	0.19	0.45	0.05
20:2 (n—6)	0.89	0.95	0.39	0.51	0.37	0.16	0.52	0.29	0.45	0.16	0.36	0.04
20:3 (n—6)	4.20	0.76	2.61*	0.91	3.23	0.54	2.93*	0.64	2.96	0.47	2.57**	0.71
20:4 (n—6)	9.30	1.72	7.27*	1.32	8.70*	2.11	9.23*	1.38	8.25	2.18	8.88**	0.66
22:4 (n—6)	0.24	0.06	0.56	0.37	0.30	0.28	0.85	1.85	0.14	0.15	0.03	0.04
22:5 (n—6)	0.11	0.05	0.03	0.04	0.09	0.02	0.08	0.08	0.08	0.10	—	
22:5 (n—3)	0.82	0.29	0.32	0.25	0.59	0.25	0.39	0.26	0.55	0.04	0.56	0.43
22:6 (n—3)	0.24	0.76	1.55*	0.71	2.15*	0.98	1.69*	0.75	2.25*	0.73	2.51**	1.08

*Significant on 0.05 level (Bartlett's test): non-prednisone-treated against prednisone-treated.
**Significant on 0.05 level: blood donors against non-prednisone-treated progressive polyarthritics.

It seems that corticosteroids have some influence on metabolism of polyunsaturated fatty acids. Therefore it is necessary during studies of fatty acid profiles in multiple sclerosis patients to separate those who are under treatment with corticosteroids and those who are not. A detailed control study with a monitored lipid diet is necessary.

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Note

Gas—liquid and column liquid chromatography for studying vitamin U metabolism in humans and animals

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In recent years the mortality rate from gastrointestinal diseases has increased all over the world, evidently due to greater environmental contamination. Normally, peptic and duodenal ulcers as well as gastritis are treated with the synthetic vitamin U preparation (D,L-S-methylmethionine sulfonium chloride) [1]. Vitamin U was first isolated from cabbage juice [2] and parsley leaves [3]. Later it was detected in various natural sources, mainly of plant origin [4—6]. This allowed a wide use of food products with a high content of vitamin U (different varieties of cabbage, beets, parsley, celery, etc.) for the prophylaxis of gastrointestinal diseases.

In view of the important biological role of vitamin U, great attention is paid to the methods of studying its metabolism in the human and animal body. Existing microbiological and chemical methods are of low specificity and are laborious and time-consuming [7—9]. Emphasis should be given to chromatographic techniques, particularly to liquid and gas—liquid chromatography.

The present paper describes the study of vitamin U assimilation in animals and humans by means of chromatographic methods.

EXPERIMENTAL

Apparatus

The experiments were carried out using a gas chromatograph Chrom-41 (Laboratory Equipment, Prague, Czechoslovakia) with a flame ionization detector. It was supplied with a glass column (150 × 0.3 cm) packed with Polychrome-1 (Olaine Chemical Producing Plant, Riga, U.S.S.R.) having 0.25—0.5 mm grains impregnated with 10% Carbowax 20M. The temperature of the column was 70°C and that of the evaporator and detector 130°C. The carrier gas was helium flowing at a rate of 30 ml/min. Hydrogen and air flow-rates were 25 and 250 ml/min, respectively.

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The spectropolarimeter used was a Jasco Model J 40-AS (sensitivity 20 × 10^{-4} degrees/cm, time constant 16 sec and chart speed 5 × 10^{-1} cm/min).

Reagents

The reagents used were: D,L-S-methylmethionine sulfonium chloride (MMSCI or MMS for free base) of 96,3% purity (Ufa Vitamin-Producing Plant, Ufa, U.S.S.R.); toluene, carbon tetrachloride and dimethylsulfide were analytical reagent grade.

Animals

The experiments were carried out on Wistar white rats weighing 200 g. MMSCI in the form of a 2% aqueous solution was administered per os at a dose of 10 mg per 100 g body weight. The rats were decapitated at different time intervals after drug administration. Immediately after decapitation blood and viscera were removed and placed on ice. The amount of MMS excreted was measured in 24-h urine samples collected from five rats.

Gas—liquid chromatography

A batch of the tissue (liver, kidneys) was homogenized in cooled borate buffer pH 9.8—10 at a ratio of 1:2 (w/v). The suspension was transferred to 10-ml vials tightly stoppered with rubber caps. Blood and urine (3—5 ml) were placed into vials and the solution was made up to 10 ml with borate buffer. The vials were quickly heated on a boiling water bath and then autoclaved for 30 min at 120°C and 0.2 MPa. After cooling 40—100 μl of *n*-octanol (to prevent foaming) were injected through the rubber stopper with the aid of a microsyringe, and the resultant dimethyl sulfide was distilled in the apparatus shown in Fig. 1. The temperature of the water bath was 80°C, the nitrogen flow-rate was 45 ml/min, and the distillation time was 30 min. Dimethyl sulfide was trapped in a toluene-containing trap cooled with a mixture of dry ice and acetone; 1 μl of toluene solution was analyzed in the gas chromatograph.

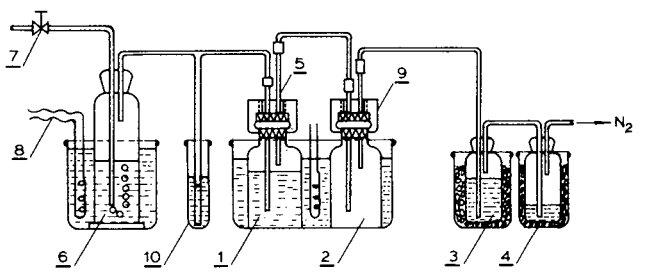

Fig. 1. Schematic diagram of the apparatus for dimethyl sulfide isolation from samples after MMS degradation. 1 = Vial with a sample homogenate, 2 = intermediate vial, 3 = trap with 40% $NaHSO_3$ cooled with ice, 4 = trap with toluene cooled with dry ice in acetone, 5 = syringe needle, 6 = Drexel bottle for nitrogen heating, 7 = fine-adjustment valve, 8 = heater, 9 = clamp, 10 = mercury cut-off seal.

Liquid chromatography

Cooled liver and kidneys were homogenized with two volumes of distilled water and supplemented with trichloroacetic acid to adjust the final concentra-

tion to 10%. The homogenates were centrifuged and the clear solution was extracted three times with sulfuric ether to remove the trichloroacetic acid. Aqueous solutions were evaporated in vacuo on a rotor evaporator at max. 40°C. The residue was extracted with 80% methanol and the precipitate discarded. The water—methanol solution was evaporated to bring the volume to 0.5—1.0 ml. The cooled urine was filtered and the filtrate evaporated in vacuo. The dry residue obtained in this way was dissolved in 5—10 ml of 80% methanol and the precipitate was discarded. The solution was concentrated to bring the final volume to 0.5—1.0 ml.

The resultant concentrates containing MMS were placed on a column packed with 15 g of silica gel (Woelm, Eschwege, G.F.R.) in 90% methanol. The column was washed with 90% methanol to eliminate completely the yellow zone. The MMS was eluted in the solvent system methanol—formic acid—water (4:1:4). Fractions of 1 ml were collected and assayed by thin-layer chromatography on silica gel plates (Silufol, Prague, Czechoslovakia) in the solvent system *sec.*-amyl alcohol—formic acid—water (4:4:2) (detection with ninhydrin). The vitamin U-containing fractions were combined and evaporated in vacuo. The quantity of isolated vitamin was measured by gas—liquid chromatography.

Circular dichroism

Circular dichroic spectra of the isolated vitamin U preparation were recorded after treatment with *o*-phthalaldehyde in the presence of mercaptoethanol [10].

RESULTS AND DISCUSSION

The gas chromatographic assay of vitamin U was performed with respect to dimethyl sulfide, its decomposition product which is formed in equimolecular quantities as a result of S-methylmethionine degradation in alkaline medium following the reaction

$$OH^- + (CH_3)_2S^+\text{-}CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOO^- \rightarrow (CH_3)_2S + HOCH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOO^-$$

The yield of dimethyl sulfide is 91% (heating at 120°C and pH 9.7—10 for 30 min).

Carbon tetrachloride was used as internal standard. The calibration curve was established and calculations were made as described previously [11]. Gas chromatograms are given in Fig. 2.

In order to determine the sensitivity and error of the method used, MMSCI was added to homogenates of different rat organs. The data obtained indicate a direct correlation between the amount of vitamin U administered and measured in the concentration range 0.02—1.0 mg/g. The lowest detectable quantity of vitamin U in the sample was 20 μg, the relative error ± 3.0%.

The gas chromatographic data of the vitamin U concentration in rat liver, kidneys and blood at different time intervals after its oral administration are

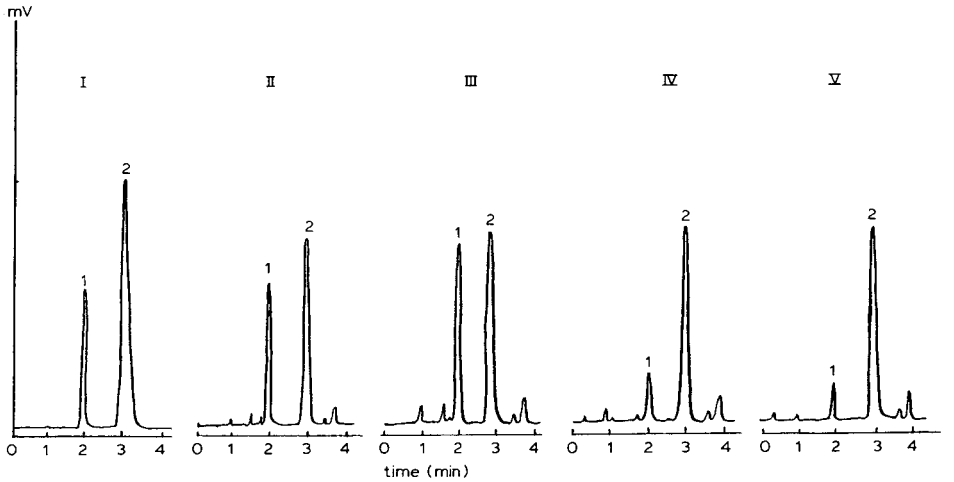

Fig. 2. Gas—liquid chromatograms of volatile substances obtained by heating homogenates of rat organs and urine. 1 = Dimethyl sulfide, 2 = carbon tetrachloride; toluene peak is not shown. I = MMSCI, II = kidneys, III = liver, IV = blood, V = urine.

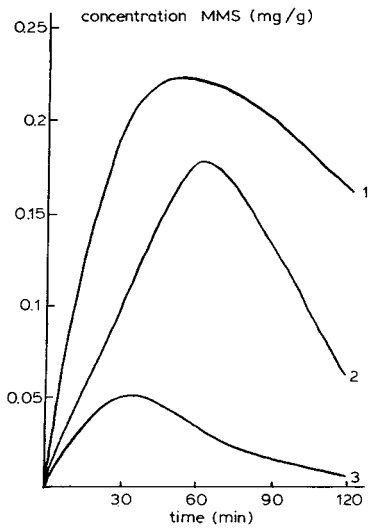

Fig. 3. The variation of MMS concentration in rat organs with time elapsed after drug administration. 1 = Kidneys, 2 = liver, 3 = blood.

presented in Fig. 3. It can be seen that the vitamin U concentration reaches a maximum in blood at 30 min and in liver and kidneys at 60 min after administration, and then decreases rapidly. On the second day after administration, only traces of the preparation can be found in blood and organs. We failed to detect vitamin U in the blood or organs of control rats fed with a standard vivarium diet. This suggests that vitamin U is actively involved in metabolic processes occurring in the animal body.

In order to verify the gas chromatographic data, vitamin U was isolated from rat organs using silica gel column partition chromatography. First, pro-

TABLE I

COMPARISON OF GAS CHROMATOGRAPHIC ANALYSIS OF VITAMIN U IN RAT ORGANS BEFORE AND AFTER PURIFICATION USING COLUMN LIQUID CHROMATOGRAPHY

Rat organ	Detected before sample purification (mg)	Detected after sample purification (mg)	Recovery after purification (%)
Liver (57 g)	2.85	2.0	70
Kidneys (13 g)	1.95	1.6	82
Vitamin preparation (1 mg)	1.0	0.8	80

teins from liver and kidney homogenates were precipitated with trichloroacetic acid. The vitamin-containing fraction was decomposed to yield dimethyl sulfide, which was determined by gas chromatography. As follows from the data obtained (Table I), the gas chromatographic technique can be used to measure vitamin U in animal tissues without preliminary preparation of the sample.

Measurements of vitamin U in the urine of rats and men given the drug at a dose of 100 mg/day showed its renal excretion at a rate of 5—6% of the dose administered. Using circular dichroism, it was demonstrated that the vitamin excreted in the urine was the D-isomer.

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Note

Urinary peptides in rheumatic diseases
Separation by reversed-phase high-performance liquid chromatography

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Urinary peptides are products of protein breakdown. Many of them contain hydroxyproline and are therefore thought to be derived from structural collagen. The presence of hydroxyproline-rich peptides in urine is brought about by the resistance of the triple-helical structure of collagen to common liver proteases. The concentration of urine hydroxyproline-rich peptides is considered to be a good indicator of collagen catabolism [1, 2].

The methods used for isolation and fractionation of urinary peptides are based mainly on liquid chromatography using an ion exchanger or molecular sieve or their combination. They are, however, rather tedious and slow and, therefore, are unsuitable for the determination of a large number of samples.

We therefore decided to test high-performance liquid chromatography (HPLC) for this purpose. Our attention was focussed at first on the higher-molecular-weight peptides (mol. wt. higher than 4000). There exist practically no data in the literature in this respect [3—6].

The diagnosis of osteoarthrosis is based on X-ray and clinical examination and no more precise method is available for determining the intensity of the osteoarthrotic process. However, as already mentioned, we suppose that more

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specific indicators of the metabolism of articular structures (especially of collagen) are present in the fraction of higher-molecular-weight hydroxyproline-containing peptides.

MATERIALS AND METHODS

HPLC instrumentation

The equipment used consisted of a reciprocating piston pump (Milton Roy, St. Petersburg, FL, U.S.A.), a "stop-flow" injector (home-made), a chromatographic cartridge-type column-system CGC [7] (a heavy-walled glass tube 150 mm × 3.5 mm I.D. inserted into a jacket made of a light alloy which, owing to the chemical reinforcement of the glass surface, can withstand pressures of up to 80 MPa) packed with silica (mean particle diameter 5 μm) chemically modified with octadecyltrichlorosilane [8] (Separon SI C 18, Laboratory Instrument Works, Prague, Czechoslovakia), UV variable-wavelength detector (Cecil Instruments, Cambridge, Great Britain) and a potentiometric recorder (Servogor 220, Goerz, Austria). The column showed 9700 theoretical plates for toluene as a test substance in methanol—water (70:30, v/v) as the mobile phase at a rate of 1 ml/min.

HPLC procedure

Twenty-four-hour samples of urine were obtained from eight healthy subjects (controls), from fifteen patients with different stage of osteoarthrosis, and from nine patients with rheumatoid arthritis of medium or high activity. Urines containing protein were excluded. A 25-ml volume of each urine was fractionated on a Bio-Gel P-4 column (Bio-Rad Labs., Richmond, CA, U.S.A.), 400 × 28 mm, equilibrated in and eluted with 0.01 *M* aqueous sodium chloride. The peaks were monitored at 220 nm (Pye-Unicam UV spectrophotometer SP 8-200). The first eluted peak (35 ml) containing peptides of higher molecular weight was used for further study. Samples were lyophilised and stored at −20°C.

A phosphate buffer (0.01 *M* KH_2PO_4, 0.1% H_3PO_4) with 25% methanol (p.a., Lachema, Brno, Czechoslovakia), pH 2.63, served as the mobile phase for isocratic HPLC separation of urinary peptides. Mobile phases with lower (over 15%) as well as higher (up to 40%) methanol contents have been also tested. However, under these conditions the separation was poor because the peptides were either heavily sorbed (in the case of lower methanol content) or eluted at very similar retention times (in the case of higher methanol content).

The eluted peptides were monitored at 210 nm, and the operating pressure was 3.2 MPa at a flow-rate of 0.12 ml/min. Samples were prepared by dissolving the lyophilised substances (approximately 1.5 mg in 30 μl of distilled water) immediately before injection; about 3 μl of this solution were injected. It was not deemed necessary to inject exactly the same volume of sample in each analysis, because the chromatograms were always evaluated in terms of relative concentrations (absorbances) of relevant components with respect to one that was used as internal standard; this procedure eliminated the necessity of absolute concentration measurements.

Peak evaluation

Five characteristic peaks of the chromatogram were selected for subsequent evaluation. One peak (the highest in all chromatograms) was designated P_0, and four other peaks showing the same retention volumes in all chromatograms were designated P_1 to P_4. The heights of individual peaks were measured and the relative values P_i/P_0 (i = 1, 2, 3, 4) were calculated. Thus, the relative heights of individual peaks could be easily compared irrespective of the injected volume and/or of the attenuation of the detector. It must be kept in mind that every peak corresponds obviously to a number of peptides with rather similar retention characteristics. The ratios thus obtained were evaluated statistically by Student's t-test and the results are presented in Table I.

TABLE I

STATISTICAL SIGNIFICANCE (t-TEST) OF DIFFERENCES BETWEEN THE GROUPS UNDER STUDY

	Controls vs. osteoarthrosis	Controls vs. rheumatoid arthritis	Osteoarthrosis vs. rheumatoid arthritis
P_1/P_0	$p > 0.05$	$p > 0.05$	$p > 0.05$
P_2/P_0	$p < 0.05$	$p > 0.05$	$p < 0.05$
P_3/P_0	$p < 0.01$	$p > 0.05$	$p < 0.05$
P_4/P_0	$p > 0.05$	$p > 0.05$	$p > 0.05$

RESULTS AND DISCUSSION

Chromatograms of the higher-molecular-weight fraction contain several peaks; five major and typical peaks were evaluated. The reproducibility of the method was good: the variation of relative peak heights did not exceed 3%. Prolonged use of a column packed with silica in a mobile phase containing inorganic buffer often leads to contraction of the bed, resulting in impaired separation efficiency and shortened retention times. This could be observed also in our study, but the decrease in retention times never exceeded 4%. The evaluation of individual peaks was not impaired by this effect.

According to Student's t-test the relative decrease of the peak P_3 is the most significant; at the 1% level in the group of osteoarthrosis, and in this case also, peak P_2 is decreased significantly at the 5% level (Table I). It is important that there exist significant differences also between the patient groups of osteoarthrosis and rheumatoid arthritis. Generally, it can be said that the variation range of P_i/P_0 ratios was rather high for all peaks in the rheumatoid arthritis group and relatively small for P_2 and P_3 in the case of osteoarthrosis (Fig. 1). Examples of chromatograms for the studied groups are presented in Figs. 2—4.

The HPLC method used in this study enabled us to fractionate larger peptides separated previously by chromatography on the Bio-Gel P-4 column. Our preliminary results show that the determination of larger peptides could

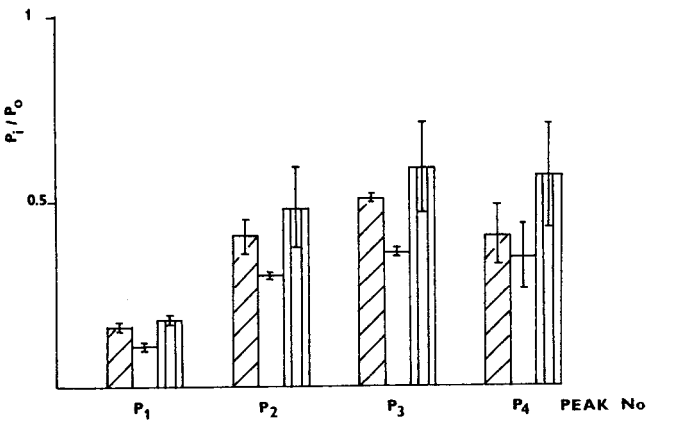
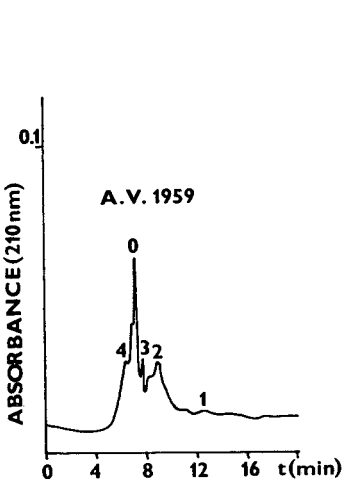


Fig. 1. Mean values and variation ranges of the relevant values P_i/P_0 for the three groups — osteoarthrosis (□), rheumatoid arthritis (▨), controls (▨).



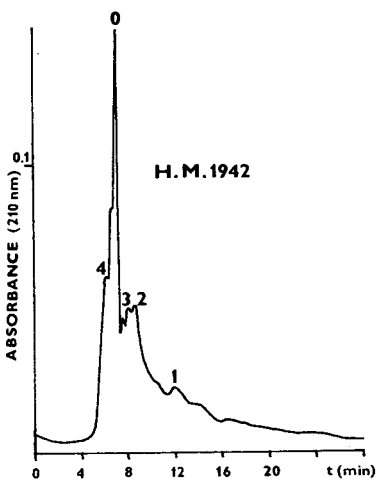


Fig. 2. Typical reversed-phase HPLC "metabolic profile" of urinary peptides of a healthy subject.

Fig. 3. Typical reversed-phase HPLC "metabolic profile" of urinary peptides of a patient with osteoarthrosis.

be a useful method in the treatment of degenerative joint diseases. We have found not only differences between controls and osteoarthrosis patients, but also between osteoarthrosis and rheumatoid arthritis patients. Further investigation of urinary peptide excretion in other rheumatic diseases is, however, necessary.

HPLC for the separation of urinary peptides was used also by Clark et al. [9]. These authors, however, were not able to find differences between urinary peptides in patients with osteoarthrosis and rheumatoid arthritis. The possible reason for the disagreement with our results could be explained by differences in the pretreatment procedure of the urinary peptide samples.

In comparison with the procedure of Szymanowicz and co-workers

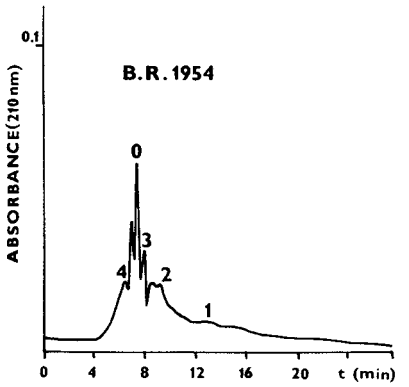


Fig. 4. Typical reversed-phase HPLC "metabolic profile" of urinary peptides of a patient with rheumatoid arthritis.

[10—12], our method is less sensitive and results in resolving several categories of peptides only. From the applicability point of view HPLC fractionation of urinary peptides is much more simple and can be used with large series of samples. On the other hand, Szymanowicz et al. [11] obtained better fractionation with smaller peptides using a combination of different liquid chromatographic methods, which according to our data are also fractionated rather well by HPLC [13]. This fraction could be also important, since according to data of Szymanowicz et al. [11] 75% of all peptides containing hydroxyproline are of lower molecular weight (up to 2000).

Investigation in this respect is in progress. We also hope that the use of a gradient may further improve the fractionation of larger peptides by HPLC. Finally, the analysis of the most important peaks seems to be necessary for elucidating their relationship to collagen.

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Note

Detection of acridine in human urine after topical coal-tar treatment

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Even at the present day coal-tar remains one of the basic dermatologic therapeutic agents, especially in the treatment of psoriasis and some types of eczema. Coal-tar used in therapy results from the pyrolytic decomposition of coal under thermodynamic conditions which enable the formation of a gamut of substances. Estimates of the number of compounds in tar are of the order of magnitude of thousands, of which about 550 substances have been identified. Besides various hydrocarbons coal-tar contains a high number of compounds with organically bound sulphur, nitrogen, and oxygen, mostly in the form of heterocycles [1].

Considering these facts it is understandable that the mechanisms of the therapeutic action of coal-tar have not yet been elucidated, especially when even the basic information about the penetration and resorption of this complex mixture of substances in the human skin is lacking.

In an endeavour to contribute to the solution of these problems and thus to a more rational exploitation of coal-tar as a therapeutic agent, we investigated resorption from coal-tar in dermatologic patients after local tar therapy by analyzing their urine.

MATERIALS AND METHODS

Material

For investigating the tar resorption we selected 28 patients (two females and 26 males), who required coal-tar treatment on an area larger than two-thirds of the body surface. In the treatment 10% and 20% tar paste was used; this means that in one application approximately 1—6 g of coal-tar was spread on the patient's skin.

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Sample preparation [2, 3]

Urine analysis was carried out always before and after the treatment, in some cases even during the treatment.

For basic orientation thin-layer chromatographic (TLC) analysis 200 ml of morning urine were taken, boiled with 50 ml of concentrated HCl, and extracted with ca. 100 ml of chloroform. The extract was then concentrated by a stream of nitrogen at 40° C to a final volume of 1 ml. A 2-μl aliquot was used for TLC.

For acridine identification organic bases were separated after transformation to sulphates by treating 100 ml of urine with 150 ml of 20% sulphuric acid. The reaction mixture was extracted by 100 ml of chloroform. Sulphates of basic substances remained in the aqueous phase, which was separated and made alkaline with 150 ml of 26% ammonium hydroxide. The released bases were then extracted into 100 ml of chloroform and the extract was concentrated to 1 ml.

Thin-layer chromatography

The urine extracts were separated on the commercial silica gel thin-layer plates Silufol (Silufol^{R}, 20 × 20 cm) and Merck (DC-Fertigplatten Kieselgel 60, 20 × 20 cm). Several solvent systems were tested. The best results were obtained with a mixture of acetic acid—benzene—diethyl ether—methanol (18:120:60:1, v/v) in the first direction, and a mixture of butanol—ammonium hydroxide (26%) (4:1) in the other direction. For detecting the polycyclic substances on chromatograms we exploited in most cases their own fluorescence in UV light (254 and 360 nm), or fluorescence quenching in the same region. Functional groups were detected by means of standard colour reactions [4].

Gas chromatography

Gas chromatography of the fractions eluted from the chromatograms was performed with a Pye GCV apparatus using two column packings. The sample volume was 5 μl.

Packing A. 5% OV-1 on Diatomite CQ (0.125—0.16 mm), column 150 cm × 4 mm I.D. Flow-rates: nitrogen 25 ml/min, air 250 ml/min, hydrogen 30 ml/min. The column temperature was 200° C, the detector temperature 240° C, the sampling temperature 300° C.

Packing B. 3% OV-17 on Diatomite CQ (0.125—0.16 mm), column 200 cm × 2 mm I.D. Gas flow-rates and temperatures as for packing A.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry was carried out with a Ribermag GC-MS R 1010 computer in the biochemical laboratories of the Medical Faculty in Dijon (France). A capillary column coated with a non-polar phase (Silar 5 CP) was used for the separation. The column bypassed the separator and led directly into the ionization source. Samples were introduced by means of a glass fibre. The temperature of the evaporation space was 250° C, the column temperature was 140° C.

RESULTS AND DISCUSSION

The basic information on polyaromatic and heterocyclic substances excreted in urine was obtained from TLC runs. This analysis was carried out for all patients. In all cases chromatograms of urine samples taken before and after tar application were compared. We thus found that in all cases investigated several fluorescing spots appeared on chromatograms of samples taken after the external application of tar, which did not occur in urine prior to the tar application (Fig. 1). The most marked one was a spot with an intense blue—green fluorescence with R_F values of 0.05 and 0.76 in the first and second direction, respectively.

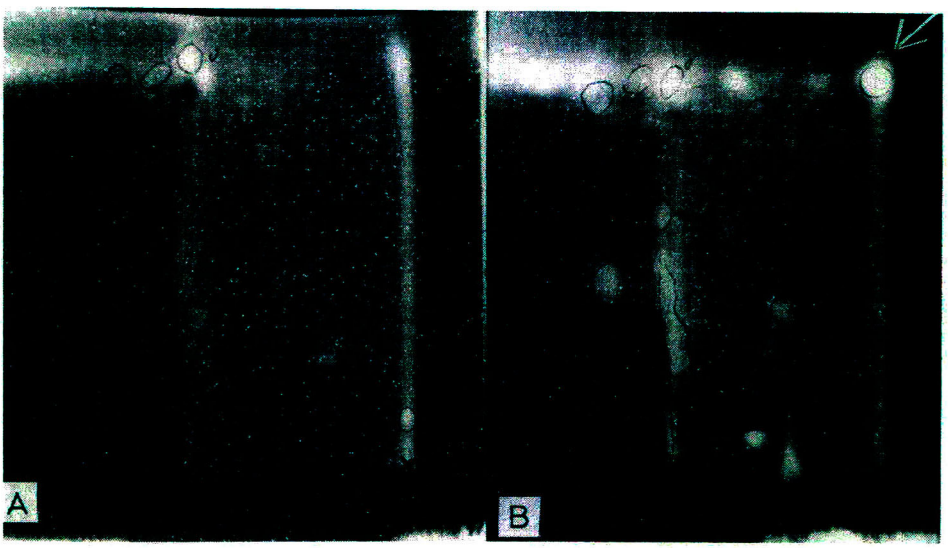

Fig. 1. Thin-layer chromatography of urine extracts: (A) before the coal-tar application, (B) after the coal-tar application. x = origin; the arrow indicates the unknown, blue—green fluorescing substance.

After comparing this unknown substance with several standards of corresponding properties it became apparent that the strongly fluorescing spot contained with high probability one or several derivatives of quinoline, isoquinoline, or acridine. Furthermore, it appeared that the found properties and chromatographic behaviour of acridine were fully identical with those of the unknown substance.

Further identification of the substance was then performed by means of gas chromatography. Organic bases isolated from both coal-tar and urine of the patients were separated on thin layers, eluted, and subjected to further separation by gas chromatography. By comparing these results with the properties of pure acridine we found that the retention times of acridine in both column packings were identical with those of the peak detected in eluates from the chromatograms, namely 2.6 and 4.9 min. The presence of acridine in urine was thus demonstrated (Fig. 2).

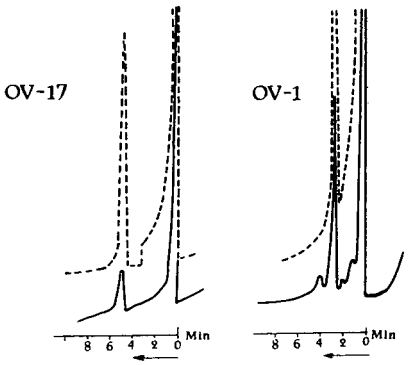

Fig. 2. Gas chromatography of acridine from urine. (——), Urine extract; (— — —), acridine standard (10^{-4} mol/l). For further details see the text.

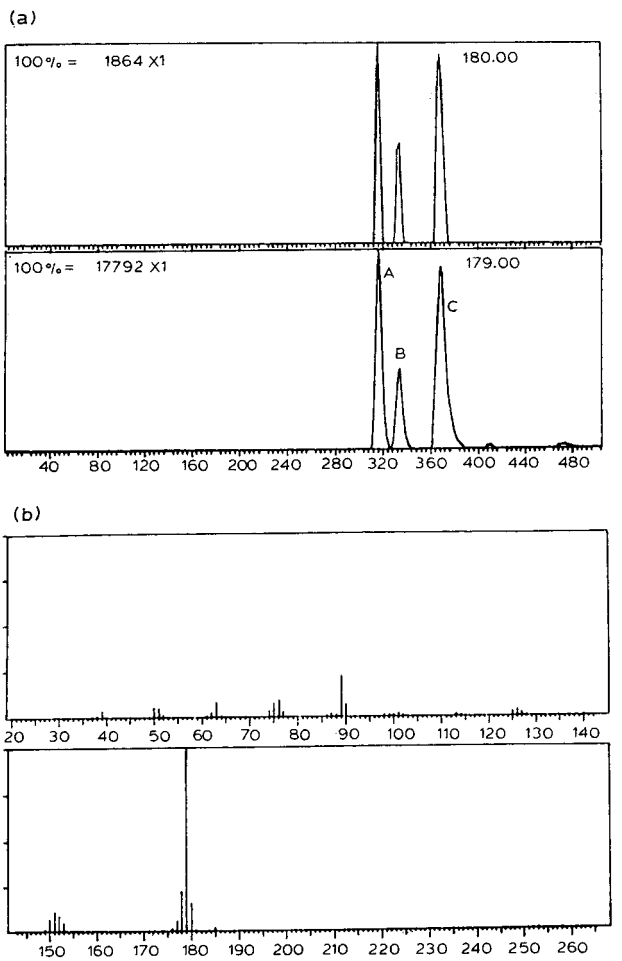

Fig. 3. Fragmentogram and mass spectrum. (a) Fragmentogram of urine extract containing acridine. (b) Mass spectrum of standard acridine, which is identical with fraction B in Fig. 3a.

Clinical verification of the demonstration of acridine in urine after percutaneous resorption was performed by a test with a paste containing acridine instead of tar. According to the data on the acridine content of coal-tar [1, 3], we prepared magistraliter a paste containing 0.12 g of pure acridine in 100 g of the paste. This corresponds to the reported 0.6% of acridine in tar if 20% tar paste is considered. The acridine paste was applied to a patient in the usual way and the excretion of acridine in urine was investigated. The acridine spot was observed on chromatograms since the first day of the application of the acridine paste, as well as after the use of coal-tar. This experiment yielded another proof for the resorption of acridine from coal-tar.

Mass spectra of standard acridine and acridine isolated from urine confirmed the identity of the two compared substances having the same retention time (Fig. 3). (The main fragment ion was *m/e* 179, the next were *m/e* 180 and 178, etc.) This finding was also in accord with data in the computer library; at the same time the computer evaluated the probability of the sought agreement by an index DI. In the given case DI was 0.279, which indicated a very high probability.

The presence of acridine in urine after the coal-tar application was convincingly demonstrated by the mass spectrographic analysis.

Acridine is present in coal-tar as one of several basic nitrogen-containing compounds. According to its content it is ranked in second place behind carbazole (coal-tar contains 1.5% of carbazole, 0.6% of acridine, 0.3% of quinoline, 0.02% of pyridine, etc.) [5].

The detection of acridine in urine is the first proof of the resorption of a coal-tar component through the skin and the investigation of this process can contribute to an improvement of coal-tar therapy by selecting the effective and relatively less toxic components of this extraordinarily complex mixture of substances.

ACKNOWLEDGEMENT

The authors are much obliged to Prof. Prudent Padieu from the Laboratoire de Biochimie in Dijon for having enabled them to carry out the analysis on the chromatograph Ribermag GS-MS R1010.

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CHROMBIO. 1533

Note

Capillary gas chromatographic method for determination of flumecinol in plasma and saliva

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Flumecinol (Zixoryn®, 3-trifluoromethyl-α-ethyl-benzhydrol [1] is a selective hepatic enzyme inducer both in animal experiments and in human clinical studies. For example, flumecinol induces the endoplasmic reticulum-bound mixed-function oxidase system of the liver [2—6]. The main indicating area of flumecinol therapy is recommended for the treatment of icterus neonatorum.

Our preliminary experiments showed that flumecinol seems to bind very intensively to plasma protein(s) [7]; therefore, the study of in vivo protein binding and the determination of the plasma flumecinol concentration in rats during chronical toxicity studies required a more sensitive method than our earlier one [8].

This new simple method is appropriate for routine analysis; the human pharmacokinetics of the drug and its in vivo protein binding could be calculated from the saliva/plasma level, and the plasma level of flumecinol can be determined in chronic toxicity experiments from 250 μl of rat plasma.

EXPERIMENTAL

Materials

Zixoryn® was manufactured by Chemical Works of Gedeon Richter, Budapest, Hungary. The specific activity of [1-^{14}C]flumecinol was 1467.4 MBq/g (39.6 mCi/g).

Potassium hydroxide and diethyl ether puriss. were from Merck (Darmstadt, G.F.R.). Diethyl ether, chloroform and methanol were carefully purified by bidistillation before use. Parafilm® was from American Can. Co., Greenwich, CT, U.S.A. All other chemicals were the products of Reanal (Budapest, Hungary) and were of analytical grade.

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Preparation of calibration curves

To 1 ml of human plasma or 2 ml of saliva, 200 ng (for 250 µl of rat plasma 100 ng) of 3-trifluoromethyl-benzhydrol (internal standard) were added. The flumecinol concentration in the calibration curves was between 10 and 200 ng for human plasma, 10 and 50 ng for saliva, and 10 and 100 ng for rat plasma samples, the flumecinol being added in methanol. To these samples 0.6 ml of 2 *N* potassium hydroxide was added and they were extracted with 5 ml of diethyl ether, shaken for 20 min and centrifuged at 3000 *g* for 15 min.

The diethyl ether was transferred to conical test-tubes and evaporated at room temperature. The dry residue was dissolved in 20 µl of chloroform under shaking and 1—2 µl were injected into the gas chromatograph.

For quantitation the ratio of peak height of drug to that of internal standard was used. The recovery of flumecinol was detected by liquid scintillation counting of [^{14}C] flumecinol.

Determination of radioactivity

Radioactivity of liquid samples was counted in a Packard Tri-Carb liquid scintillation spectrometer Type 2660. The [^{14}C] flumecinol in aqueous samples (0.25—2 ml) was counted in 5 ml of Insta-Gel® (Packard, Downers Grove, IL, U.S.A.) and in the diethyl ether samples (5 ml) in 5 ml of toluene-based liquid scintillation solution (5 g of PPO, 0.1 g of dimethyl POPOP, 1000 ml of toluene). For the determination of the absolute activity the external standard—channel ratio method was used.

Gas—liquid chromatography

A Hewlett-Packard 5736A gas chromatograph equipped with a flame ionization detector (FID) was used. The electronic parameters were set at maximum sensitivity: range 1, attenuation 1 for the plasma and saliva samples.

Nitrogen of high purity was used as carrier and auxiliary gas. The methyl silicone Sp 2100 — Carbowax 20M deactivated — flexible fused-silica capillary column was 25 m × 0.20—0.21 mm I.D. (Hewlett-Packard). The capillary column was operated at 160°C (human and rat plasma) and 150°C (human saliva) with a nitrogen flow-rate of 1.6 ml/min. The capillary column pressure was 196.6 kPa (28.5 p.s.i.) (plasma) and 207.0 kPa (30 p.s.i.) (saliva). The flow-rate was 18.2 ml/min at the split vent and 1.5 ml/min at the septum vent. The flow-rates of the detector gases were 40 ml/min for hydrogen and 150 ml/min for air. The temperature settings were as follows: capillary injector port in split mode 250°C (split insert was unpacked), FID 300°C.

In vivo experiments

For the pharmacokinetic examination healthy volunteers received Zixoryn® (flumecinol) in a single 100-mg oral dose; for the saliva kinetics 400 mg of Zixoryn were administered orally. Venous blood and saliva samples were obtained up to 72 h (on 14 occasions). Salivary flow was stimulated by chewing Parafilm® until at least 3 ml had been collected. The rats received Zixoryn mixed in the daily food in a dose of 125 mg per kg per day during the chronic toxicity studies.

Sodium citrate (3.8% w/v) was added to the human blood samples in a ratio of 1:9, to prevent coagulation. The plasma and saliva samples were kept deep-frozen until analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of human plasma extract. The retention time for internal standard (3-trifluoromethyl-benzhydrol) was 3.1 min, and for flumecinol 4.1 min.

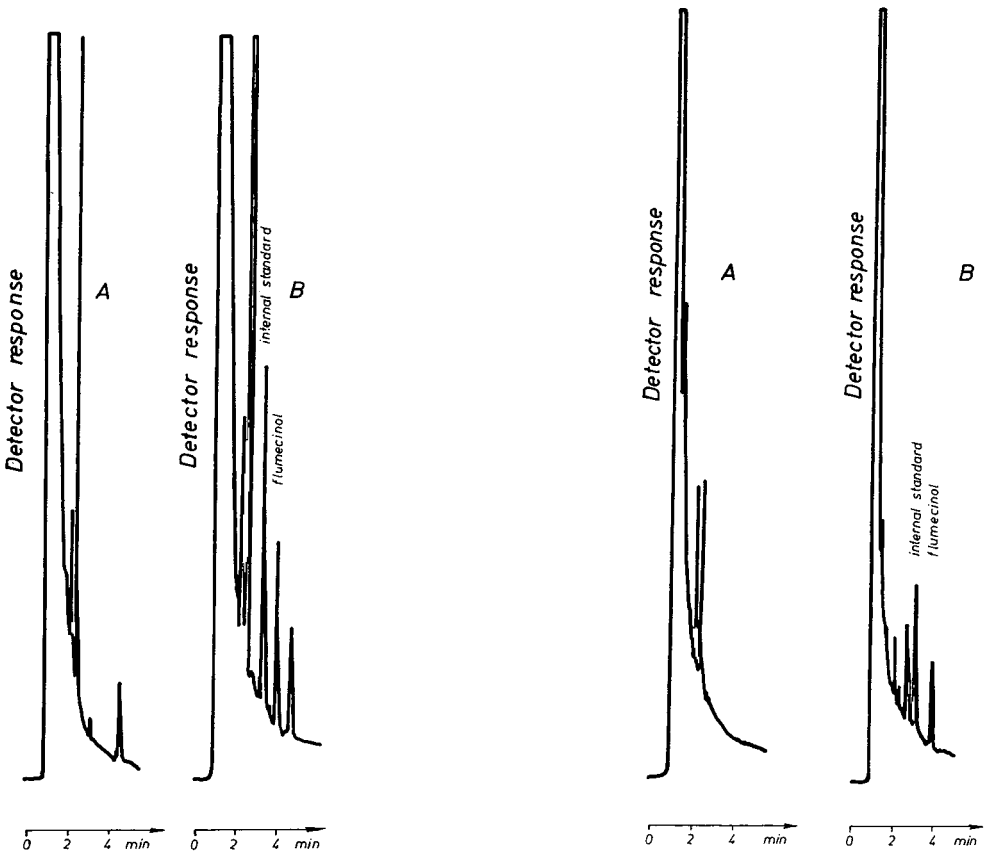

Fig. 1. Chromatograms of human plasma extract: (A) control plasma; (B) plasma obtained 2.5 h after oral administration of flumecinol.

Fig. 2. Chromatograms of rat plasma extract: (A) control plasma; (B) plasma obtained after 125 mg per kg per day oral administration of flumecinol.

Fig. 2 shows a chromatogram of rat plasma extract. The retention times are the same as for human plasma chromatograms. The method can also be applied to human saliva; Fig. 3 shows a typical chromatogram of human saliva extract. The endogenous compounds can be separated at lower temperature

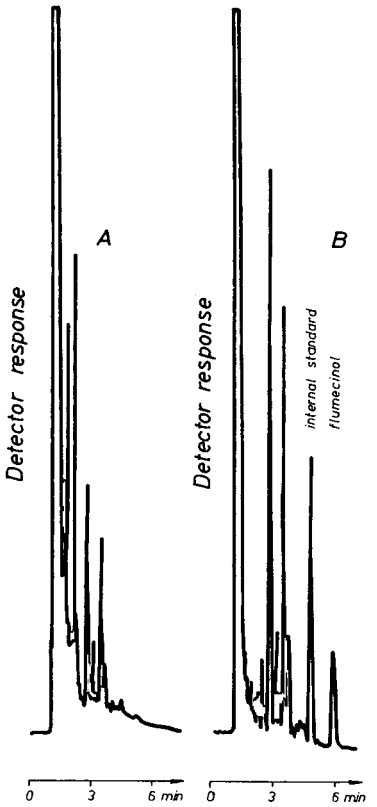

Fig. 3. Chromatograms of human saliva extract: (A) control saliva; (B) saliva obtained 3 h after oral administration of flumecinol.

and therefore the retention times are longer (4.8 and 6.0 min) than in the case of plasma samples.

The calibration curves obtained with human and rat plasma and saliva show a good linearity within the range 10—200 ng/ml flumecinol (Fig. 4). The correlation coefficient of the calibration curve for human plasma was 0.990317, for saliva 0.990028, and for rat plasma 0.994828.

Recovery of flumecinol from plasma and saliva samples was checked using radiolabelled drug and was found to be 78.11 ± 3.452% (S.D.), 75.03 ± 1.056% and 88.10 ± 2.442% for human plasma, saliva and rat plasma, respectively.

In Fig. 5 the concentration—time curves are shown for human plasma and saliva, demonstrating that the method allows monitoring of plasma and saliva concentrations up to the 72nd and 30th hour, respectively.

The advantages of the extraction and flexible fused capillary gas—liquid chromatographic method are that it has a sensitivity of 0.5 ng/ml and permits the determination of flumecinol in a volume as small as 250 μl of rat plasma too. From the same volunteers the saliva/plasma concentration ratio could be determined and the in vivo protein binding of flumecinol could be calculated. The retention time for flumecinol determination is short (4.5 min).

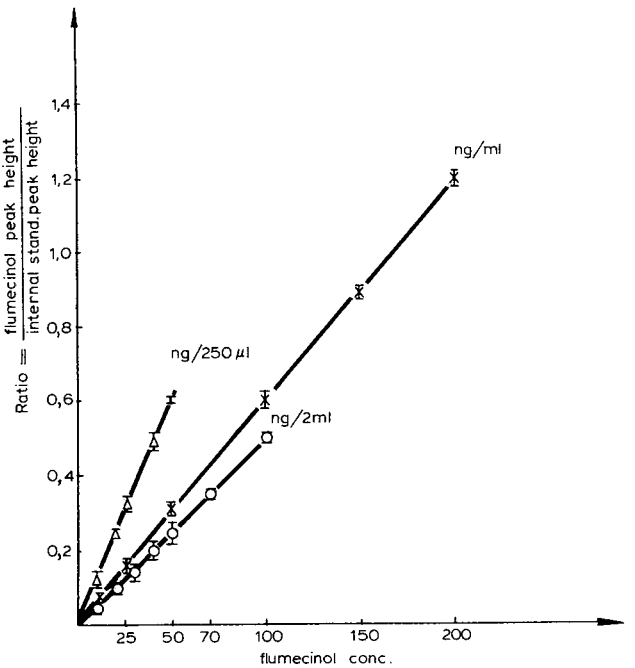

Fig. 4. Calibration curves for human plasma (x——x), human saliva (○——○) and rat plasma (△——△) (mean ± S.D.).

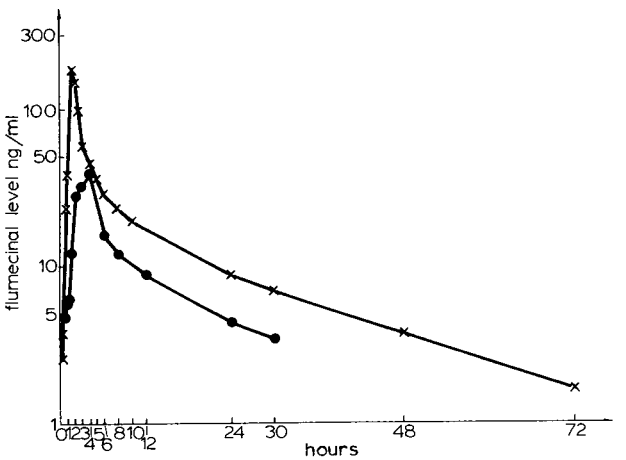

Fig. 5. plasma (x——x) and saliva (○——○) flumecinol concentration—time curves after oral administration of 100 mg (plasma) and 400 mg (saliva) of flumecinol to healthy volunteers.

ACKNOWLEDGEMENTS

Thanks are due to J. Szammer (Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, Hungary) for synthesis of the labelled flumecinol and to Mrs I. Kókai and Mr. T. Baracs for their excellent technical assistance.

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CHROMBIO. 1530

Note

Monitoring of ethimizol and its metabolites in serum or saliva by means of high-performance liquid chromatography

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There is an extensive and rapidly growing utilization of high-performance liquid chromatography (HPLC) in the monitoring of many drugs to control dosage in order to achieve the optimum therapeutic effect and safety. The concentration of drugs is determined in various body fluids — whole blood, serum, plasma, and saliva as well. Samples of saliva may be taken painlessly and without the risk of iatrogenic infection and this substantiates the attempts to control the drug dosage on the basis of its salivary concentration.

Ethimizol (I), bis-methylamide of 1-ethylimidazole-4,5-dicarboxylic acid (see Fig. 1), is an original Soviet preparation [1]. In clinical practice ethimizol is mainly used in the treatment of respiratory disorders [2, 3].

The aim of this paper is to demonstrate that for monitoring purposes serum data of ethimizol and one of its metabolites (M_1) can be substituted by saliva data.

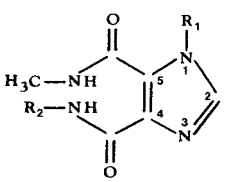

Fig. 1. Structural formula of ethimizol (I: $R_1 = C_2H_5$, $R_2 = CH_3$), antiffeine (II: $R_1 = CH_3$, $R_2 = CH_3$), and metabolite 1 (M_1:$R_1 = C_2H_5$, $R_2 = H$).

EXPERIMENTAL

Subjects

Four healthy male volunteers (for age and weight see Table I) participated in this study. After an overnight fast each received a single oral dose of 2 mg/kg ethimizol as a tablet. Blood was drawn 2, 3, 4, 5, and 6 h after ethimizol ad-

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ministration. Saliva was collected at the same time intervals. (The salivary flow was stimulated by chewing a piece of Parafilm.) Both serum and saliva were stored frozen until analysis.

Sample treatment

Serum or saliva sample (2.0 ml) diluted with a 2.0 ml aqueous solution (1.0 μg/ml) of the internal standard antiffeine (Fig. 1, II) was applied onto a disposable Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). (The cartridge was conditioned before use by washing with 5 ml of methanol followed by 5 ml of water.) After the diluted sample had passed through the cartridge, the packing was washed with 2 ml of water. The remaining water was blown off. The cartridge was connected to a disposable micro-column packed with silica gel (ca. 0.2 g) and the compounds under analysis were eluted with 4 ml of acetonitrile from the cartridge packing (C_{18}) through the silica sorbent into a conical glass vial. The acetonitrile eluate was evaporated to dryness under a stream of nitrogen at 50°C. The residue was redissolved in 20 μl of chloroform and 10 μl of centrifuged solution were chromatographed [4].

Chromatographic technique

A Spectra-Physics SP 8000 high-performance liquid chromatograph equipped with a variable-wavelength photometric detector (Model SP 770) was used. A stainless-steel column (25 cm × 4.6 mm I.D.) was packed with LiChrosorb SI-100, particle size 5 μm (Merck, Darmstadt, G.F.R.). The eluent, a mixture of *n*-heptane—dichloromethane—methanol—triethylamine (85:10:4.75:0.25, v/v) was pumped at a rate of 1.2 ml/min. Detection was effected at 262 nm. All separations were carried out at ambient temperature.

RESULTS AND DISCUSSION

A slightly modified sample treatment procedure developed previously [4] was used to preserve clogging of the HPLC column with any non-elutable co-preseparated endogenous compounds. As shown in Fig. 2, the preseparated blank human serum or saliva gave only a small peak of a compound (maybe caffeine which is eluted at 8.0 min), which did not significantly influence the evaluation of the peaks of I, (II), and M_1, which eluted at 6.3, (7.6), and 10.2 min, respectively. (The evaluation of the peaks of further ethimizol metabolites, eluted after 12 min until about 18 min, was initially omitted.)

Serum or saliva samples (2.0 ml) containing ethimizol (0.1 μg/ml) and metabolite 1 (1.0 μg/ml) were processed as described above in order to determine the within-day reproducibility of the method. The coefficients of variation calculated from quadruplicate determinations of I and M_1 in serum or saliva were 7.3% and 3.4%, respectively, or 6.9% and 3.6%, respectively.

The half-lives, calculated from the individual concentration—time curves of ethimizol (M_1) in serum or saliva, are listed in Table I. There is excellent agreement between the half-life values determined from serum and from saliva. Each corresponding pair of concentration dependence declines nearly in parallel and thus the saliva-to-serum concentration ratio ([Sa]/[Se]) of ethimizol (M_1) is almost constant (see Table I). The mean [Sa]/[Se] ratio for M_1 is significantly higher than that for I.

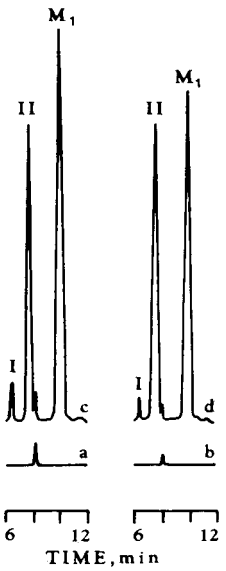

Fig. 2. Normal-phase HPLC elution profile of preseparated blank human serum (a) or saliva (b), and a serum (c) or a saliva (d) sample obtained 2 h after ethimizol administration. The chromatograms are recorded between the start of the ethimizol elution curve and the end of an HPLC analysis run time (12 min).

TABLE I

SERUM (Se) AND SALIVA (Sa) HALF-LIVES OF ETHIMIZOL AND METABOLITE 1, AND THEIR [Sa]/[Se] CONCENTRATION RATIOS

Subject			Half-life of I in:		[Sa]/[Se] ratio of I	Half-life of M_1 in		[Sa]/[Se] ratio of M_1
Code	Age (years)	Weight (kg)	Se (h)	Sa (h)		Se (h)	Sa (h)	
J.R.	28	74	0.78	0.82	0.42—0.47	3.41	3.46	0.88—0.89
Z.K.	29	80	0.67	0.72	0.51—0.67	1.82	1.85	0.86—0.89
L.L.	33	77	0.97	0.97	0.40—0.40	3.22	3.47	0.84—0.89
V.S.	36	65	1.33	1.35	0.60—0.63	2.50	2.51	0.84—0.84

The results presented demonstrate that the chromatogram of the saliva sample fully substitutes the serum HPLC analysis not only qualitatively but also quantitatively. The saliva data may be used reliably for the determination of the terminal half-life of I (M_1) after a single oral administration of ethimizol tablets in a commonly used therapeutic dose. Since the concentration of many drugs (metabolites) in saliva equals their free (non-protein-bound) concentration in plasma, the saliva levels might be more relevant for their pharmacologic action than the corresponding serum (plasma) concentrations [5]. In patients under long-term treatment, the validity of controlling ethimizol dosage by saliva rather than serum analysis has still to be verified.

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CHROMBIO. 1531

Note

Bestimmung von Glibenclamid mit Hochleistungs-Flüssigkeits-Chromatographie in Humanplasma

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Forschungsabteilung 2 des Direktionsbereiches Forschung und Entwicklung des VEB Pharmazeutisches Kombinat GERMED Dresden, Stammbetrieb VEB Arzneimittelwerk Dresden, DDR-8122 Radebeul (D.D.R.)

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Institut für Klinische Pharmakologie der Medizinischen Akademie "Carl Gustav Carus", DDR-Dresden (D.D.R.)

Glibenclamid, 1-{4-[2-(5-Chlor-2-methoxybenzamido)-ethyl]-phenylsulfonyl}-3-cyclohexylharnstoff (Fig. 1) stimuliert die Insulinsekretion durch die Beta-Zellen des endokrinen Pankreas. Glibenclamid (Maninil®) wird in der Regel als Antidiabetikum bei Erwachsenen- und Altersdiabetes eingesetzt. Die orale Dosis liegt im allgemeinen zwischen 5 und 10 mg/Tag.

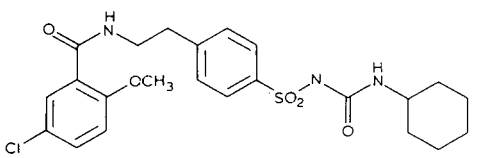

Fig. 1. Glibenclamid.

Mit Berücksichtigung in der Literatur beschiebener Methoden sollte eine Methode zur quantitativen Bestimmung von Glibenclamid in Humanserum gesucht werden. Auf Grund der grossen Selektivität und Empfindlichkeit schien uns für diesen Zweck die Hochleistungs-Flüssigkeits-Chromatographie (HPLC) besonders geeignet zu sein.

UV-spektrophotometrische [1], kolorimetrische und fluorimetrische [1, 2] Methoden sind nicht genug empfindlich bzw. selektiv. Radioimmunologische

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Untersuchungen [3—7] sind zwar empfindlich, aber bedingt durch auftretende Metaboliten nicht spezifisch genug und störanfällig. Der Einsatz gaschromatographischer Methoden [8—11] hat sich durch die notwendigen aufwendigen Derivatisierungen nicht bewährt; ausserdem lässt die Selektivität zu wünschen übrig. Zufriedenstellende Ergebnisse bei pharmakokinetischen Untersuchungen am Menschen und Hund inbezug auf Empfindlichkeit, Störanfälligkeit, Einfachheit und Schnelligkeit werden mit der HPLC erzielt [12—17].

Da bei den in der Literatur beschriebenen Methoden Glibenclamid bzw. Tolbutamid (innerer Standard) nicht exakt von Verunreinigungen des Serums, die besonders stark bei älteren Patienten auftreten, abgetrennt werden konnten, suchten wir nach einem empfindlichen Verfahren, das gleichzeitig eine exakte Trennung des Glibenclamid bzw. Tolutamid auch von stark auftretenden Verunreinigungen gewährleistet.

EXPERIMENTELLER TEIL

Die Versuche wurden mit dem Flüssigkeits-Chromatograph 3 der Firma Pye Unicam durchgeführt. Die Trennung erfolgte an LiChrosorb RP-18-Säulen

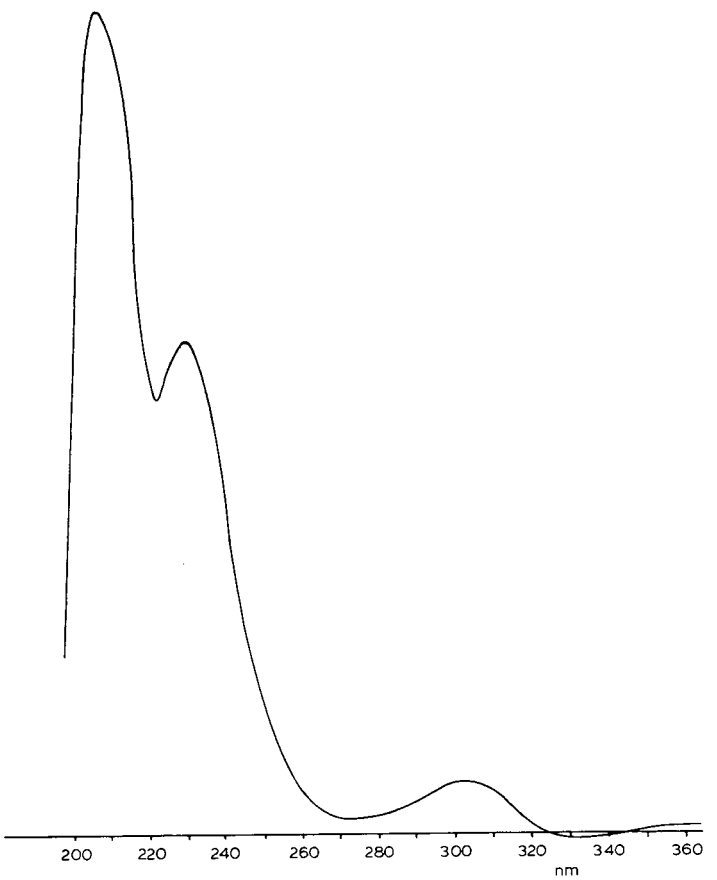

Fig. 2. Absorption von Glibenclamid in mobiler Phase.

(250 mm × 4.6 mm I.D.) Merck, Partikelgrösse 5 μm. Als mobile Phase dienten Acetonitril—5 m*M* Phosphorsäure, Acetonitril—Phosphatpuffer pH 6 und Acetonitril—Perchlorsäure unterschiedlicher Zusammensetzung. Die Fliessgeschwindigkeit betrug 2 ml/min. Die Detektion erfolgte bei 230 nm (s. Fig. 2). Bei niedrigerer Wellenlänge [16] konnten wir keine befriedigenden Ergebnisse erhalten.

Die Extraktion des Wirkstoffes aus dem Humanserum wurde wie nachstehend beschrieben vorgenommen: 1 ml Serum wird mit 0.5 ml Phosphatpuffer (pH 6) and 150 mg 1-Butyl-3-(*p*-tolylsulfonyl)-harnstoff (Tolbutamid) (Fig. 3) als innerer Standard versetzt und zweimal mit je 4 ml Äther—Chloroform (6:2) extrahiert. Die vereinten Extrakte wurden im Luftstrom zur Trockne eingeengt und der Rückstand in der mobilen Phase gelöst. Zur Ermittlung der Empfindlichkeit und der Wiederfindungsrate werden Konzentrationsreihen von Glibenclamid im Bereich von 10—300 ng/ml in mobiler Phase (300 ng Tolbutamid) und nach Extraktion aus Humanserum und Pufferlösung pH 6 (150 ng Tolbutamid) bestimmt.

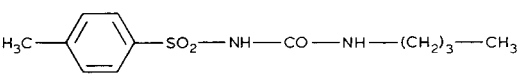

Fig. 3. Tolbutamid.

ERGEBNISSE

Im Serumextrakt vorhandene Verunreinigungen lassen sich an LiChrosorb RP-18-Säulen mit Acetonitril—12 m*M* Perchlorsäure (47:53) als mobile Phase bei einer Fliessgeschwindigkeit von 2.0 ml/min von Glibenclamid und Tolbutamid abtrennen (Fig. 4—6). Nach 9 min Fliesszeit lässt man innerhalb 2 min den Acetonitrilgehalt auf 100% ansteigen (Exponent 0.1), anschliessend äquilibriert man die Säule 8 min mit der mobilen Phase. Dieses Trennsystem ist dem in der Literatur beschriebenen, bei denen Glibenclamid im wesentlichen an Reversed-Phase-Säulen isokratisch mit mobilen Phasen die aus Acetonitril—Phosphatpuffer oder Phosphorsäure-Gemischen unterschiedlicher Zusammensetzung bestehen, weit überlegen. Glibenclamid und Tolbutamid werden auch in stark verunreinigten Seren sicher von den Verunreinigungen abgetrennt.

Die Injektion erfolgt über eine 20-μl Dosierschleife. Die Retentionszeit beträgt für Tolbutamid etwa 6 min und für Glibenclamid 13 min. Die Eichkurven von Glibenclamid und aus Serum extrahiertem Glibenclamid sind im Bereich von 10—300 ng/ml linear. Die Nachweisgrenze liegt bei 10 ng/ml (Fig. 7) und ist damit ausreichend zur Ermittlung der Bioverfügbarkeit von Glibenclamid (Fig. 8). Bei der Extraktion werden 93% des Glibenclamid wiedergefunden. Bedingt durch die exakte Abtrennung des Glibenclamid und Tolbutamid von Verunreinigungen wurde eine sehr niedrige Standardabweichung, die unter den bisher in der Literatur angegebenen Werten liegt, erzielt (Tabelle I).

Zur Überprüfung der Methode wurde der Blutspiegel von Glibenclamid bei einem älteren diabetischen Patienten über 9 Stunden bestimmt. Der Patient

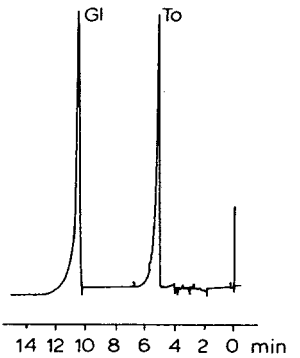

Fig. 4. Chromatogramm vom Kontrollserum.

Fig. 5. Chromatogramm von Glibenclamid (Gl) und Tolbutamid (To).

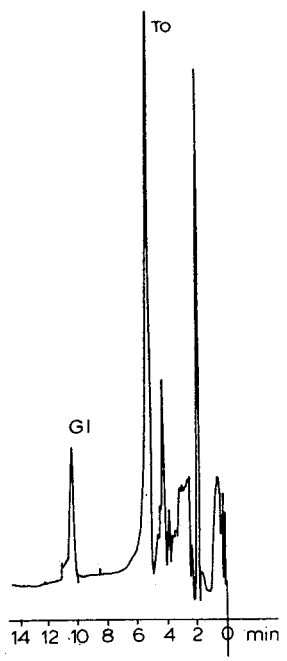

Fig. 6. Chromatogramm von Glibenclamid (Gl) und Tolbutamid (To) aus Serum extrahiert.

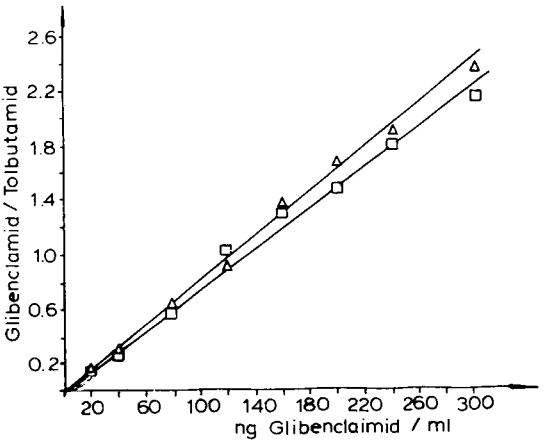

Fig. 7. Eichkurven von Glibenclamid aus Puffer extrahiert (□) und Glibenclamid aus Serum extrahiert (△).

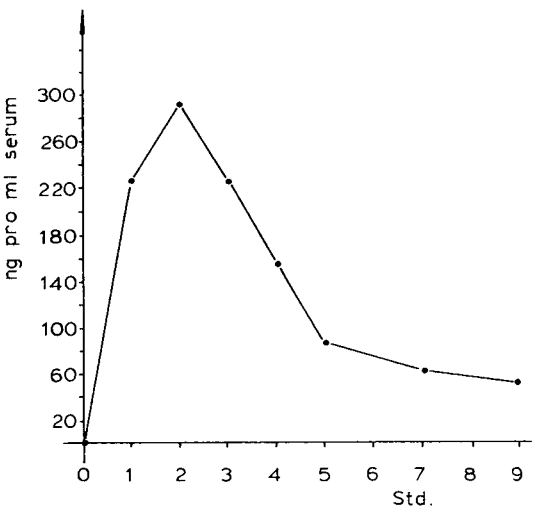

Fig. 8. Bioverfügbarkeit von Glibenclamid.

erhielt 5 mg Glibenclamid oral verabreicht (Fig. 8). Die beschriebene Methode eignet sich, bedingt durch ihre einfache Probenvorbereitung und relativ schnelle Elution für Serienanalysen. Sie kann im Rahmen pharmakokinetischer Untersuchungen sehr gut zur Bestimmung von Glibenclamid in Humanseren eingesetzt werden, und zeichnet sich durch Selektivität, Empfindlichkeit und Präzision aus.

TABELLE I

STANDARDABWEICHUNG BEI DER BESTIMMUNG VON GLIBENCLAMID AUS HUMANSERUM

Vorgegeben (ng/ml)	Gefunden (ng/ml)	*n*	*s*	*s* (%)
10	9.8	4	± 0.957	± 9.8
20	19.8	6	± 1.329	± 6.7
40	40.2	6	± 1.329	± 3.3
80	80.0	6	± 4.940	± 6.2
120	119.5	6	± 2.258	± 1.9
160	160.0	6	± 4.655	± 2.9
200	200.0	6	± 2.608	± 1.3
240	240.0	6	± 7.183	± 3.0
300	300.0	6	± 7.548	± 2.5

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Note

Rapid method for determination of cimetidine in biological fluids by high-performance liquid chromatography using Extrelut extraction

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Cimetidine as a specific histamine H_2 receptor antagonist has been introduced recently as Histodil (RG) in Hungary for the treatment of gastric and duodenal ulcers. The degree of inhibition of gastric acid depends on the drug concentration in blood; therefore it was necessary to examine the pharmacokinetics of Histodil and to compare its bioavailability with Tagamet (SKF). Several extraction and high-performance liquid chromatographic (HPLC) methods have been developed for the determination of cimetidine and its metabolites in blood, plasma and urine [1—7] using different organic solvents for extraction (e.g. ethyl acetate, octanol, dichloromethane etc.).

We have developed also a rapid extraction method to study the bioavailability of both Tagamet (SKF) and Histodil (RG) after oral (p.o.) and intravenous (i.v.) administration to healthy volunteers. The HPLC method of Lee and Osborne [2] was used with some modifications. Our method can be used with success for the analysis of cimetidine.

EXPERIMENTAL

Clinical study

This was a balanced three-way cross-over study in which each of six volunteers received each of the following regimens at 2-week intervals. Regimen A: 200 mg of Histodil, i.v. Regimen B: 2 × 200 mg of Histodil. Regimen C: 2 × 200 mg of Tagamet tablets p.o. Heparinized blood samples were collected before and at 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10 and 24 h after the dose.

The blood samples were centrifuged as soon as possible; thereafter the plasma samples were stored at −20° C.

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Extraction procedure

Cimetidine and metiamide (internal standard) can be extracted from plasma into ethyl acetate using an Extrelut (Merck, Darmstadt, G.F.R.) column. Extrelut is a granular support material and the lipophilic compounds can be extracted by organic solvents from aqueous phase. To prepare the column, Pasteur pipettes 14 cm × 0.5 cm I.D. were filled to a height of 9 cm with Extrelut (500 mg). Then 1 μg of metiamide and 0.1 ml of 6 *N* sodium hydroxide were added to 0.8 ml of plasma. This alkaline plasma sample was applied to the top of the column and allowed to soak for 15 min. The compounds were eluted by 5 ml of ethyl acetate (Merck) collected in a tube. The organic phase was evaporated in a stream of nitrogen at 40°C. For analytical purposes 60 μl of plasma extract dissolved in 0.5 ml of eluent were injected onto the analytical column.

Recovery studies were performed at both high and low concentrations (0.03—0.7 mg/l). Percentage recovery was determined by comparing the peak heights of cimetidine and metiamide extracted from plasma samples to standard stock solutions injected directly onto the column. In order to study the intra- and inter-assay variability, a plasma sample was assayed four times on the same day, and the calibration curve was prepared by using 3—5 parallel samples for each point.

Chromatographic conditions

A Hewlett-Packard 1081 B chromatograph equipped with a variable-wavelength UV detector (Labor MIM) and a 3380 S integrator was used. The separation was performed on a 200 × 4.6 mm I.D. HP Si-100 LiChrosorb (10 μm particle size) column. The mobile phase consisted of acetonitrile—methanol—water—25% ammonium hydroxide (250:30:10:0.4, v/v). A flow-rate of 1 ml/min was maintained. The absorbance of the effluent was monitored at 228 nm.

RESULTS AND DISCUSSION

We found that the final recovery of compounds was 75% for metiamide and 95% for cimetidine (S.D. ± 8.65 and ± 6.24, respectively). The recovery of metiamide was lower, but the value proved to be adequate for routine determination of cimetidine in plasma.

The limit for safe quantitation was found to be 0.03 mg/l. The extraction procedure was rapid, the eluate did not contain emulsions and 5 ml of ethyl acetate were enough for quantitative estimation of the drug. We had to analyse the extracts on the same day, because cimetidine and metiamide stored in ethyl acetate at +6°C are not stable after 24 h.

The intra-assay variation was negligible. The calibration curves were linear within the concentration range of interest (30—700 μg/l). The regression line had a correlation coefficient of 0.970885 with a standard error of 1.6×10^{-4}, in the case of 29 samples.

Fig. 1 shows the structure of the compounds. Fig. 2 illustrates chromatograms of three plasma samples. In the chromatogram obtained after extracting 0.8 ml of blank human plasma (Fig. 2a), no additional peaks are seen which

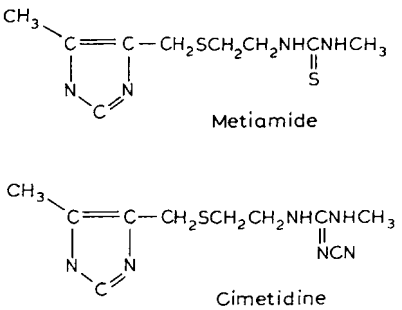

Fig. 1. Structures of cimetidine and metiamide.

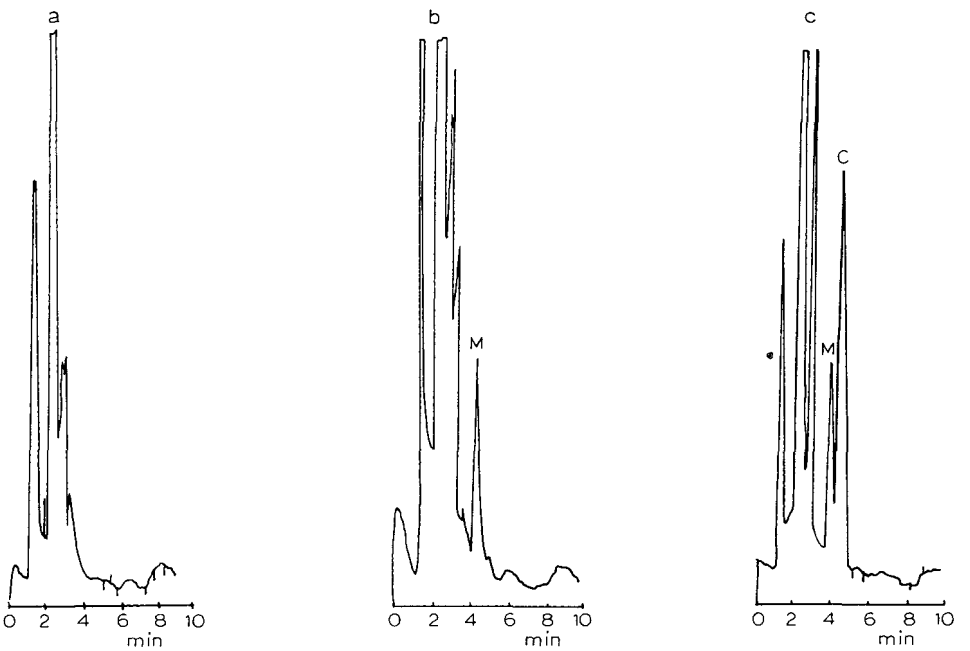

Fig. 2. Chromatogram of plasma extracts: (a) blank human plasma; (b) extract of plasma containing 1 μg of metiamide; (c) plasma extract after the administration of 400 mg of cimetidine p.o.

could interfere with the determination of cimetidine and metiamide. Fig. 2b represents extracts of plasma containing 1 μg of metiamide, and Fig. 2c is a chromatogram obtained after extracting 0.8 ml of plasma from a healthy volunteer 2 h after administration of 400 mg cimetidine p.o. Cimetidine and the internal standard (metiamide) were well resolved with retention times of 4.9 and 4.1 min, respectively.

Fig. 3 shows blood concentration—time curves following both oral and intravenous administration of Histodil tablets and injection, and Tagamet tablets. The analysis of the results was performed by a TPA/i 1001 computer. We used

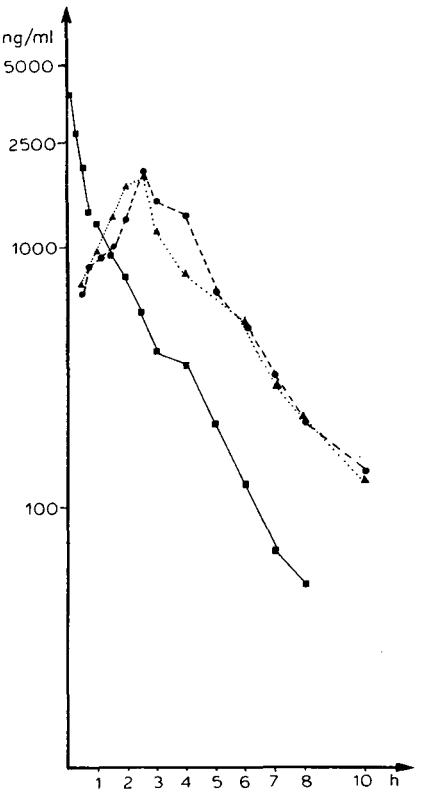

Fig. 3. Blood concentration—time curve following oral administration of 2 × 200 mg of Tagamet and Histodil tablets, and 200 mg of Histodil i.v. The curves show the mean blood plasma levels of cimetidine. (■), Histodil i.v.; (▲), Tagamet p.o.; (●), Histodil p.o.

a two-compartment model to describe the cimetidine plasma concentration kinetics.

Our extraction method with Extrelut has several advantages over methods that incorporate a classic extraction procedure for cimetidine and its metabolites [1—7]. The method is rapid and requires less organic solvent compared to the procedures mentioned above. The eluate does not contain emulsion; therefore there is no need for further purification. The evaporation is carried out in the same vials in which the eluent is collected. The sensitivity (0.03 mg/l) is higher than in the previous procedures [1—7].

Our method was applied to pharmacokinetic investigation of different cimetidine products in humans. The method can be used routinely for clinical monitoring of cimetidine but it does not allow the detection of its metabolites.

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CHROMBIO. 1538

Note

Some quantitative aspects of UV detection in capillary isotachophoresis as applied to bioavailability studies

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The analysis of drugs in biological fluids for the purpose of bioavailability assessment requires sensitive and selective methods for the detection of the compound of interest. Analytical isotachophoresis combined with UV detection may be one of the methods of choice. The objective of this study is to propose a new reliable means of quantitation useful in conjunction with absorptiometric detection in analytical isotachophoresis and to demonstrate its applicability to the determination of trace amounts of biologically active compounds in complex matrices.

The concept of zone area as an integrated zone absorbance useful in zone electrophoresis or elution chromatography cannot be directly transferred to isotachophoresis because of the rather different zone shapes in these techniques [1]. In isotachophoresis the concentration profile of the zone along the longitudinal axis of the separation compartment is almost rectangular even for very short zones [1].

THEORY

If a sufficiently small amount of UV-absorbing substance is introduced as a sample, it may produce a zone shorter than the UV detector aperture width, but still possessing quite a rectangular concentration profile [1, 2]. Indeed, this is a very typical situation in isotachophoresis. As first noticed by Arlinger [3], the step height of such a short zone is no longer constant, but it becomes

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dependent on the physical zone length or on the amount of the compound injected. Partial analysis of the step height behaviour, given by Svoboda and Vacík [2], has revealed that for such short zones the step height expressed in terms of absorbance D

$$D = 1 - T \tag{1}$$

(T denoting transmittance) becomes a linear function of the amount injected and may thus be used for quantitation. The apparent zone length (read as the zone width in its half height) becomes independent on the amount injected and becomes equal to the detector aperture width:

$$D_{peak} = D_0 \cdot L/d \tag{2a}$$

$$w = d = \text{constant} \tag{2b}$$

The various interrelations between physical zone length L, maximum zone absorbance D_{peak}, apparent zone length w, and integrated absorbance are summarized in Fig. 1 together with the schematic appearance of the zone at various physical zone lengths. In both eqn. 2a and Fig. 1, d denotes the detector aperture width. Using the model of rectangular detector aperture and planar zone boundaries, the straightforward calculation of integrated zone absorbance is easily done, yielding the following expressions relating the physical zone length and integrated absorbance S (zone area), respectively

$$S = D_0 \cdot L \tag{3a}$$

$$S = D_0 \cdot L + D_1 \cdot d/2 \tag{3b}$$

where D_0 denotes the correct response [3] of the compound of interest and D_1 the correct response of the zone following or preceding the zone of interest.

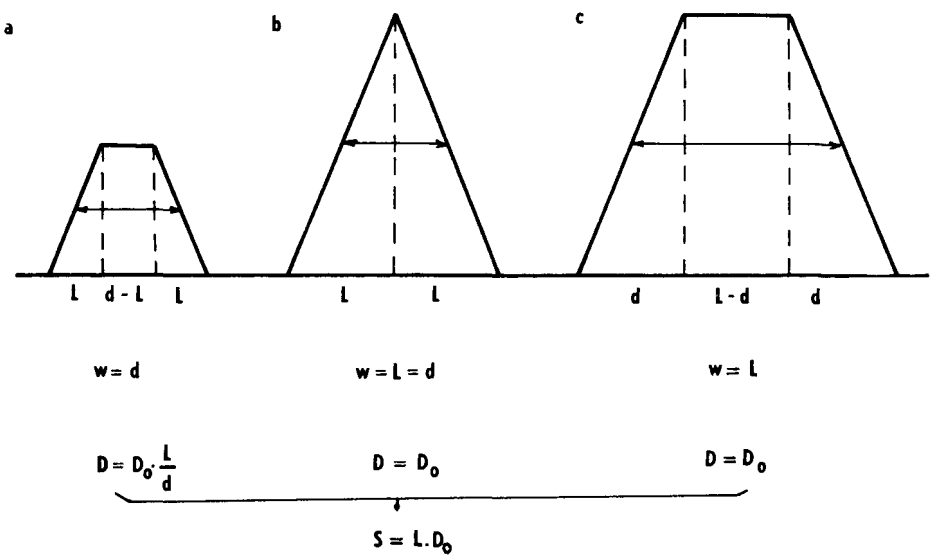

Fig. 1. Interrelations between various zone parameters: (a) zone length shorter than detector aperture width; (b) zone length equal to the aperture width; (c) zone length greater than the aperture width. For explanation of symbols see text.

Eqn. 3a holds for a single UV-absorbing zone passing the detector aperture, eqn. 3b holds for a strongly absorbing short zone followed or preceded by a less absorbing long zone of compound 1 (possibly internal standard).

More involved models including circular detector aperture and paraboloidal zone boundaries were analyzed numerically to verify the usefulness of zone area as a means of quantitation in more real circumstances [4]. The results of these simulations are exemplified in Table I, where slopes and intercepts of regression lines relating the calculated zone area and physical zone length

$$S = a \cdot L + b \tag{4}$$

are given. In each case the model yielded linear dependence with insignificant intercept and slope close to correct response (cf. eqn. 3a).

TABLE I

ZONE AREA AS A FUNCTION OF ITS PHYSICAL LENGTH

$S = a \cdot L + b$. Single absorbing zone passing through the detector.

Aperture	Zone boundary	a	s_a*	$10^2\ b$	$10^2\ s_b$*
Circular	Planar	8.444	0.006	0.4	1.1
Circular	Paraboloidal	8.41	0.04	3	6
Rectangular	Paraboloidal	8.06	0.03	2	5

*Standard error of the estimates of a and b (as calculated from the Monte Carlo simulation [4]).

EXPERIMENTAL

The compound under study [α-arylalkylamino-ω(N_q-aryl-1,q-diaza(substituted)cycloalk-1-yl) alcohol] is denoted here as VULM 120; it was synthesized in the Drug Research Institute (Modra, Czechoslovakia). It possesses three tertiary amine functions, two of them being dialkylarylamine groups, the third being of the trialkylamine type. Its molecule carries two relatively weak isolated aromatic chromophores and its net charge in the vicinity of pH 5 is about 1.5 electron charges. Considering the modest absorptivity of the compound under study, a low absorbing internal standard (tetraphenylarsonium ion) was chosen. A detailed scheme of sample preparation prior to isotachophoresis is shown in Fig. 2.

Isotachophoresis was done using the instrument constructed in the Drug Research Institute, Modra, equipped with a 28 cm × 0.5 mm I.D. FEP capillary (Kablo Vrchlabí, Czechoslovakia) and filter photometric UV detector. A high-pressure mercury arc lamp (HQE 40; Narva, G.D.R.) and 254-nm interference filter (UV KIF 254; C. Zeiss, Jena, G.D.R.) were used. The leading electrolyte consisted of sodium acetate brought to pH 5 with acetic acid and diluted to give a final Na^+ concentration of 2 mmoles/l. After dilution, the pH was checked and readjusted to 5 ± 0.05 by addition of either sodium acetate (2 mmoles/l solution) or a few drops of concentrated acetic acid. No additives to this electrolyte were used. The terminating electrolyte used was ca. 0.2 mole/l

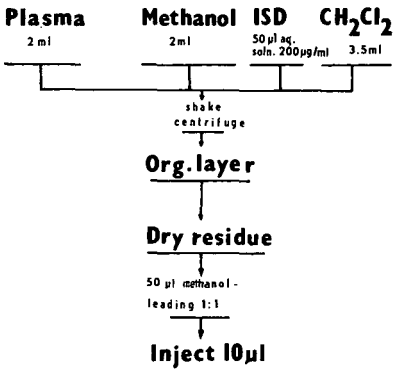

Fig. 2. Scheme of sample preparation prior to isotachophoresis. The dichloromethane extract was reduced to dryness on a water-bath at 55—65° C. ISD = internal standard (200 µg/ml aqueous solution of tetraphenylarsonium chloride).

glycine. All chemicals used were p.a. grade preparations (Lachema, Brno, Czechoslovakia) except for tetraphenylarsonium chloride (p.a.; Fluka, Buchs, Switzerland) and dichloromethane (Merck, Darmstadt, G.F.R.; Uvasol grade). Typical recordings of sample and blank runs are shown in Fig. 3. In both cases 2 ml of either control or spiked plasma were subjected to the sample preparation procedure given in Fig. 2. and 10 µl of the resulting solution were analyzed.

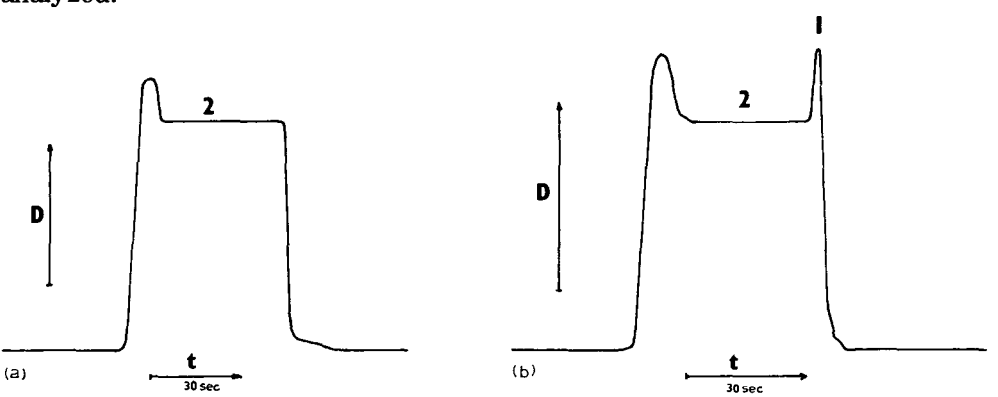

Fig. 3. Isotachopherograms of (a) typical blank and (b) sample runs. 1 = VULM 120, 2 = internal standard; driving current 20 µA. In both cases 2 ml of plasma were extracted.

The results were quantitated using the internal standard method in terms of the zone area ratios

$$c_{\text{plasma}} = a(S_{120}/S_{\text{TPA}}) + b \qquad (5)$$

where c_{plasma} is the unknown plasma concentration, S_{120} and S_{TPA} are the zone areas of VULM 120 and tetraphenylarsonium cation, respectively. The standard curve, obtained by analyzing rat plasma samples spiked with known amounts of VULM 120, is shown in Fig. 4. The curve is linear from the detection limit up to the highest concentration tested (6 µg/ml plasma). The detection limit was

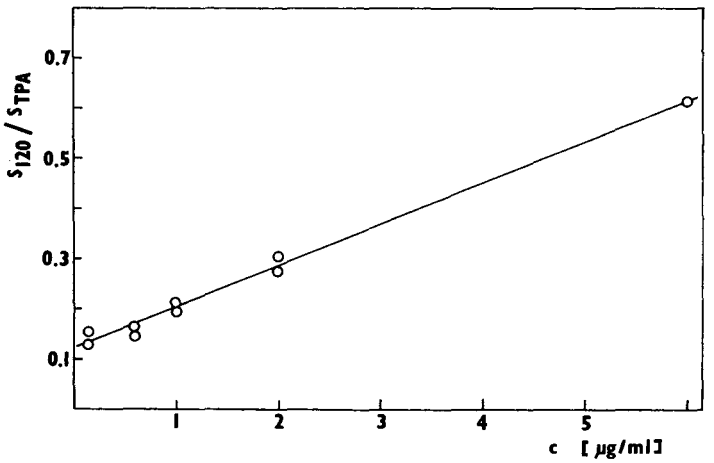

Fig. 4. Standard curve obtained by analyzing plasma samples spiked with known amounts of VULM 120. c denotes concentration of VULM 120 in plasma; S_{120}/S_{TPA} = zone area ratio (see text).

TABLE II

SOME REPRESENTATIVE RESULTS OF PLASMA CONCENTRATION OF VULM 120

$c_{plasma} = (12.4 \pm 0.2) \cdot (S_{120}/S_{TPA}) - (1.54 \pm 0.02)$; $n = 9$. All concentration data are in µg/ml.

Concentration expected (c_0)	Concentration found (c)	σ*	σ_e**	n***
0.40	0.39	0.04	0.052	6
0.50	0.53	0.07	0.053	3
1.00	1.03	0.04	0.061	3

*Standard error estimated from the spread of the measured data.
**Standard error estimated from the calibration curve.
***Number of measurements.

estimated from the spread of the standard curve points and it is about 80 ng/ml plasma. The slope and intercept of the standard curve together with their standard errors and some representative data are given in Table II.

DISCUSSION

Based on the reported results, the zone area (in terms of integrated zone absorbance) may be considered a very useful means of quantitation in capillary isotachophoresis, improving the sensitivity and accuracy even in such highly demanding tasks as analysis of trace amounts of biologically active compounds in the complex matrices of biological fluids. Even though the UV-spike method based on step height measurement gave rise to some analytical methodologies with detection limits in the low picomole range (see, for example, refs. 2 and

5), it suffers from serious disadvantages. The linear relation may be affected by the non-rectangular shape of detector aperture. Moreover, both the UV-spike and classical zone-length methods become very inaccurate if the physical zone length approaches the detector aperture width. The use of zone area, which is a monotonic function (linear in most cases) of the physical zone length (or the amount injected) regardless of the physical zone length, may help to overcome these difficulties.

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END OF SYMPOSIUM PAPERS

PUBLICATION SCHEDULE FOR 1983

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

MONTH	D 1982	J	F	M	
Journal of Chromatography	252 253/1 253/2	254 255 256/1	256/2 256/3 257/1	257/2 258 259/1	The publication schedule for further issues will be published later.
Chromatographic Reviews					
Biomedical Applications		272/1	272/2	273/1	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint 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 review articles, see page 2 of cover under Submission of Papers.

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

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

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