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REVIEW

COMPUTER-AIDED MOBILE PHASE OPTIMIZATION AND CHROMATOGRAM SIMULATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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1. INTRODUCTION

Several different methods of computer- or microprocessor-aided mobile phase optimization in high-performance liquid chromatography (HPLC) have recently become available. Some can be run off-line using programs designed for personal microcomputers and others are integral routines in microprocessor-controlled chromatographs. The purpose of this review is to outline some of the salient features of these different systems, and to compare them with the computer chromatogram simulation method (CCSM) developed and tested in our laboratories. The procedures under review are listed in Table 1. While this list will certainly not remain comprehensive for long, the methods it contains are broadly representative of the various semi-empirical and systematic strategies that can be adopted with the goal of multi-solvent mobile phase optimization in view.

2. STRATEGIES FOR OPTIMIZATION

Most strategies for the optimization of HPLC conditions are based on the well verified relationship [1]

$$R_s = \tfrac{1}{4} \cdot (\alpha - 1) \cdot (\sqrt{N}) \cdot \frac{k'}{k'+1} \qquad (1)$$

where R_s = resolution factor, N = plate number, k' = solute capacity factor and α = selectivity factor (k'_2/k'_1).

At the present time approximately 100 000 theoretical plates per m represent the maximum efficiency attainable with conventional packed columns, and, as is well known, because resolution is a function of $\sqrt{N}$, any increase in plate number results in a proportionately smaller increase in effective resolution. Increases in resolution obtained by increasing solute capacity factors obviously compromise sensitivity of detection. Consequently, most efforts at enhanced resolution have been directed at changing the selectivity factor (α), either by altering the stationary phase or the mobile phase, or both. There are, however,

some difficulties in ensuring reproducibility of separations when different stationary phases are used for selectivity, particularly when reversed-phase (RP) packings are employed, and complex separations are attempted which may depend on some degree of mixed-mode chromatography for success. Selectivity through mobile phase modification is therefore preferable in principle, and in practice. Mobile phase modification includes, of course, the commonly adopted methods of gradient (i.e., non-isoeluotropic) elution. This is usually employed simply to reduce the time of analysis and increase sensitivity, but can also be used to generate retention data with which to predict retention times in corresponding isocratic systems (and vice versa), aspects of the theory and practice of which have been extensively discussed by Snyder and co-workers [2,3].

The ideal system of chromatographic optimization should enable the chromatographer to achieve the maximum attainable separation of all components of complex multi-component mixtures of solutes within the minimum time of analysis possible. The advent of the microcomputer has recently placed within the reach of all chromatographers a variety of possible computational methods for achieving this goal. Under ideal circumstances, the best method would involve the direct (a priori) prediction of chromatographic behaviour from the individual chemistry of each solute in relation to the mobile phase composition. While it is, for example, possible to predict the retentions of, for example, small peptides and polypeptides in reversed-phase chromatography from their amino acid composition [4, 5], the general utility of this approach is severely limited [4,6].

In more typical cases of smaller molecules the complexity of the interactions between such solutes and the mobile and stationary phases is such [7] that deterministic models of their chromatographic behaviour and solvent theory have been of limited value for optimizing complex multi-component separations, particularly with respect to the reversed-phase mode which has essentially come to dominate HPLC on account of its reproducibility and versatility. A deterministic procedure that has been adapted for microcomputer-aided mobile phase optimization in isocratic RP-HPLC has, nevertheless, been recently developed by Jinno and Kawasaki [8]. Their method (Retention Prediction System) is based on the type of quantitative structure—retention relationships (QSRR) that derive from principles elaborated in gas—liquid chromatography (GLC). It is, therefore, based on the assumption that the free energy of retention of a molecule can be derived from a linear combination of retention energies of its constituent functionalities. It is also assumed that there will be no changes in the stationary phase (due for example to column conditioning) that will modify QSRR. In Jinno and Kawasaki's [9] latest approach the correlations between log k' and physicochemical parameters such as π (hydrophobic parameter), P (partition coefficient), F (correlation factor), χ (molecular connectivity index), L/B (shape parameter) and V_W (Van der Waals volume) have been reduced to log P and F, enabling the calculations of these correlations by linear multi-regression analyses to be carried out on a 16-bit microcomputer. The database for such predictive procedures obviously requires a large number of experiments and compounds, and empirical correction factors have had to be included even with the closely related substituted polycyclic aromatic hydrocarbons studied by Jinno and Kawasaki [9] in order to obtain

a reasonably useful correlation between predicted and observed retentions as a function of mobile phase compositions. The application of this procedure to other less ideal solutes may, however, be less satisfactory.

In the majority of instances, trial and error methods of reversed-phase optimization have predominated with in general the selection of the best binary mobile phase on the basis of individual organic modifier selectivities [10,11]. However, as separations of increasing complexity have been required, multi-solvent mobile phases have been used with increasing frequency because of the different specific selectivities that can be obtained with individual organic modifiers [12]. Intuitive or non-systematic optimization of three-component mobile phases is both time-consuming and ineffective, for four components it is virtually impossible, and methods of sequential approximation and/or stochastic prediction employing computer-aided factor design or mixture design strategies become necessary. The former are often applied to discrete variables (e.g. pH, temperature) and the latter to related variables such as solvent concentrations. Table 1 includes examples of both of these optimization strategies.

TABLE 1

METHODS OF MICROCOMPUTER- OR MICROPROCESSOR-AIDED MOBILE PHASE OPTIMIZATION IN HPLC

Method	System	Type	Reference	
			Authors	No.
COF	Off-line	Isocratic	Glajch et al. (1980)	20
Window diagram	Off-line	Isocratic	Sachok et al. (1980)	32
ORM (SENTINEL)	Integral	Isocratic	Glajch et al. (1982)	35
ISOOPT	Integral	Isocratic	Berridge (1982)	25
TERNOPT	Integral	Isocratic	Berridge (1982)	25
GRADOPT	Integral	Gradient	Berridge (1982)	25
Search and stop	Off-line	Isocratic	Drouen et al. (1982)	19
OPTIM (I)	Integral	Isocratic	Bradley and Gillen (1983)	27
OPTIM (G)	Integral	Gradient	Bradley and Gillen (1984)	28
OPEX	Off-line	Gradient	Sabate et al. (1983)	26
IMGE	Off-line	Gradient	Kirkland and Glajch (1983)	21
SMGE	Off-line	Gradient	Kirkland and Glajch (1983)	21
PEAKIN/SAS	Off-line	Isocratic	Issaq (1984)	36
RPS	Off-line	Isocratic	Jinno and Kawasaki (1984)	8,9
CCSM-I	Off-line	Isocratic	D'Agostino et al. (1984)	39

3. CRITERIA FOR OPTIMIZATION AND PARAMETERS FOR EVALUATION OF CHROMATOGRAMS

In order to use some form of automated method for optimization it is necessary to derive an objective parameter to define the 'goodness' of chromatograms. There is, as yet however, no universally accepted measure. A variety of different quantitative measures of the quality of peak separation have been proposed. Those commonly used are usually defined either in terms of separation/resolution of either all peaks collectively, or alternatively the least well

resolved pair, together with, in some instances, a term which relates to the total time of analysis required to effect a given separation. The resultant parameter or function constitutes the response with which the quality of an experiment is evaluated when using, for example, a simplex method [13] as a multi-dimensional search procedure for localising the optimum conditions. From a stochastic point of view the use of only the least well resolved pair as the response has certain limitations for multi-component separation in HPLC. A criterion which operates on all solutes, either by summating individual peak pair resolution values, or by calculating their product, is, in our opinion, preferable.

3.1. Chromatographic response function

The chromatographic response function (CRF) is a criterion which has been used in several forms but which can be essentially defined as peak separation parameter (Fig. 1). It was originally suggested by Kaiser [14] and developed as a summated function for multi-component separations in GLC by Morgan and Deming [15], who introduced the use of a logarithmic as distinct from geometric peak separation factor to enhance the sensitivity of the CRF value to poorly separated peaks. It was applied to HPLC by Watson and Carr [16], who included a term to measure excess analysis time, as in the form given in eqn. 2.

$$\mathrm{CRF} = \Sigma \ln(P_i/P_d) + \alpha(T_m - T_l) \qquad (2)$$

where P_i is the experimental peak separation, P_d is the desired peak separation (common to all pairs of peaks), T_l is the actual analysis time, T_m is the acceptable analysis time and α is an arbitrary weighting factor adjusted to achieve an operationally satisfactory balance between resolution and analysis time (typical values are 0.1—0.01).

In calculating the CRF, only pairs of peaks that do not give effectively baseline resolution ($P<1$) will contribute to the overall value, and under ideal conditions, e.g. $P_i>P_d$ for all peaks and $T_l<T_m$, CRF is zero (see Fig. 1). However, this criterion may not provide a particularly robust separation, particularly when there are large differences in adjacent peak height. For this reason optimization criteria based on a peak resolution parameter in which separations greater than baseline may be optimized are generally to be preferred on

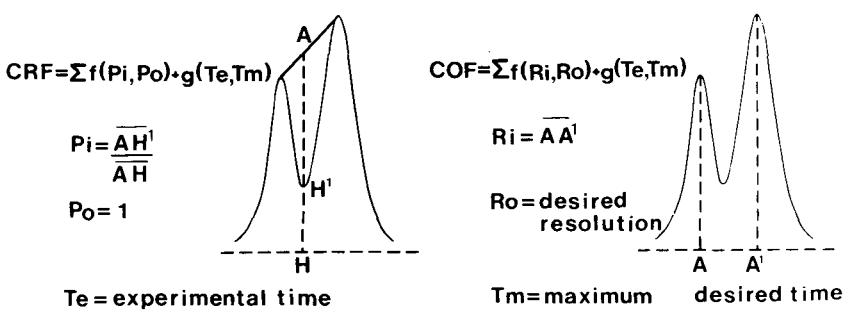


Fig. 1. Peak separation and peak resolution based parameters. CRF and COF are shown as examples.

account of their greater flexibility in an interactive context. Furthermore, although Wegscheider et al. [17] have introduced a 'noise' term into CRF calculations (which they calculate as a product rather than a sum), peak separations according to the CRF criterion may, in practice, be difficult to measure accurately because of baseline drift, baseline noise or large differences in peak heights. Most CRF-type calculations have involved the assumption that separations of all components are equally desirable, although this is often at variance with real chromatographic requirements.

3.2. Relative resolution product

The relative resolution product (RRP) defined by Schoenmakers et al. [18] and Drouen et al. [19] measures greater than baseline separations, and in its original form makes the assumption that all solutes are of equal importance, although weighting factors could be included. The latest form taken by this criterion is given in eqn. 3.

$$r = \prod_{i=1}^{n-1} R_{s_{i+1,i}} \Big/ \left[\left(\sum_{i=1}^{n-1} R_{s_{i+1,i}} \right) / (n-1)^{n-1} \right] \tag{3}$$

where $R_{s_{i+1,i}}$ is the resolution factor for adjacent peaks i and $i+1$, defined as $R_s = (V_{i+1} - V_i)/(w_i + w_{i+1})$ where V is the elution volume and w the 2σ peak width.

The RRP (r) reaches a maximum of 1 (the optimum) when all R_s values (resolution factors) are equal, with the result that it describes a situation when all peaks are evenly distributed throughout the chromatogram. It is, however, reduced to a minimum (0) when any two peaks are co-eluted. This, together with the lack of a peak priority term whereby the separation of peaks of particular analytical importance can be protected, constitutes a serious limitation of this criterion. Furthermore, because it considers only the relative and not the absolute positions of peaks it does not, in its original form, distinguish between alternative solutions with the same r values but different analysis times. A modification of eqn. 3 was consequently proposed by Drouen et al. [19] to take into account the observed different overall analysis times encountered when using nominally isoeluotropic ternary mobile phases. This consists of incorporating a factor in the denominator of the equation representing an imaginary peak ($i = 0$) eluting at the desired starting point of the chromatogram (t_0) such that, if the first actual peak elutes later than this point (for example $k' = 1$), the value of r is reduced. In this form the RRP aims at an even distribution of peaks early in the chromatogram.

3.3. Chromatographic optimization function

The chromatographic optimization function (COF) [20], on the other hand, has interactive features which commend it for stochastic (i.e. statistical) predictive approaches to chromatographic optimization. Like the RRP it is a peak resolution rather than a peak separation parameter (Fig. 1), but includes in its original form weighting factors for both time of analysis (like CRF) and peak

resolution priority (eqn. 4):

$$\mathrm{COF} = \sum_{i=1}^{k} A_i \cdot \ln\left(\frac{R_{s_{i+1,i}}}{R_{s_{id}}}\right) + B(T_m - T_l) \tag{4}$$

where R_{s_i} = peak resolution, $R_{s_{id}}$ = desired peak resolution, T_m = maximum analysis time acceptable, T_l = actual analysis time and A_i and B are arbitrary weighting factors.

Under optimized conditions the COF approaches zero from a negative direction and only when peak priority weightings are all set to unity does it reduce to a form akin to CRF (eqn. 2). As discussed below, however, the full form of the COF has not been utilised in all applications for automated optimization.

3.4. Chromatographic optimization coefficient

In our own approach to computer-aided optimization by off-line methods we have used an extended version of the full COF formula which we have termed the chromatographic optimization coefficient (COC) (eqn. 5). As described below it is used as a coefficient to calculate quality of chromatograms, real and predicted, and not as a function whose variation is directly correlated with mobile phase composition as in the original method of Glajch et al. [20]. The COC is, like COF, a peak resolution parameter and includes weighting factors for peak priorities as well as a term reflecting the total analysis time.

$$\mathrm{COC} = \sum_{i=1}^{n} \; i \neq j \; \sum_{j=1}^{n-1} A_i \cdot A_j \cdot \ln\left(\frac{R_{s_{i,j}}}{R_{s_{i,jd}}}\right) + \sum_{j=1}^{n} B \cdot \sqrt{A_i} \cdot \frac{T_m - T_i}{T_m} \tag{5}$$

where n = number of compounds, A_i and A_j are weighting factors for each compound, $R_{s_{i,j}}$ = resolution between peaks i and j, $R_{s_{i,jd}}$ = desired resolution between i and j, T_m = desired total analysis time, T_i = retention time of peak i, and B is a weighting factor for analysis time (typically 0.1). If $T_i < T_m$ then $T_m - T_i = 0$; if $R_{s_{i,j}} > R_{s_{i,jd}}$ then $R_{s_{i,j}} = R_{s_{i,jd}}$.

Like COF, the COC is the sum and not the product of individual peak pair resolutions and is not unduly influenced by a single instance of eclipsed peaks, although as a logarithmic resolution function it remains sensitive to incompletely resolved solutes.

By insertion of appropriate parameters (A_i, $R_{s_{i,jd}}$, T_m, B) optimum conditions can be selected in terms of peak resolution, total analysis time or any combination of these parameters. When the COC is implemented as part of the automated computer chromatogram simulation method (CCSM), the chromatographer has interactive control over peak priority weightings and desired total analysis time, and must enter the desired resolution in the form of the experimentally determined peak width for each component of the mixture (i.e. as a function of the efficiency of the system with respect to each solute), or a greater value as he deems appropriate, depending on the robustness of the predicted separation.

4. METHODS OF OPTIMIZATION

4.1. Isoeluotropic versus non-isoeluotropic optimization

Optimization procedures can be operationally divided into those for isocratic separations and those for gradient elution conditions. Table 1 includes examples of both, as indicated. However, they are more logically divided into those which involve an empirical pre-selection of overall solvent strength and which then search for a local optimum involving an isoeluotropic multi-component mobile phase and those which systematically optimize a multi-component mobile phase without prior selection of solvent strength. Among the latter non-isoeluotropic (or multi-eluotropic) methods are those which allow systematic optimization of multi-solvent gradient elution (e.g. SMGE [21] and CCSM-Gradient, see below), as well as a fully systematic optimization of solvent strength for isocratic separations with mixtures of two (PEAKIN/SAS) or three (CCSM-I) organic modifiers in water.

4.2. Sequential approximation with factorial design versus statistical prediction with mixture design

In addition to discriminating between isoeluotropic and non-isoeluotropic optimization systems, two main design approaches can be distinguished among the procedures listed in Table 1. The first involves a method of successive approximation requiring an initially undefined number of experimental chromatograms before an optimum is found, and the second is a stochastic predictive approach based solely on data from a limited pre-defined number of chromatograms. The nature of the equations used to define the relationship between solute behaviour and chromatographic conditions is also significantly different in various methods with linear, quadratic and higher-order polynomial regressions used in different cases, with implications with regard to precision with which optima are defined, and the speed with which they are found.

4.3. Sequential approximation methods

4.3.1. Simplex optimization

Some optimization procedures have chosen sequential simplex methods for attaining the best response. A simplex is a geometric figure defined by the number of vertices equal to the number of dimensions of the factor space plus one [13]. Sequential simplex optimization is a geometric search pattern technique which evaluates the response of a system from a set of points forming a simplex in the factor space and tracks the optimum by continually forming new simplices by reflecting one point in the hyperplane of the remaining points, according to the rules proposed by Spendley et al. [22]. The latter have their origin in a consideration of how the evolutionary operation of the steep ascent optimization procedure of Box could be automated. The response is defined as a surface in $(n + 1)$-dimensional space, where n is the number of independently variable experimental factors (flow-rate, temperature, pH, solvent concentration, etc.). Thus, in a typical case of ternary solvent optimiza-

tion with pre-determined solvent strength there are two such variables and the response surface lies in three-dimensional space, with the simplex requiring three initial experimental points and mapping as a triangle in two-dimensional factor space (Fig. 2). With the aid of function minimization techniques [23], the simplex can be made to contract towards the optimum (provided this is unique). In a quaternary solvent system for RP-HPLC in which solvent strength (i.e. water concentration) is an independent variable the appropriate simplex is

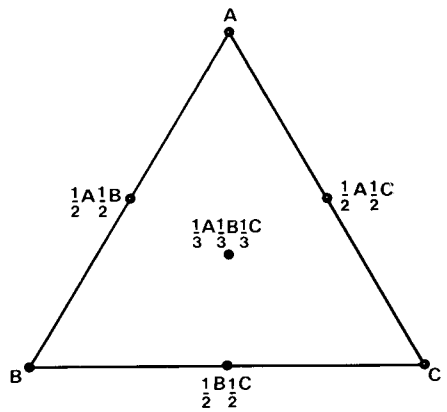


Fig. 2. Seven-point simplex lattice for mixture design optimization of a ternary mobile phase (after Glajch et al. [20]), where A, B and C are three solvents in normal phase or three binary organic modifier—water mixtures of equivalent solvent strengths in RP-HPLC.

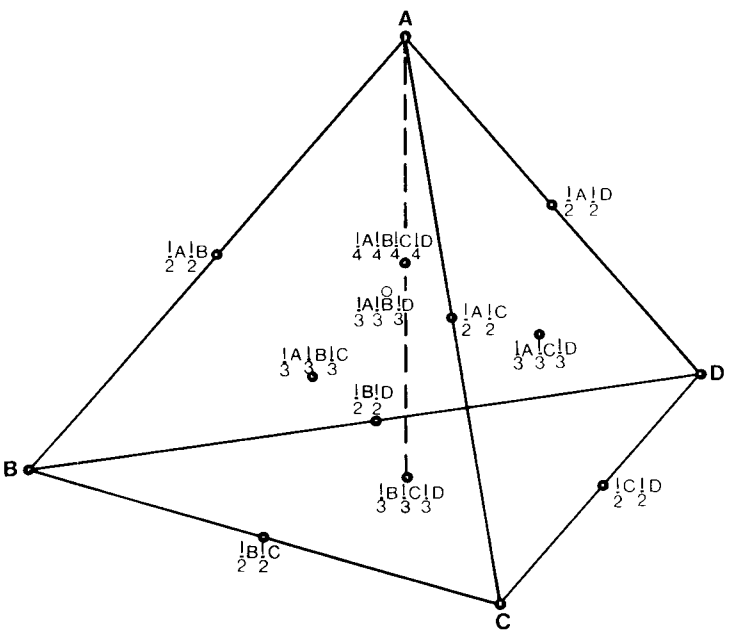


Fig. 3. Fifteen-point simplex lattice for mixture design optimization of a quaternary mobile phase in normal-phase HPLC showing the properties of the four different solvents in each experiment.

a tetrahedron (Fig. 3), with the response surface mapping in a four-dimensional hyperspace ($n + 1 = 4$). Function minimization techniques or sequential simplex optimization runs of different sizes are essential for localizing true optima in this situation. The problems associated with applying simplex algorithms for numerical optimization to experimental optimization have been extensively considered by Deming and Parker [13].

In their original experiments Morgan and Deming [15] used a 3^2 factorial design to optimize carrier gas flow-rate and temperature in GLC. As with most practical applications of simplex designs, it was necessary to place certain limits on these variables. This was achieved by assigning an undesirable arbitrary response value (CRF = −100) to any vertex of the simplex that violated this boundary. Anything up to 25—30 vertices (i.e. chromatograms) were generated in the search for the optimum, and the likely existence of multiple optima in the factor space domain, due to changes in peak retention order [24], means that these techniques cannot guarantee that the global optimum has been achieved. Nevertheless, sequential optimization is the basis of several methods for mobile phase optimization in HPLC, both in the form described here and in other semi-predictive techniques designed to limit the number of experiments required for full sequential optimization.

4.3.2. ISOOPT, TERNOPT and GRADOPT

These three closely related methods, devised by Berridge [25] for fully automated optimization are all based on a sequential 3^2 factorial simplex design. Response is measured by a CRF parameter, (eqn. 2), with the addition of a further term (L^x) in respect of peak number and dictated by the unattended on-line operation of these programs, controlling the chromatograph. The large step size simplex method of Yarbro and Deming (see ref. 13) is used with Nelder and Mead's [23] algorithm for function minimization. In ISOOPT flow-rate and binary composition are optimized, in GRADOPT solvent strength and gradient duration, while in TERNOPT a three-component isocratic mobile phase (two variables) is defined. The only computational difference is that in ISOOPT and TERNOPT boundary violations result in a rejection of corresponding experimental coordinates by assignment of CRF=−100, while in GRADOPT the simplex contracts to the relevant boundary to facilitate a more rapid convergence on the optimum. Despite this, however, up to fifteen sequential chromatograms are required for GRADOPT and up to thirty in ISOOPT and TERNOPT, even when optimizing conditions for a small number of solutes (e.g. four). None of the solute mixtures illustrated [25] involved crossovers, which as explained above can result in multiple local minima in factor space which these sequential simplex techniques are poorly equipped to handle.

OPEX [26] is another comparable multi-factorial sequential simplex method, based on resolution of only the least well resolved pair of solutes. These techniques are clearly of limited value for global optimization and are time-consuming even for simple problems in HPLC. This has lead to the development of techniques in which sequential approximation and stochastic prediction are combined to accelerate the process.

4.4. Sequential approximation with stochastic prediction

4.4.1. OPTIM

A simple example of this combined approach is the 'adaptive intelligence' ROM chip that constitutes the OPTIM system of Spectra-Physics. It is based on what is essentially a 2^2 simplex progress for binary optimization combined with a statistical predictive method for defining a ternary (three-component) optimum. Its criterion for optimization (XA) has not been precisely defined but is probably of the CRF type, being a function of separation times and the resolution of the worst resolved pair plus peak number term [27]. Having established a binary (A+B) optimum to within 1—2% in a step-wise manner it calculates the appropriate isoeluotropic A+C mixture, runs it and the 50:50 (A+B+C) mixture and by an unspecified computation predicts a ternary optimum. The binary mode is, however, time-consuming and total optimization times of 10—18 h for a twelve-component mixture are reported [27]. Its application to gradient elution has been recently described [28] and consists of substituting gradient slope for binary composition in the first stage of optimization, the starting point of the binary mobile phase being determined by the criterion that less than four peaks elute within a time equivalent to a k' of 4. It is capable of optimizing only a one-step linear gradient profile.

4.4.2. Window diagrams

Several of the semi-predictive techniques designed to overcome some of these problems are based on a window diagram approach. This originated in the studies of Laub and co-workers [29,30] on liquid phase component optimization in GLC, a problem which has obvious similarities with mobile phase optimization in HPLC, but deals with a simpler relationship between volatility and the effects of liquid phase composition on retentions than is the case for mobile phase composition and solute retention in the latter situation. Nevertheless, the window diagram is a more effective means of localising a global optimum if (and this is the key) an appropriate mathematical function can be found which accurately defines the relationship between retentions and the variable under consideration. In the experiments of Laub and co-workers [29,30] there was a single variable and a linear relationship was assumed. The window diagram is created from a graphical representation of all individual analyte pair relative retentions as a function of the variable, with intermediate values derived by linear interpolation from two experimental data sets for the limiting conditions. The intersections of the relative retention curves delimit a series of windows of accessibility. The highest point of the tallest window corresponds to the chromatographic conditions which should give the best possible separation of the two worst resolved pairs of analytes, with all other pairs separated better. This approach was transferred to HPLC with the substitution of the separation factor ($S = 2R_s/N^{\frac{1}{2}}$) for the relative volatility by Jones and Wellington [31], who also used it for mono-factorial optimization (pH). The window diagram method was also extended to non-simultaneous multifactorial analysis in RP-HPLC by Sachok et al. [32], when the windows become multi-dimensional in nature. Its limitations in this context include not only the greatly increased numbers of experimental chromatograms required,

but also a requirement for a more accurate correlation of chromatographic variables with predicted retentions for intermediate values than is possible with linear regression.

4.4.3. Search and stop

The method of Schoenmakers et al. [18] and Drouen et al. [19] for isoeluotropic mobile phase optimization is a mono-factorial sequential procedure but with certain modifications to the form of the regressions. The overall solvent strength in search and stop is chosen on the basis of a preliminary gradient elution chromatogram which establishes the range of polarities in the sample (equivalent to the SCOUT step in the SENTINEL procedure). Operationally search and stop starts with retention data from two pre-determined isocratic experiments (usually two different binary mobile phases chosen from three preliminary experiments) and linear regressions between solvent composition and ln k' values are calculated in the first instance. From these a so-called phase selection diagram (equivalent to a window diagram) is created using the relative resolution product (eqn. 3) as a function of solvent composition. The method continues with the generation of an experimental chromatogram under mobile phase conditions predicted as optimum (i.e. highest value of r) by this criterion. If the observed retentions differ significantly from the predicted values they are used to construct a corrected phase selection diagram from which a new, and hopefully improved, optimum is predicted. The iteration stops when the next optimum offers no further improvements. In an example of a five-component mixture a total of five to six chromatograms was required before this stage was achieved.

A significant disadvantage of search and stop is the fact that the RRP does not necessarily result in the most effective separation being chosen as optimal, as the results of Drouen et al. [19] themselves illustrate. Another major problem with this method is that if the variation of ln k' with solvent composition deviates significantly from linearity then the convergence to the optimum may be slow requiring many additional chromatograms. To compensate for this effect, in its latest form [19] search and stop runs these sequential chromatograms at a composition shifted from the linearly predicted optimum to a value which partially approximates a quadratic expression for ln k' versus mobile phase.

4.5. Stochastic prediction without sequential approximation

Mixture design experiments based on simplex designs can also be used for statistical predictive methods of mobile phase optimization that do not involve sequential approximation. The essential steps in this approach have been defined by Snee [33] as (1) generation of data using a pre-planned experimental design, (2) finding a mathematical model to fit this data using statistical curve-fitting techniques and (3) examining the response—surface contours to determine the best value. The advantages inherent in this approach are that they require a limited pre-defined number of experimental chromatograms and can be used for assembling a model of the chromatographic system in question that can, in principle, permit the behaviour of all solutes to be accurately pre-

dicted within the mobile phase envelope defined by the simplex, without recourse to further experiments, thus enabling the global optimum to be located. Their main disadvantage is that a relatively extensive series of polynomial regressions must be examined before coefficients that accurately describe the retentions as a function of solvent composition can be obtained.

4.5.1. Chromatographic optimization function (COF) method

The first attempt at this solely predictive approach was that of Glajch et al. [20]. The experimental design selected for isoeluotropic optimization was a ten-point design described by Snee [33], subsequently modified to a seven-point design as illustrated in Fig. 2, the other three points being relegated to a means of checking experimental error. These designs were based on the Snyder [2] solvent—selectivity triangle concept in which the combined specific selective effects of three different mobile phases are used to obtain the maximum resolution. Thus, unlike some of the ternary optimization methods described above [18,19,25,27], this scheme allows in the reversed-phase mode selection of a four-component mobile phase, i.e. three different organic modifiers and water, which obviously enhances its potential for resolving complex mixtures. The optimization criterion for the original COF method was based on eqn. 4, although A_i was generally set to unity for all peaks and B to zero so that the time term of the equation was eliminated. A single polynomial regression of a pre-determined quadratic form was used to describe the variation of the computed COF value as a function of mobile phase composition (i.e. solvents A, B, C versus COF). This procedure has, however, several distinct limitations, the most notable of which was that it only works if all peaks have the same relative retention order in all mobile phases, that is, if there is peak cross-over the COF value may not reflect this change. Furthermore, as no peak number term was included, solutions with co-eluting peaks may give better COF values than those in which more peaks are detected [20]. Nevertheless, in cases in which no peak order change was observed, useful prediction of optimum conditions was obtained, validating the general concept of mixture design statistical methods for optimization. Direct correlation of COF with solvent composition results, however, in a relatively high level of imprecision when applied to more than five to six solutes [20]. Attempts to extend the COF method to accommodate the problems were deemed cumbersome, however, and an alternative approach, the overlapping resolution map (ORM) was developed. This is essentially a simultaneous multi-factorial application of the window diagram approach with the use of quadratic regressions.

4.5.2. Overlapping resolution map (ORM)

This procedure also utilises the seven-point solvent selectivity triangle-based simplex design as its source of experimental data, and like COF is based on mathematical mixture design models (simplex—lattice) originally proposed by Scheffe [34]. It relies on comparing the resolution of every pair of peaks obtained for each solvent mixture by calculating the resolution surface of the solvent triangle by fitting the data to a second-order polynomial regression

equation [20,35]. A desired minimum resolution is chosen and the area of the triangle representing that separation for each pair delineated, the resultant overlapping resolution map thus comprising the intersection of Venn diagrams for acceptable resolution for all compounds. It extends the window diagram technique to the simultaneous analysis of ternary (four-component) mobile phases in the RP mode as distinct from binary solvent mixtures. In its original form [20] the ORM did not generate a unique optimum, but simply served to define an area corresponding to limits of mobile phase composition within which acceptable resolution of all peaks can be obtained, if this is possible, i.e. a window of accessibility. It handles peak cross-overs (provided that peaks are correctly identified in the experimental chromatograms) but assumes equivalence of priority for all peaks under consideration; minor peaks must be totally excluded if an improved separation of major components is required.

Subsequent modifications to the ORM procedure [35] relate essentially to its operation in the normal-phase mode and subsequent implementation as a fully automated optimization protocol (SENTINEL, DuPont) and include the definition of a unique optimum, by an unspecified procedure. This presumably involves the assignment of a numerical COF-type value to the sum of predicted solute pair resolutions. There remains, however, no facility for assignment of differing peak priorities and there is no systematic optimization of analysis time, other than that inherent in the selection of an overall solvent strength on the basis of a preliminary binary gradient chromatogram (SCOUT in SENTINEL terminology).

4.5.3. PEAKIN-SAS

This stochastic predictive procedure developed by Issaq [36] is based on a similar approach to ORM with peak interval as the criterion of optimum resolution and using a ten-point simplex lattice as the data base. If differs only in that it has been designed as a non-isoeluotropic system not requiring pre-selection of solvent strength and is limited in this form to mixtures of two organic modifiers and water, using a full cubic form of the regressions searched.

4.5.4. Computer chromatogram simulation method (CCSM-I)

It is clear that none of the above methods affords a complete solution to the problem of systematic optimization of the mobile phase. Each suffers from one or more defects, either in terms of assuming a specific and possibly inappropriate mathematical relationship between solute retentions and mobile phase composition, and/or lack of facilities to control optimization with respect to time of analysis or to the real analytical importance of individual peaks.

Our approach to this problem was to develop a coherent predictive statistical method which can cope with any change in retention order, with any number of solutes, which incorporates a peak priority weighting factor, and which can optimize analysis time in an interactive manner, according to the requirements of the individual chromatographer.

The CCSM is based on the use of the COC (eqn. 5) as the optimization criterion. It is a statistical mixture design method which also employed the seven-point simplex (Fig. 2) used by Glajch and co-workers [20,35] for ternary mobile phase optimization. It differs from the COF method (see *4.5.1.*) in that

the retention times for each component of the solute mixture are first transformed by the computer program into logarithmic retention indices ($\ln T_i/\ln T_0$) using one component of the mixture as an internal standard. Log retention indices give the best fit to a Gaussian distribution and reduce variance of data from replicate chromatograms obtained at different times. Furthermore, we cannot see any advantage in using peak interval ($\Delta T_0/4$) [20,35], a secondary variable which is geometrically increased in relation to peak number, as distinct from log retention indices, as the dependent variable. Calculation of the latter involves no loss of real chromatographic information and thus enables a complete simulation of a chromatogram to be assembled by the computer. The use of capacity factors does not improve the fit to a normal distribution and does not reduce variance due to time-related changes in, for example, column characteristics. Because calculation of capacity factors requires an additional step, it is not included in the CCSM procedure.

The next step is calculation by a basic algorithm of the best polynomial multiple regression for each solute (solvents A, B, C, D versus $\ln T_i/\ln T_0$). The a priori restriction of calculated polynomials to a pre-determined quadratic form by Glajch and co-workers [20,35] is not, in our view, justified. The complexity of solvent–solute–stationary phase interaction in HPLC may require even more complex mathematical models [37,38] for enhanced precision. In principle any continuous response surface can be represented by a polynomial, if enough terms are included. In our experience, treatment of water simply as a diluent of the organic modifiers is not valid with respect to a number of solutes. We have opted for selection from all possible incomplete 6th degree equations, with the best for each retention index selected by evaluation of correlation coefficients (r_{ij}). Analysis of data from experiments with mixtures of ten corticosteroid hormones [39] resulted in a significant improvement in precision, compared with other quadratic regressions calculated according to the COF method [39]. Provided that uncontrolled changes in column performance do not supervene, this must result in an increase in the likelihood that the predicted optimum will yield an improved separation in practice.

After insertion of desired separation parameters (A_i, $R_{s_{i,jd}}$, T_m) by the chromatographer our program continues by simulating a large number of individual chromatograms at a pre-selected solvent composition interval until the entire mobile phase envelope defined as in Fig. 1 has been explored. COC values are thus calculated for chromatographic retentions predicted for all possible three- and four-component mobile phases, and the global optimum, together with any alternative local minima identified and displayed as well as the predicted retention times for these compositions (see Fig. 7). If the predicted separation is clearly inappropriate (for example because the most important solute is minimally resolved), then the simulations can be repeated with a new set of priority weightings, which will be reflected in the calculated COC values. In our experience not more than two or three such iterations are required to generate a satisfactory solution, and with prior experience of the chromatographic behaviour of the compounds in question, it is often possible to achieve a satisfactory optimum in a single run of the program, lasting approximately 2–3 h for mixtures of about fifteen compounds. If a good

separation is predicted, then attempts can be made to reduce analysis time by further simulations with a reduced value for T_m. The effective use of this method in optimizing the separation of a natural mixture of ten polar steroid hormones has recently been described [39].

Fig. 4 shows in a graphical form the solutions that the program found which yielded better separations than the best of the experimental chromatograms that provided the database. As can be seen, two local optima were discovered, one involving a four-component and another a three-component mobile phase, the latter giving the lower COC values and thus better overall separations. The actual chromatograms with this mobile phase and the predicted retentions are illustrated in Fig. 5. The error index calculated as the mean difference between all predicted and observed retentions was 6%.

We have also used this program in an attempt to optimize the reversed-phase separation of a group of naturally occurring catechol oestrogens, their metabolites and their precursors. The selectivity patterns obtained with four different binary mobile phases are illustrated in Fig. 6, and the experimental data for the seven chromatograms using acetonitrile, methanol and tetrahydrofuran as the organic modifiers (after Snyder [2]) are shown in Fig. 7, together with their computed COC values. With this mixture of solutes and solvents the program in fact identified a binary mixture (acetonitrile—water) as the optimum. As shown in Fig. 6, this failed to resolve only two compounds (Nos. 2 and 3) which were, however, resolved with tetrahydrofuran—water. When we weighted the priority values for 2 and 3, we could not, however, identify a solution in

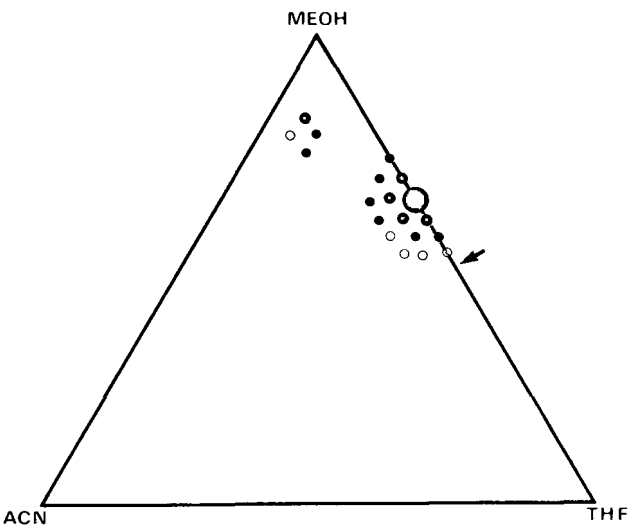


Fig. 4. Solutions afforded by CCSM-I for mixture of polar corticosteroids using a seven-point simplex. The points indicated and their size show the COC values for all mobile phases giving better predicted separations than the best of the experimental chromatograms (indicated by arrow). The best predicted solution corresponded to a three-component mobile phase comprising methanol—tetrahydrofuran—water (22:4:74) although note the four-component local minimum also found. The data for this optimization and the identities of the steroids are given in ref. 39.

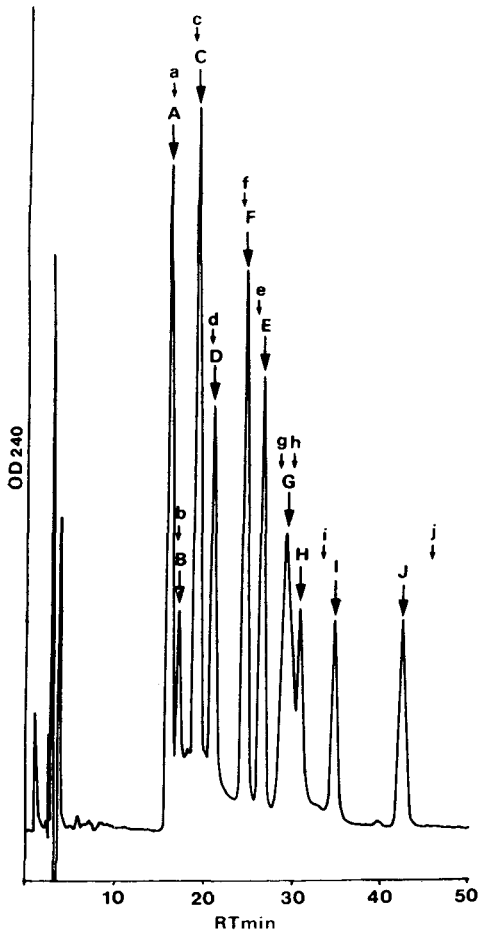
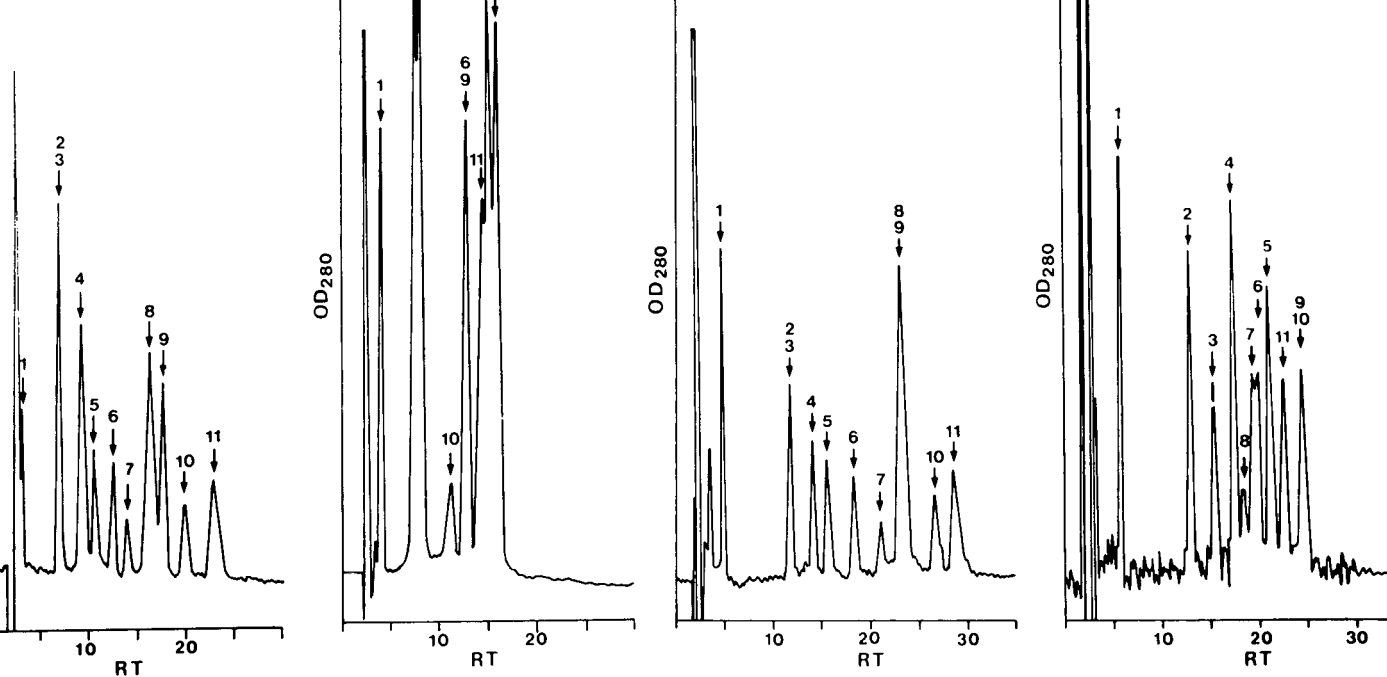


Fig. 5. Predicted and actual separations given by the mobile phase optimized in Fig. 4. The UV trace shows the actual separation with compounds (designated by upper case letters), the arrows with corresponding lower case letters show the retention times predicted by the program for each component of the mixture, with a mean error of 6%.

which complete resolution of all peaks was predicted when using the seven-point procedure with empirically chosen pre-defined overall solvent strenghts.

In the above form, the CCSM method still has important limitations; notably, there is an arbitrary selection of overall solvent strength for the iso-eluotropic optimization. Furthermore, because of non-additive solvent strength effects obtained with mixed organic modifiers [12] predicted absolute retentions may deviate significantly from those observed, if regressions are calculated on the basis of a seven-point design (Fig. 2), although relative retentions will be accurately predicted and in general the effective optimum will be found. Nevertheless, to encompass organic modifier—water interactions and their effects on absolute retention, we have extended the procedure, basing it on the same algorithm, but permitting a true optimization of a quaternary mobile phase with solvent strength as an independent variable (four components, three variables). In this modification a total of twelve pre-determined



6. Binary solvent selectivities for a mixture of oestrogens, catechol oestrogens and their metabolites. The chromatograms sh

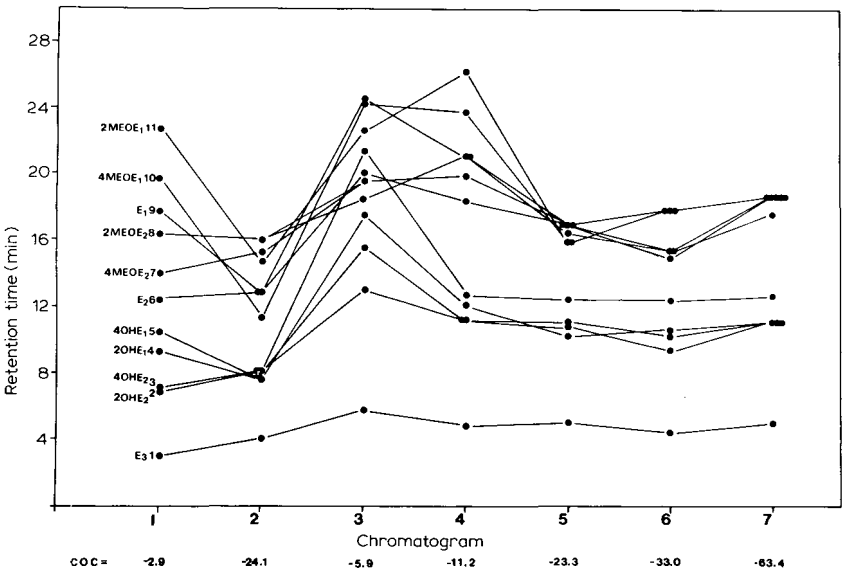


Fig. 7. Data from seven-point simplex for optimization of the isocratic separations of a mixture of oestrogens and catechol oestrogens and their metabolites shown in Fig. 6, together with corresponding COC values for each of the seven experimental chromatograms; with equal priority weightings and a desired analysis time of 25 min.

chromatograms (illustrated in Fig. 8) are required for RP-HPLC, in which solvent strengths bracketing the likely optimum are employed and very water-rich and water-poor mobile phases are excluded. Our program has, therefore, now been extended to this form of database. Introducing the water—solvent product as an independent variable in the regression calculations can result in up to a five-fold increase in the precision with which absolute retentions are predicted by CCSM.

The modified CCSM procedure can also be applied to normal-phase optimization in adsorption liquid chromatography (LC), in which it is possible that even greater specific selective effects may occur as a consequence of the more widely differing relevant properties of solvents used in this mode, i.e. non-localising, localising dipole and localising base solvents, compared with the mixtures of proton acceptor, proton donator and dipole solvents appropriate for bonded-phase LC [40]. As Glajch and Kirkland [41] have recently re-emphasized, the possibility of using quaternary solvent mixtures is a pre-requisite for utilizing the total range of potential selectivity effects in LC. For normal-phase isoeluotropic optimization with a mixture of four solvents a total of fifteen pre-determined chromatograms provides the data base in CCSM, corresponding to the full tetrahedral simplex lattice (Fig. 3).

The CCSM-I program has, up to the present, been effected on an 8-bit Apple IIe computer, the program itself occupying about 12K of RAM in BASIC and computations of optimized mobile phases taking anything up to 4—6 h each using a non-compiled version. Significant improvements have, however, obtained with use of machine code routines and other time-saving manoeuvers. These have enabled different solutions involving different priority weightings, etc. to be generated more rapidly (50—80 min) for comparison.

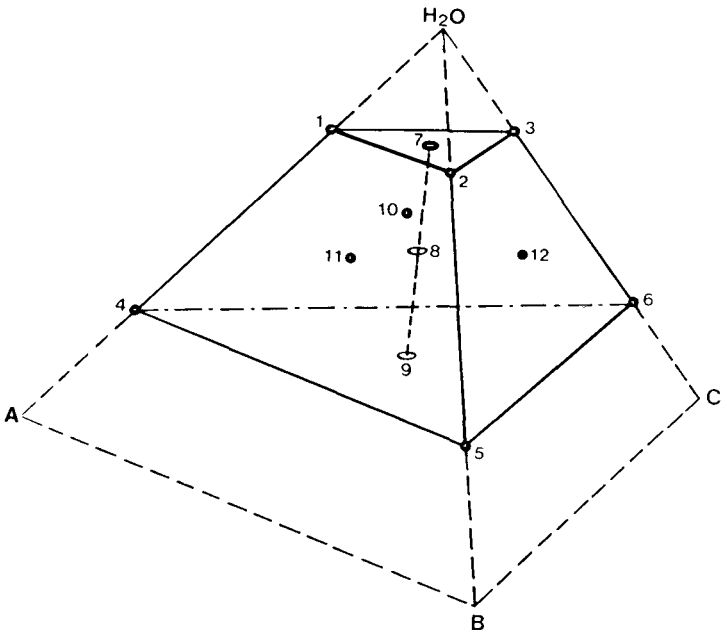


Fig. 8. Twelve-point design for systematic optimization of solvent strength in isocratic RP-HPLC without preselection of solvent strength, where A, B and C are three different organic modifiers, and the overall maximum and minimum solvent strengths are defined by the overall polarity of the solute mixture. This database can also be used for the optimization of a continuous two-pump gradient profile to separate the same solutes.

4.6. Optimization of gradient elution

The simplest form of automated gradient elution optimization has already been mentioned, viz. GRADOPT, OPEX and OPTIM [9] in which solvent strength and gradient duration are optimized without reference to solvent selectivity, which must be separately optimized beforehand in the isocratic mode. Simultaneous multi-factorial optimization by sequential simplex techniques and by window diagram methods is subject to various practical drawbacks, which have already been discussed. In principle, therefore, stochastic predictive methods based on an appropriate simplex lattice, combined with appropriate equations for describing solute relative retentions offer the most economical route to simultaneous optimization of gradient profiles and multi-solvent selectivities.

The most comprehensive approach to this goal of selective multi-solvent gradient elution (SMGE) optimization thus far has been that of Kirkland and Glajch [21,41]. Thus, although numerous studies of ternary gradient elution in practice have been carried out, notably by Jandera et al. [42], their theoretical treatment has been confined to linear gradients and has neglected mutual interactions between the two organic modifiers and water. The theoretical prediction of optimal ternary gradients from binary gradient data that they propose has not, as yet, been generally verified experimentally. Their system is, furthermore, implicitly confined to three-component mobile phases, thus limiting the range of potential specific selective effects that can be deployed.

Kirkland and Glajch [21], by contrast, have approached the problem from the standpoint of the solvent selectivity triangle with its four components. The first extension of the system was to develop a design for iso-selective multi-solvent gradient elution (IMGE) in which solvent strength but not separation selectivity (i.e. organic modifier ratios) is altered during the run [21]. To generate the requisite data for statistical optimization a series of seven iso-selective linear gradients are run, extending the selectivity triangle to a prism [41]. These data are used to calculate the coefficients of the quadratic equations that describe resolution contour plots for all peak pairs, exactly as in the ORM method of isoeluotropic optimization [35], except that peak pair resolution is defined in terms of absolute retention times, rather than on the basis of peak separations. This enables retention times for all peaks to be estimated through the complete solvent selectivity prism, and thus the global optimum with respect to linear iso-selective four-component gradients to be calculated, with a reported precision of 1—2% [41].

Thus far, however, the fully selective SMGE concept has not been realised as a systematic procedure. As an interim approach Glajch and Kirkland [41] have adopted a semi-empirical procedure in which different iso-selective gradients are selected for groups of compounds of similar polarity by inspection of iso-selective gradient runs obtained for IMGE, and these are then linked, empirically, in a discontinuous step-selective gradient with a linear solvent strength increase. A practical disadvantage of this method is that it can result in large fluctuations in detector baseline when abrupt changes of solvent composition are effected, and does not take into full account the effects of initial conditions on relative retentions of later eluting solutes. To overcome this problem and to attain the maximum flexibility it is necessary to incorporate continuous selectivity changes (preferably non-linear) in conjunction with non-linear changes in solvent strength. Kirkland and co-workers have not, as yet, provided details of how this is to be achieved.

It is clear, nevertheless, that when dealing with mixtures which contain several groups of solutes with similar selectivity factors and in which the overall polarity range is wide that a solution to systematic optimization must be sought in non-isoeluotropic designs. A program (CCSM-Gradient) has now been developed which uses the same twelve-point database illustrated in Fig. 8 to select the most appropriate binary (two-pump) gradient. The rationale of CCSM-Gradient is that a gradient can be divided into a series of instantaneous isocratic conditions for which the positions of each compound within the column can be calculated provided that certain physical characteristics of the system such as dead volume and column void volume are known. In this manner it is possible for the computer to systematically simulate the effect of various gradient profiles on elution volumes using data from a pre-defined limited number of isoeluotropic experiments. The result is an optimization with respect to a continuous gradient profile as distinct from a linked series of isoeluotropic conditions.

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6. SUMMARY

Systems for computer- and microprocessor-aided optimization of the mobile phase in high-performance liquid chromatography (HPLC) are reviewed and compared with the computer chromatogram simulation method (CCSM). CCSM was developed and tested in our laboratories for the off-line interactive optimization of four-component isoeluotropic mobile phases for HPLC. It is based on a statistical mixture design method which requires a limited predefined number of experimental chromatograms and it predicts solute retention times for systematically simulated chromatograms, selecting the best according to user-defined separation priorities. The application of similar procedures to gradient elution are discussed.

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GAS CHROMATOGRAPHIC PROFILING OF PHENOLIC ACIDS IN URINE OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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SUMMARY

Phenolic acids are analysed within the profile of the organic acids in urine of patients with cirrhosis. For the following constituents an increased urinary excretion is observed: 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, vanillic acid, homovanillic acid, 4-hydroxymandelic acid, 4-hydroxy-3-methoxyphenylpropionic acid and *p*-cresol.

The phenols are metabolites of tyrosine and are produced in the liver, in extrahepatic tissues and by intestinal microorganisms. They are suggested as biochemical control parameters for the metabolizing function of the liver, for the effect of therapy and for the existence of portal-systemic venous collaterals.

INTRODUCTION

Tyrosine metabolism is the source for a number of phenolic acids, especially 4-hydroxy compounds. The acids are produced in the liver, in extrahepatic tissues and by intestinal microorganisms. Livercirrhosis is accompanied by increased levels of phenolic substances in serum and urine [1, 2]. It is assumed that the phenols, or the metabolic situation leading to these components, contribute to the development of hepatic encephalopathy or coma [2]. Since the detoxifying function of the cirrhotic liver is limited, therapeutic measures are taken to reduce the production of bacterial metabolites. The therapy is aimed, in particular, at ammonia, but also at that part of the phenols which is formed in the intestine. Reduced dietary proteins, neomycin and lactulose are chosen for therapy.

Because patients in coma respond poorly to all forms of therapy, early recognition and treatment of hepatic encephalopathy are essential. The phenols appear to be useful biochemical parameters to control the metabolic situation

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of patients with cirrhosis in order to reduce the risk of hazards, and to follow the effect of therapy.

On the basis of a diazo method, phenols were determined by a colorimetric group reaction [3]. Separated phenolic compounds in serum and urine were investigated by paper, thin-layer (TLC) and gas chromatographic (GC) methods [1, 2, 4]. The efficiency of the separations obtained was not satisfactory. This report describes a gas chromatographic profiling method which allows shifts to be detected in the relative concentrations of the separated phenolic acids within the pattern of the urinary organic acids.

EXPERIMENTAL

Patients and specimens

Urine samples (24 h) were collected from seven patients with liver cirrhosis and three normal controls. Five of the seven patients were treated conservatively with low-protein diet, neomycin and lactulose; two patients had undergone surgery and obtained a splenorenal and portacaval shunt, respectively. Aliquots of 60 ml of urine were kept frozen at −20° C until they were analysed.

Sample preparation

The sample preparation procedure, which has been described in detail before [5], included the O-methyloximation of urinary oxocarboxylic acids, the extraction of the organic acids by an anion-exchange procedure, the methylation of the acids with diazomethane and the pre-fractionation of the derivatives by preparative TLC. Instead of using 10-ml samples, volumes of 30 ml and 60 ml were employed in this study. The amounts of isopropanol and O-methylhydroxylamine hydrochloride were increased proportionally. The TLC plates used had a silica gel thickness of 2 mm. Fraction 2 of the TLC pre-fractionation was divided into two equally broad subfractions 2a and 2b.

Gas chromatographic and mass spectrometric analysis

Fractions 2a, 2b and 3 were analysed by GC. The identity of the substances labelled in the chromatograms was confirmed by gas chromatography—mass spectrometry (GC—MS). For the GC separations a Model 3700 gas chromatograph with flame-ionization detector (Varian, Darmstadt, F.R.G.) was used. The operating conditions were as follows: 25 m × 0.2 mm I.D. glass capillary column, coated with OV-17 (Perkin-Elmer, Überlingen, F.R.G.); carrier gas, nitrogen at 4 ml/min; column temperature, 40° C for 10 min, then programmed to 230° C at 2° C/min; injector block temperature, 250° C; sample size, 1 μl at a splitting ratio of 1:20.

For the GC—MS analyses a combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian-MAT, Bremen, F.R.G.) was used employing an open coupling system between the gas chromatograph and the mass spectrometer. By automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15—300 and stored on magnetic tape. The GC conditions were the same as described for the GC separations, except that helium was used as the carrier gas. The MS conditions were as follows: ionization by electron impact; ionization energy, 70 eV;

accelerating voltage, 3 kV; multiplier voltage, 2.25 kV; emission current, 300 μA; resolution, 600.

RESULTS AND DISCUSSION

Aspects of the method

The phenolic acids are analysed within the profile of other organic acids. Figs. 1, 3 and 5 show the chromatograms of the fractions 2a, 2b and 3 of the organic acids in urine of a patient with cirrhosis. The corresponding profiles of a control person are depicted in Figs. 2, 4 and 6. In comparison with previous methods for phenolic compounds [1—3], the profile concept offers the

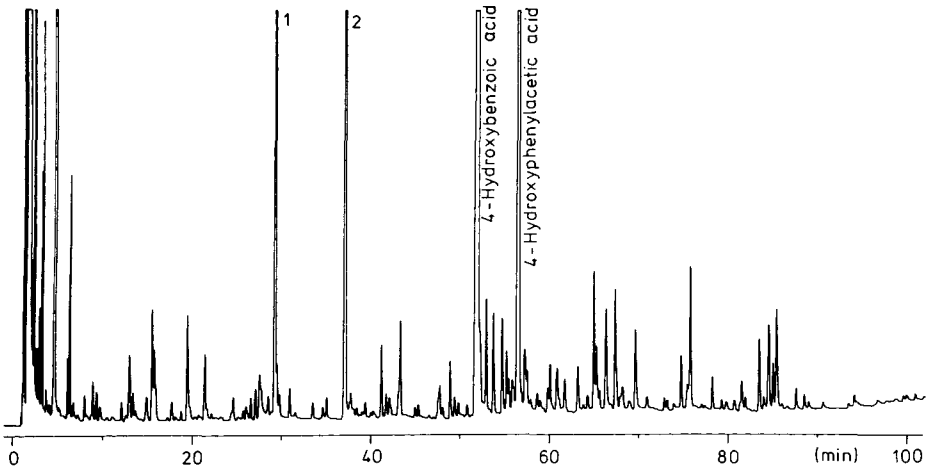

Fig. 1. Gas chromatogram of fraction 2a of the organic acids in urine of a patient with cirrhosis. Numbered peaks: 1 = benzoic acid, 2 = phenylacetic acid. All phenolic acids in Figs. 1—6 are methylated at the carboxyl group and at the —OH group.

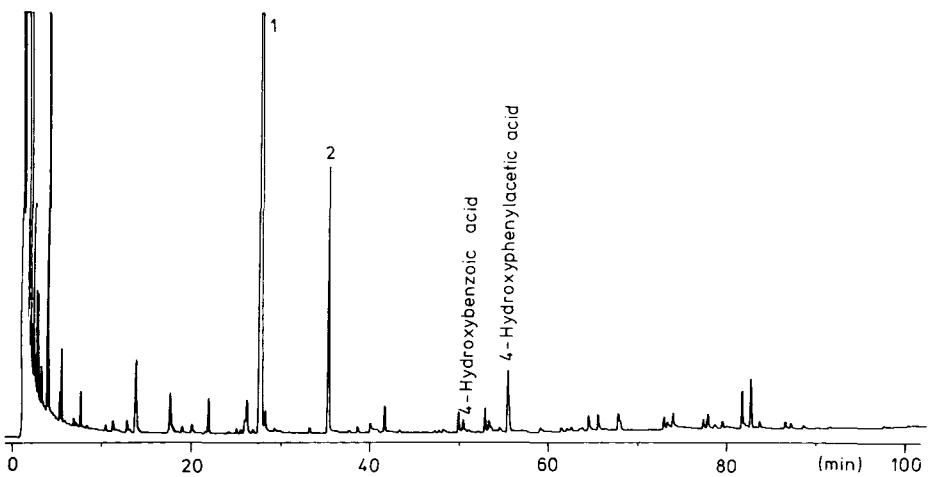

Fig. 2. Gas chromatogram of fraction 2a of the organic acids in urine of a healthy individual. Numbered peaks as in Fig. 1.

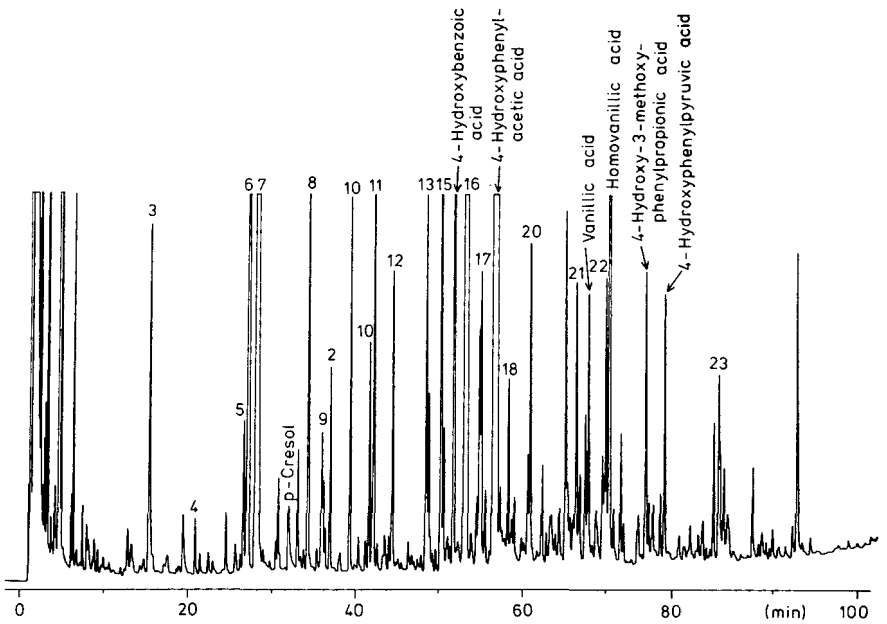

Fig. 3. Gas chromatogram of fraction 2b of the organic acids in urine of a patient with cirrhosis. Numbered peaks: 2 = phenylacetic acid, 3 = pyruvic acid, 4 = methylmalonic acid, 5 = ethylmalonic acid, 6 = succinic acid, 7 = 5-methylfurancarboxylic acid, 8 = glutaric acid, 9 = 3-methylgutaric acid, 10 = 3-methylglutaconic acid, 11 = adipic acid, 12 = 3-methyladipic acid, 13 = 3,4-methyleneadipic acid, 15 = anthranilic acid, 16 = 2-oxoglutaric acid, 17 = suberic acid, 18 = 2-oxoadipic acid, 20 = azelaic acid, 21 = sebacic acid, 22 = 5,6-decynedioic acid, 23 = indoleacetic acid. All oxocarboxylic acids in Figs. 3 and 4 are O-methyloximated at the carbonyl group.

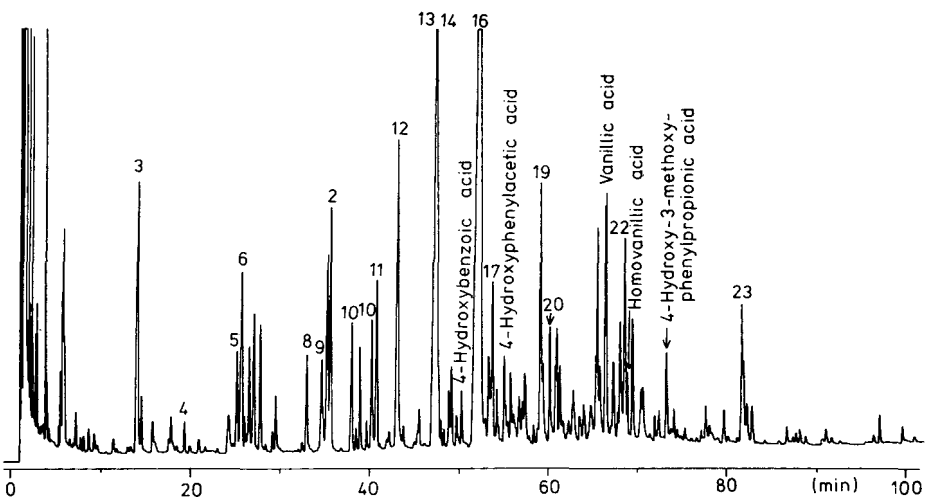

Fig. 4. Gas chromatogram of fraction 2b of the organic acids in urine of a healthy individual. Numbered peaks as in Fig. 3; additional peaks: 14 = pimelic acid, 19 = 3,4-methylenesuberic acid.

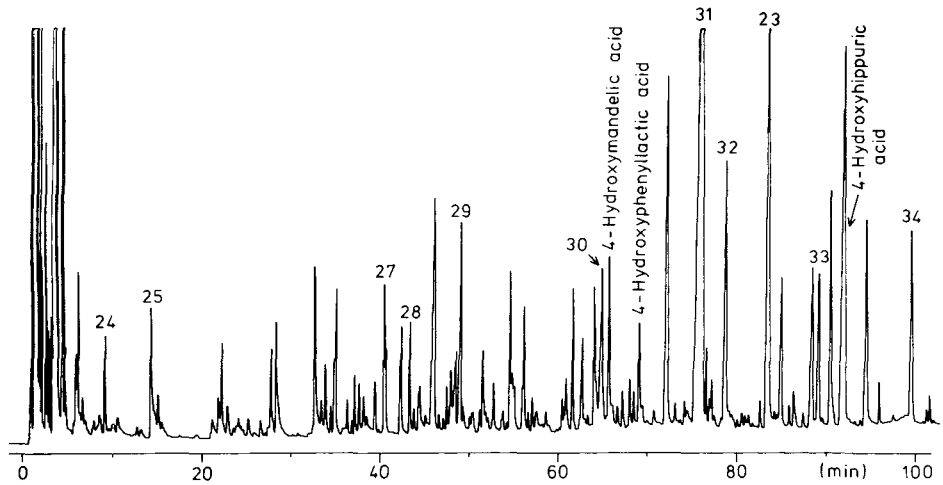

Fig. 5. Gas chromatogram of fraction 3 of the organic acids in urine of a patient with cirrhosis. Numbered peaks: 23 = indoleacetic acid, 24 = 3-hydroxyisovaleric acid, 25 = 3-hydroxyisobutyric acid, 27= 3-hydroxy-3-methylglutaric acid, 28 = 2-isopropyl-2-hydroxy-succinic acid, 29 = mandelic acid, 30 = furoylglycine, 31 = hippuric acid, 32 = vanillyl-mandelic acid, 33 = 3-hydroxyhippuric acid, 34 = phenylacetylglutamic acid.

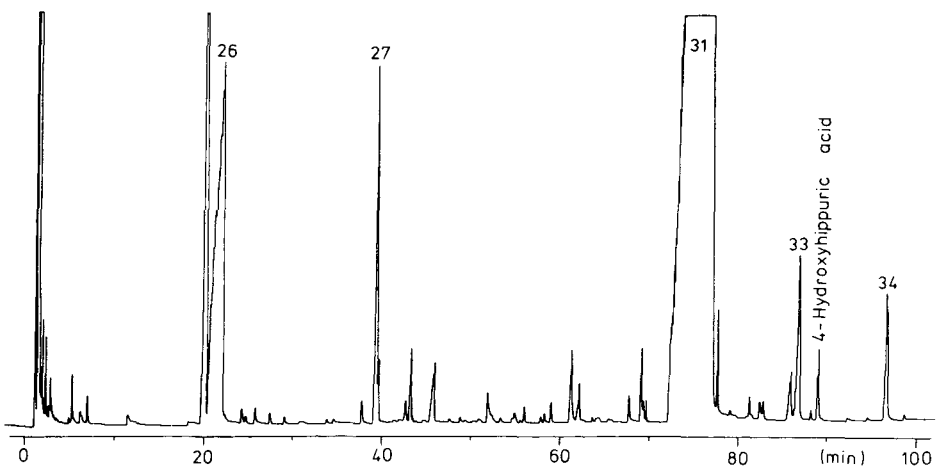

Fig. 6. Gas chromatogram of fraction 3 of the organic acids in urine of a healthy individual. Numbered peaks as in Fig. 5; additional peak: 26 = phosphoric acid (as trimethyl ester).

advantage that several phenolic acids can be analysed simultaneously and that besides the phenolic substances other metabolites can also be examined.

In cirrhosis and other liver diseases the conjugation capacity of the liver can be observed. The glycine conjugates furoylglycine, hippuric acid, 3-hydroxyhippuric acid and 4-hydroxyhippuric acid and the glutamic acid conjugate phenylacetylglutamic acid appear in fraction 3. In the cases studied so far, it is observed that in liver cirrhosis the conjugation with glycine and glutamic acid is functioning, except that the amount of hippuric acid excreted in urine is lower than in normal subjects. Because of their low volatility, the conjugates with glucuronic acid and sulphate (e.g. of phenol and *p*-cresol)

cannot be directly examined by this method. However, they can be indirectly analysed after hydrolysis of the urine with β-glucuronidase—arylsulphatase.

Other groups of non-phenolic metabolites that can be studied by the profiling technique in conjunction with the neomycin therapy of patients with liver cirrhosis, are methyl-branched dicarboxylic acids and acids with a cyclopropane ring. These substances are presumably formed by ω- and β-oxidation from bacterial fatty acids [6]. From the few cases followed so far, there is an indication that with the neomycin therapy applied the excretion of these acids (e.g. 3-methyladipic acid, 3,4-methyleneadipic acid and 3,4-methylenesuberic acid) is decreased compared to unbranched dicarboxylic acids. This can be explained by lower production of bacterial fatty acids and reduced bacterial metabolism.

A disadvantage of the described profiling technique is that primarily it is not an exact quantitative method. It allows comparative studies and the recognition of pronounced changes in the relative concentrations of different components in the profile, such as the high urinary excretion of phenolic acids in patients with liver cirrhosis as compared to normals. Quantitative data would require suitable internal standards and several calibration standards for each component to be quantified.

The pre-fractionation step is useful to improve the separation of the compounds and thereby the specificity. Phenolic acids of higher polarity, such as 4-hydroxymandelic acid, 4-hydroxyphenyllactic acid and 4-hydroxyhippuric acid are constituents of fraction 3 (Fig. 5) and are well separated from other components. 4-Hydroxy-3-methoxyphenylpropionic acid and 4-hydroxyphenylpyruvic acid, which appear in fraction 2b (Fig. 3), would be covered by large amounts of hippuric acid, a component of fraction 3, if pre-fractionation were not performed. The subfractionation into 2a and 2b which leads to a splitting of the total amounts of 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid into two fractions can be omitted when only phenolic acids are being studied. It should be carried out, however, when branched-chain oxocarboxylic acids are being studied as well. They are enriched in fraction 2a. In accordance with decreased concentrations of branched-chain amino acids in the blood of cirrhotic patients, the urinary excretion of their metabolites, i.e. the branched-chain oxocarboxylic acids, is low.

In the reaction of phenolic acids with diazomethane, the carboxyl group as well as the phenolic hydroxyl group are methylated. For this reason it is not possible to differentiate by MS between $-OCH_3$ groups that are originally present in the molecule and those that are formed from —OH groups in the methylation step. To establish the original structure of the substances, in one analysis deuterated diazomethane was used in the sample preparation, making MS differentiation possible.

Phenolic acids in liver cirrhosis

As demonstrated in Figs. 1—6, the urinary excretion of the following phenolic substances is increased in patients with liver cirrhosis as compared to normal controls: 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, vanillic acid, homovanillic acid, 4-hydroxymandelic acid, 4-hydroxy-3-

methoxyphenylpropionic acid, and the non-carboxylic compound *p*-cresol. Between the seven cirrhotic patients studied, a considerable variance in the elevation of each single component was observed, the increases of 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid usually being the most pronounced.

The common structural feature of all the substances is the 4-hydroxyl group, which is explained by their common precursor, the amino acid tyrosine. It has been known for a long time that the metabolism of tyrosine is abnormal in cirrhosis. In the basal state and after oral administration of tyrosine, the plasma tyrosine levels [7, 8] and the excretion of the metabolite 4-hydroxyphenylpyruvic acid [8] were elevated. Increased excretion was also observed for 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid [1, 2].

The degradation of tyrosine is accomplished in the liver, in extrahepatic tissues and by microorganisms of the intestine. In liver cirrhosis the hepatic homogentisic acid pathway is partially blocked by defects of the enzymes tyrosine transaminase, 4-hydroxyphenylpyruvic acid oxidase and homogentisic acid oxidase [8]. In particular, as a consequence of the second enzyme defect, the accumulation of 4-hydroxyphenylpyruvic acid and its metabolites 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid is explained. All three phenolic acids can also be produced from tyrosine by intestinal bacteria [9]. Probably a pure bacterial metabolite is an oxidation product of 4-hydroxyphenylacetic acid, i.e. 4-hydroxybenzoic acid, which is partially conjugated with glycine to form 4-hydroxyhippuric acid. The intestinal origin of 4-hydroxybenzoic acid is supported by our findings that a cirrhotic patient who received parenteral nutrition after portacaval shunt surgery, excreted very low amounts of this acid. The excretion reached a high level when a normal diet was resumed. A further purely bacterial product is *p*-cresol [9, 10]. In experiments with rats it was shown that its urinary excretion is reduced when the intestinal flora is suppressed by oral neomycin administration [11]. Vanillic acid, homovanillic acid and 4-hydroxymandelic acid are metabolites formed in extrahepatic tissues. 4-Hydroxy-3-methoxyphenylpropionic acid may originate from the bacterial 4-hydroxyphenylpropionic acid by further extrahepatic metabolization.

Using the described profiling method, changes in the excretion of all these phenolic constituents can be observed. On the basis of their different origins, the phenols would supply information on the metabolizing function of the liver of cirrhotic patients and on the effect of therapy with neomycin, lactulose and reduced dietary proteins. Since the bacterial products of tyrosine accumulate when they do not reach the liver for further metabolization, these intestinal phenols are biochemical indicators for portal-systemic venous collaterals. In contrast to previous techniques, the method presented appears suitable for detailed and systematic studies.

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N-METHYLATION AND N,N-DIMETHYLATION OF AMINO ACIDS

AN ARTIFACT PRODUCTION IN THE ANALYSIS OF ORGANIC ACIDS USING DIAZOMETHANE AS DERIVATIZING AGENT

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SUMMARY

In the fractions of the methyl esters of urinary organic acids seventeen N-methylated or N,N-dimethylated amino acid methyl esters are identified by gas chromatography—mass spectrometry. It is shown for twelve amino acids that their amino group reacts with diazomethane to form these derivatives. Using deuterated reagents, in particular deuterated diazomethane, in the sample preparation procedure during the organic acid analysis, it is shown that the N-methylated and N,N-dimethylated amino acids are artifacts from diazomethane and are not biochemical N-methylation products.

INTRODUCTION

Diazomethane is frequently used as derivatizing agent in the gas chromatographic (GC) and gas chromatographic—mass spectrometric (GC—MS) analysis of organic acids. Its general applicability to various classes of the acids has been demonstrated by several authors [1—6]. Methylation with diazomethane is easy to perform, the resulting derivatives are stable and their MS fragmentation is usually informative and interpretable. Since diazomethane reacts with acidic hydrogen, carboxylic groups and phenolic —OH groups are methylated. In addition, other kinds of reactions can occur with diazomethane. It may react with carbonyl compounds and olefinic double bonds. Methylation of alcoholic hydroxyl groups, such as in hydroxydicarboxylic acids, with diazomethane has also been observed. This has to be taken into account when the substance is used as derivatizing agent.

The reaction of diazomethane with the amino group of amino acids is not necessarily expected, because the hydrogens at the nitrogen atom are not acidic. However, during our analyses of organic acids a series of substances was

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found in the acid fractions, whose mass spectra led to the class of N-methyl- and N,N-dimethyl-substituted amino acids. Some examples of this group of components have been described previously [4, 7]. This paper deals with a systematic study of the formation of N-methylated and N,N-dimethylated amino acids with diazomethane.

EXPERIMENTAL

Materials

The following chemicals were used: alanine, valine, leucine, isoleucine, threonine, methionine, cysteine, tyrosine, tryptophan, aspartic acid and glutamic acid from E. Merck (Darmstadt, F.R.G.), phenylalanine, Diazald® (N-methyl-N-nitroso-*p*-toluenesulphonamide), carbitol-d_1 [2-(2-ethoxyethoxy)-ethanol-d_1], a 30% solution of sodium deuterooxide in ^2H_2O, methanol-d_1, and ^2H_2O from EGA-Chemie (Steinheim, F.R.G.), and a 95% solution of formic acid-d_2 from A. Hempel (Düsseldorf, F.R.G.).

Samples

Urine samples were collected (24 h) from healthy individuals and analysed directly after the collection period.

Analysis of organic acids in urine

The sample preparation and the GC and GC—MS investigations were performed according to our previously described procedure [8]. The reference compounds were analysed according to the same GC and GC—MS conditions as the urinary components.

Synthesis of the methyl esters of N,N-dimethylalanine and N,N-dimethylphenylalanine

The selective methylation of the amino groups of alanine and phenylalanine was carried out as described by Bowman and Stroud [9]. The obtained N,N-dimethylamino acids were transformed into their methyl esters by the following procedure: 1 mg of each of the amino acid derivatives was reacted in srew-capped vials (Macherey-Nagel, Düren, F.R.G.) with 1 ml of a solution of 10% acetyl chloride in methanol for 10 min at 110°C.

Reaction of amino acids with diazomethane

One milligram of each amino acid was separately mixed with 5 ml of methanol, and the mixture was warmed up to facilitate dissolving of the amino acid. An ethereal solution of diazomethane prepared from N-nitroso-N-methylurea, was added until the N_2- development stopped and the yellow colour of the reaction mixture persisted. The reaction mixture was allowed to stand overnight (12 h) before it was concentrated under a stream of nitrogen.

Reaction of the urinary organic acids with deuterated diazomethane

The regular sample preparation procedure was used for the urinary acids except that the following changes were made: after the application of two equal portions of urine to the two anion-exchange columns, each column was

washed with 100 ml of isopropanol—^2H_2O (2:1) and subsequently with 100 ml of methanol-d_1. The acidic components were eluted with 200 ml of a solution of 4% formic acid-d_2 in methanol-d_1. The methylation was performed with deuterated diazomethane, which was prepared by the procedure of Campbell [10].

RESULTS AND DISCUSSION

Occurrence of N-methylated and N,N-dimethylated amino acids in the analysis of organic acids

In the GC—MS analyses of the organic acids a series of mass spectra was obseved in the fractions 2b and 3a—3d which suggested the presence of N-methylated and N,N-dimethylated amino acids. The substances occurred in low amounts, but were regularly found. Figs. 1—5 show the organic acid fractions in which the discussed components are labelled according to their GC—MS identifications. Derivatives of twelve amino acids are detected, the N-N-

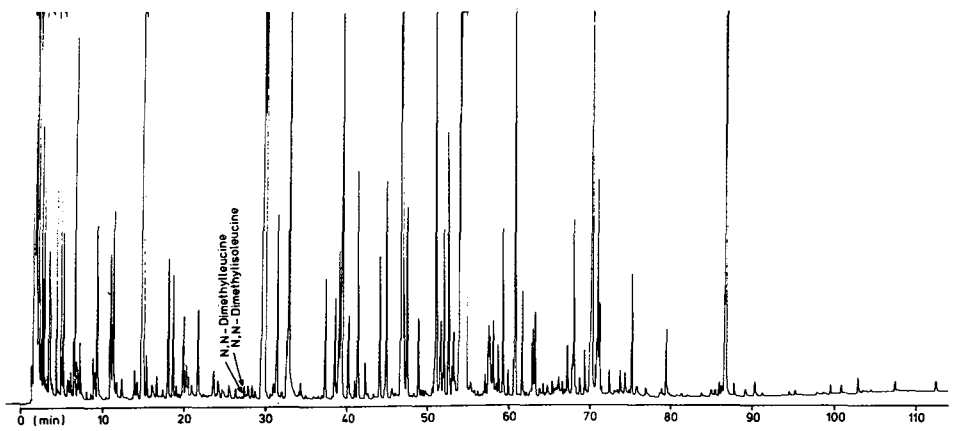

Fig. 1. Gas chromatogram of fraction 2b of the methyl esters of the organic acids in urine of a healthy individual.

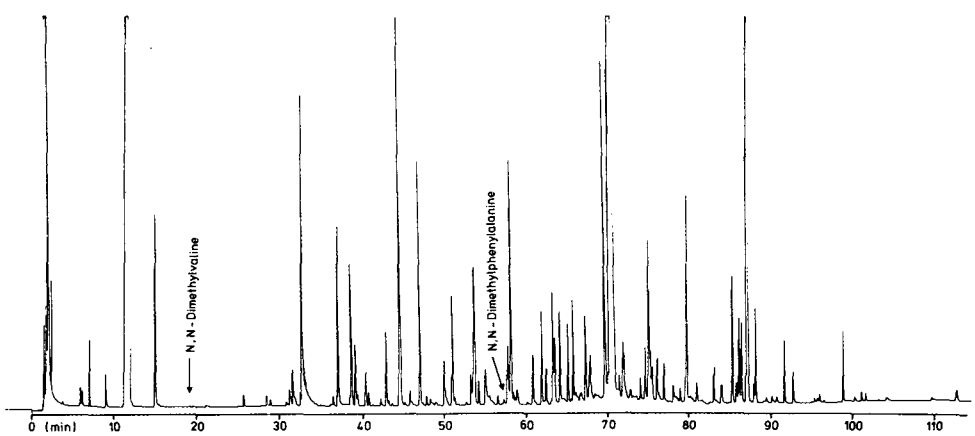

Fig. 2. Gas chromatogram of fraction 3a.

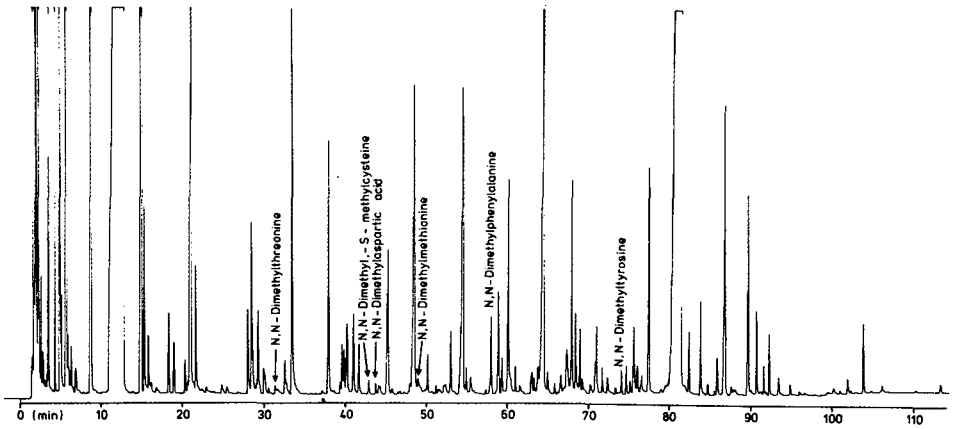

Fig. 3. Gas chromatogram of fraction 3b.

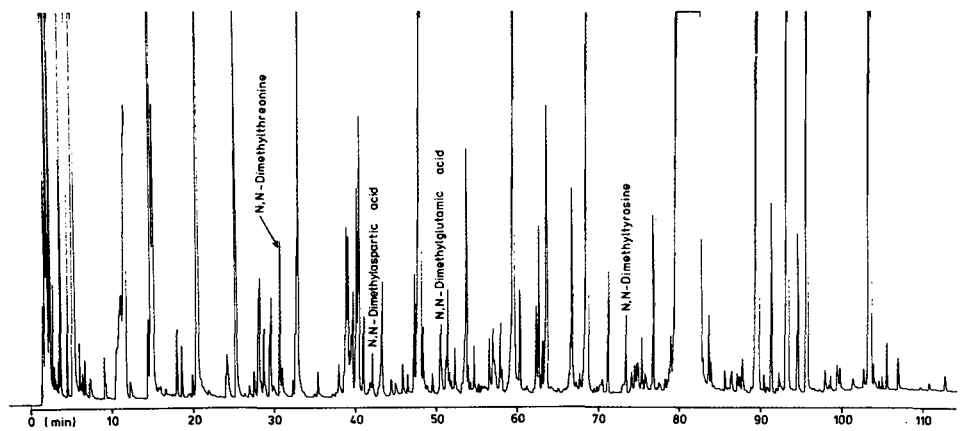

Fig. 4. Gas chromatogram of fraction 3c.

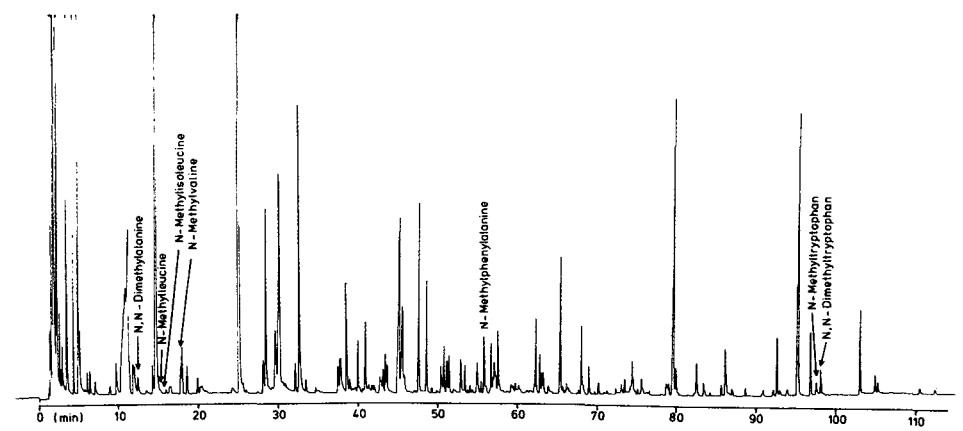

Fig. 5. Gas chromatogram of fraction 3d.

dimethylated products being found in all of the cases, the monomethylated substances only from five amino acids. Altogether seventeen peaks in the chromatograms of the organic acids correspond to derivatives of amino acids.

Synthesis of the methyl esters of N,N-dimethylated alanine and phenylalanine

To prove the assumption that the seventeen observed components are indeed methyl derivatives of amino acids, alanine and phenylalanine, as examples, were transformed into N,N-dimethylated amino acid methyl esters without using diazomethane. The reaction of the amino group with formaldehyde, hydrogen and palladium on activated charcoal [9] selectively forms the dimethylated product. To avoid diazomethane in the entire synthesis, the carboxyl group was methylated with methanol—acetyl chloride. The GC behaviour and the MS fragmentation of the synthetic substances were identical with those of the derivatives in the organic acid profiles.

Reaction of amino acids with diazomethane

The reaction of amino acids with diazomethane was assumed to be the source of the proposed amino acid derivatives in the acid profiles. To test their reaction behaviour, reference substances of the twelve amino acids expected from the mass spectra to occur in the organic acid fractions were reacted with diazomethane.

As demonstrated for leucine in Fig. 6, the amino acid is partially transformed into the N-methylated and the N,N-dimethylated substances. The sequence of the retention times of amino acid (A), N-methylated product (N) and N,N-dimethylated product (NN) varies for the different amino acids. For alanine, cysteine and aspartic acid the order A—N—NN is observed, for valine, leucine, isoleucine and phenylalanine the sequence is N—A—NN, and for

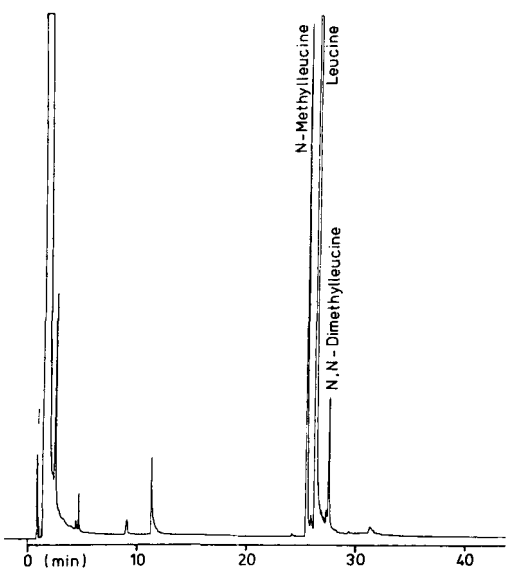

Fig. 6. Gas chromatogram of the products of the reaction between leucine and diazomethane.

threonine, methionine, tyrosine and tryptophan N—NN—A. In the experiment with the reference amino acids the amount of the N-methylated amino acid formed is larger than that of the N,N-dimethylated derivative. Other ratios are observed in the organic acid profiles. The N-methylated derivatives, which are the more polar substances, are found in lesser amounts than the N,N-dimethylated products or are not detected at all. Presumably they are not effectively extracted from the silica gel after the TLC pre-fractionation.

Mass spectrometric fragmentation

The mass spectrometric fragmentations of the N-methylated and N,N-dimethylated amino acid methyl esters are characterized by the loss of the carbomethoxy group and by the ion *m/e* 102 from the monomethylated substances and the ion *m/e* 116 from the dimethylated compounds (Fig. 7). These fragments usually occur with high abundance or as base peaks. The molecular peaks are of low intensity, in some cases not distinguishable. In Table I the fragments and their intensities of 23 reaction products of amino acids with diazomethane are summarized. The seventeen substances found in the fractions of the organic acids had the same mass spectra and showed the same

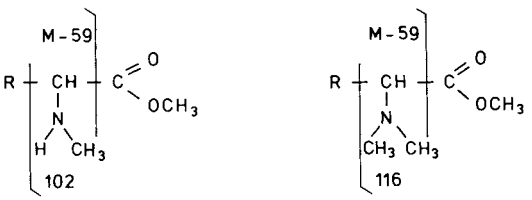

Fig. 7. Mass spectrometric fragmentation of N-methylated and N,N-dimethylated amino acid methyl esters.

TABLE I

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF N-METHYLATED AND N,N-DIMETHYLATED AMINO ACIDS

Substance	MW**	Fragments (intensities)
N-Methylalanine	117	117(9), 102(6), 70(2), 59(22), 58(100), 56(50), 42(38)
N,N-Dimethylalanine*	131	131(16), 116(5), 73(23), 72(100), 70(22), 56(31), 42(45)
N-Methylvaline*	145	102(67), 87(10), 86(100), 71(16), 70(10), 56(13), 42(28)
N,N-Dimethylvaline*	159	159(1), 116(50), 100(100), 85(18), 84(15), 70(10), 56(19), 42(38)
N-Methylleucine*	159	102(22), 101(9), 100(100), 84(2), 70(2), 58(41), 42(20)
N,N-Dimethylleucine*	173	173(3), 116(16), 115(13), 114(100), 98(2), 58(10), 42(7)
N-Methylisoleucine*	159	102(76), 100(90), 84(3), 70(16), 69(18), 42(100)
N,N-Dimethylisoleucine*	173	173(1), 116(80), 115(12), 114(100), 85(15), 70(7)
N-Methylthreonine	147	147(3), 132(6), 103(69), 102(100), 97(8), 88(97), 70(50), 42(88)
N,N-Dimethylthreonine*	161	146(4), 117(32),116(100), 102(50), 84(15), 58(57), 56(17)
N-Methyl-S-methylcysteine	163	163(2), 104(76), 102(100), 89(9), 70(14), 57(42), 56(19), 42(70)
N,N-Dimethyl-S-methylcysteine*	177	177(2), 118(35), 116(100), 103(3), 98(1), 84(10), 71(57), 70(19), 56(49)
N-Methylmethionine	177	177(3), 130(5), 129(10), 118(78), 102(27), 70(86), 61(100)
N,N-Dimethylmethionine*	191	191(9), 133(15), 132(100), 116(22), 84(90), 70(21), 61(81)
N-Methylphenylalanine*	193	191(1), 134(37), 102(100), 91(10), 77(8), 65(6), 63(2)
N,N-Dimethylphenylalanine*	207	148(32), 116(100), 91(11), 84(4), 77(9), 65(4), 63(2), 56(11)
N-Methyltyrosine	223	223(0.5), 164(15), 122(25), 121(44), 102(100), 91(7), 77(9), 65(6)
N,N-Dimethyltyrosine*	237	178(15), 163(4), 121(9), 117(10), 116(100), 91(5), 77(4), 65(3)
N-Methyltryptophan*	232	232(2), 173(4), 130(100), 116(1), 102(16), 77(9), 63(1)
N,N-Dimethyltryptophan*	246	246(4), 216(1), 187(14), 131(8), 130(30), 116(100), 101(1), 77(5), 63(1)
N-Methylaspartic acid	175	143(2), 117(9), 116(100), 102(40), 85(5), 84(54), 59(11)
N,N-Dimethylaspartic acid*	189	189(2), 131(9), 130(100), 116(28), 98(30), 88(20), 71(12), 56(9)
N,N-Dimethylglutamic acid*	203	203(2), 172(8), 145(10), 144(100), 116(8), 85(9), 84(69), 70(8), 56(6)

*Substance was found in the organic acid fractions.
**Molecular weight.

GC retention behaviour as the corresponding products from the reference amino acids. The spectra of the methyl esters of N-methylleucine, N,N-dimethyltyrosine, N,N-dimethyltryptophan [4], N,N-dimethylphenylalanine, N-methylglutamic acid [7] and N,N-dimethylglutamic acid [7, 11] have been previously published.

As is to be expected, the phenolic —OH group in the two tyrosine reaction products is methylated. The —SH group in the cysteine derivatives is transformed into $-SCH_3$ by diazomethane as well.

Proof for artifact production

The question of whether the N-methylated and N,N-dimethylated amino acids identified in the course of the analysis of organic acids are exclusively artifacts or in part biochemical N-methylation products of amino acids, was answered by the experiment with deuterated substances. The mass spectra of N-methylvaline methyl ester in a urinary acid fraction treated with regular reagents (Fig. 8) and of the corresponding product in a urinary acid fraction reacted with deuterated reagents, in particular deuterated diazomethane (Fig. 9), exemplify the findings. By GC—MS scanning of the N-methylvaline peak within the chromatogram of the organic acids treated with deuterated reagents, the characteristic fragment *m/e* 102 is not seen at all. Instead, a shift mainly to *m/e* 108 is observed. This shift by six masses originates from $-C^2H_3$ in the carbomethoxy group and $-C^2H_3$ (or $-CH^2H_2$ plus ^2H) at the nitrogen. The accompanying fragments *m/e* 107 and *m/e* 109 in Fig. 9 demonstrate that the exchange of deuterium for hydrogen is not uniform at the $-NH_2$ group. For the second characteristic fragment of N-methylvaline, i.e. $M-COOCH_3$, a shift is observed from *m/e* 86 (Fig. 8) to *m/e* 89 (Fig. 9). From these observations it can be concluded, that N-methylvaline was not originally present in the urine. It is an artifact from diazomethane and not of biochemical origin. The same result was found in the deuterium experiment for other N-methylated and N,N-dimethylated amino acids.

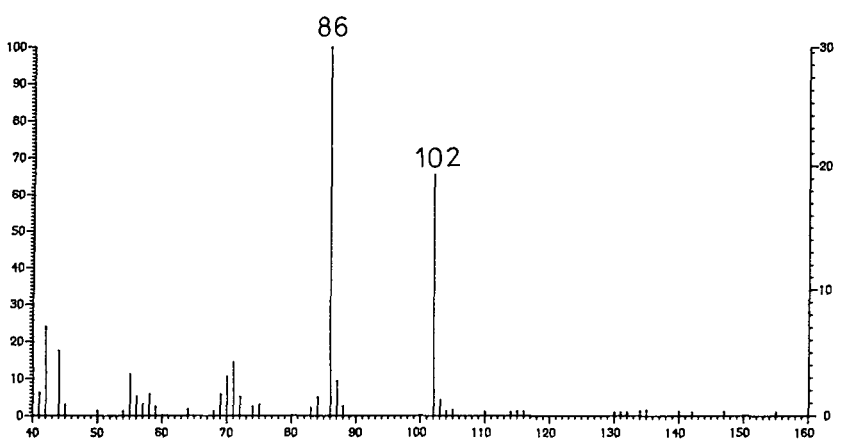

Fig. 8. Mass spectrum of N-methylvaline detected in fraction 3d of the organic acid methyl esters prepared with regular reagents.

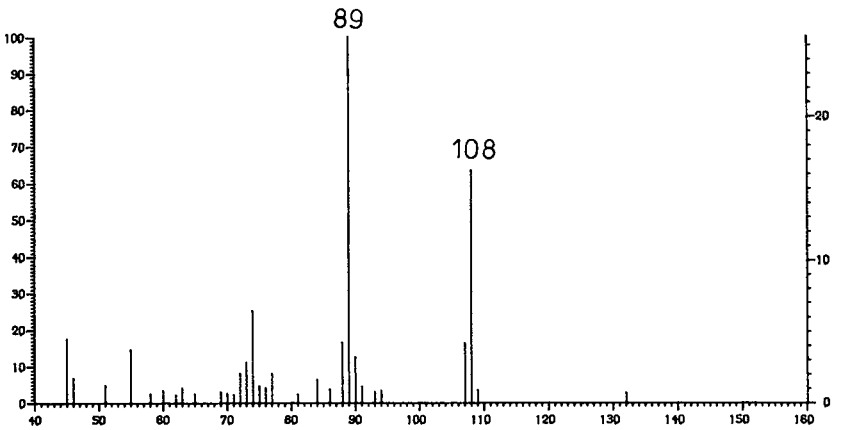

Fig. 9. Mass spectrum of N-methylvaline detected in fraction 3d of the organic acid methyl esters prepared with deuterated reagents.

The high reactivity of diazomethane, which is one of the reasons for its use as derivatizing agent in organic acid analysis, is at the same time its limitation. The investigator has to be aware, especially when he analyses lower-concentrated constituents, that a number of substances, such as the described amino acid derivatives, are artifacts from the procedure.

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CHROMBIO. 2413

COMPARATIVE EVALUATION OF THE RADIOENZYMATIC METHOD FOR THE DETERMINATION OF URINARY HISTAMINE WITH A MASS SPECTROMETRIC ASSAY

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SUMMARY

Considerable inaccuracy and unreliability have recently been demonstrated to be associated with the widely used radioenzymatic methods for the determination of histamine in biological fluids. Urine appears to inhibit the methylation of histamine by histamine N-methyltransferase such that the radioenzymatic assay underestimates the concentration of histamine present in urine. Directly comparing the radioenzymatic assay with a recently developed reference method using mass spectrometry for the determination of urinary histamine, up to 34-fold differences in the levels of urinary histamine were found with the two methods.

INTRODUCTION

The physiological and pathophysiological importance of histamine is well recognized. Because of its biological importance, a variety of methods has been developed to quantify histamine in biological fluids. Such methods include bioassay [1, 2], single and double isotope radioenzymatic assays [3—7], high-performance liquid chromatography [8, 9], and manual and automated fluorometry [10—13]. One of the most widely employed methods for the determination of histamine is the radioenzymatic assay. Although both the single and double isotope radioenzymatic methods were generally considered to be associated with an acceptable degree of accuracy, recent studies have suggested that substantial inaccuracy may at times be associated with these methods [14, 15].

Quantification of the urinary excretion of histamine is a valuable diagnostic indicator of the disorder mastocytosis, a disease we have recently found to be

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much more common than previously recognized [16—18]. Because of the importance of using such determinations in the diagnosis of mastocytosis, the accuracy of the radioenzymatic method for the determination of histamine in human urine was examined and considerable inaccuracy was encountered which could not be readily rectified. Therefore efforts were directed towards the development of a more accurate and reliable method for quantification of urinary histamine. Because it is generally accepted that one of the most accurate methods for determination of biological compounds is stable isotope dilution assay with quantification by mass spectrometry (MS), we have recently adapted this methodology for the measurement of urinary histamine [19]. This gas chromatographic (GC) method employs negative-ion chemical-ionization MS which is associated with much greater sensitivity than previous methods reported for the determination of histamine using different methods of ionization [20—22]. The lower limits of detection of histamine with this method are in the range of 100—500 fg injected on-column [23]. The precision of the assay has a coefficient of variation of 2.5% and the accuracy of measuring histamine in urine is 97.6% [19].

Because the radioenzymatic method for quantification of urinary histamine has gained widespread use, it seemed to be a potentially valuable exposition to briefly outline some of the problems encountered with this particular method and to comparatively evaluate the radioenzymatic method for quantification of urinary histamine with the GC—MS method.

MATERIALS AND METHODS

Materials

Pentafluorobenzyl bromide and diisopropylethylamine were obtained from Pierce (Rockford, IL, U.S.A.). Histamine dihydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). [$\alpha,\alpha,\beta,\beta$-$^2H_4$]Histamine dihydrochloride was obtained from Merck Isotopes (Montreal, Canada). Poly I-110 GC column packing was purchased from Applied Science Labs. (State College, PA, U.S.A.). *S*-[Methyl-^{3}H]adenosyl-L-methionine (80 Ci/mmol) and *S*-[methyl-^{14}C]-adenosyl-L-methionine (56 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Gas chromatograph and mass spectrometer conditions

GC—MS analysis was performed using a Hewlett-Packard 5982A gas chromatograph—mass spectrometer modified to detect negative ions. Conditions: electron energy 25 eV, interface temperature 250°C, internal source temperature 225°C, direct inlet line, emission current 300 μA, methane as reagent and carrier flow gas, analyzer manifold pressure $1.6 \cdot 10^{-3}$ Pa, injection port temperature 250°C, conversion diode potential —3 kV. Analysis was performed using a 60-cm packed column of 3% Poly I-110 operated at 250°C.

Radioenzyme assays of urinary histamine

The single isotope radioenzymatic assay was used essentially as described by Beaven et al. [3] except that in some experiments *S*-[^{3}H]adenosylmethionine was used rather than *S*-[^{14}C]adenosylmethionine. Results reported are the mean of duplicate or triplicate determinations of a single assay.

Mass spectrometric assay of urinary histamine

The stable isotope dilution GC—MS assay was used as recently described [19]. Briefly, to 1 ml of urine is initially added 40 ng of $[^2H_4]$histamine followed by addition of 150 μl of 1 *M* sodium hydroxide and extraction into 2 ml of butanol. Then 2 ml of heptane are added, mixed, centrifuged and the butanol—heptane layer is separated from the aqueous layer and extracted with 100 μl of 1 *M* hydrochloric acid. The aqueous hydrochloric acid phase is evaporated under a stream of nitrogen and the residue dissolved in 30 μl acetonitrile, 10 μl diisopropylethylamine, and 15 μl of a 25% solution of pentafluorobenzyl bromide in acetonitrile. After 30 min at room temperature excess reagents are evaporated under a stream of nitrogen and the residue is dissolved in 150 μl of 10% sodium carbonate followed by extraction with 250 μl methylene chloride. The upper aqueous layer is aspirated, discarded and residual sodium carbonate removed by washing the upper part of the vial with distilled water. The methylene chloride is evaporated under a stream of nitrogen and the residue dissolved in ethyl acetate for injection and analysis by GC—MS. Quantification is accomplished by selected-ion monitoring of the ratio of intensity of the *m/z* 430 and *m/z* 434 ion peaks for $[^2H_0]$- and $[^2H_4]$-$(CH_2C_6F_5)_3$-histamine, respectively.

RESULTS

Because of our interest in determining the urinary excretion of histamine as a diagnostic indicator of the disease mastocytosis, the accuracy of the single isotope radioenzymatic assay for urinary histamine was initially examined by measuring the concentration of histamine present in urine collected from five patients suspected of having mastocytosis before and after the addition of 60 ng/ml histamine. These results are listed in Table I. In each instance, the radioenzymatic assay underestimated the amount of histamine that was added to these urines. The degree of underestimation was quite variable between different urine samples but in each the magnitude of underestimation was substantial. Thus, it appeared that urine collected from these patients interfered greatly with the radioenzymatic determination of histamine.

TABLE I

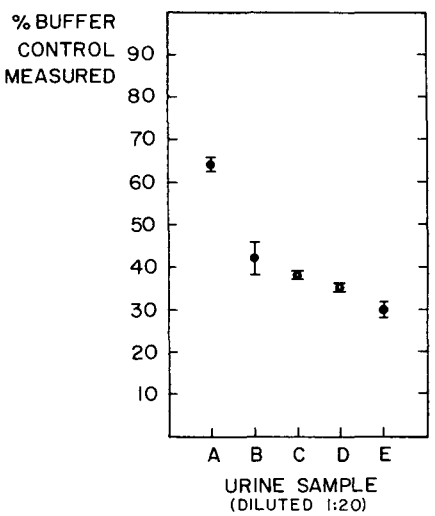
URINARY HISTAMINE MEASURED BY RADIOENZYMATIC ASSAY (ng/ml)

Concentrations of histamine were measured by single isotope radioenzymatic assay in urine obtained from five patients suspected of having mastocytosis. Histamine was measured before and after the addition of 60 ng/ml histamine and the percentage of the added histamine detected by the assay calculated.

Patient	No addition of histamine	Addition of 60 ng/ml histamine	Percentage of added histamine measured
1	2	12	17
2	20	41	35
3	7	40	55
4	8	24	27
5	3	18	25

Whether urine collected from five normal individuals also interfered with the radioenzymatic assay, as was found with the urine obtained from the mastocytosis patients, was then examined. In this experiment, the urine was initially diluted 1:20 prior to the assay with 0.1 *M* phosphate buffer, pH 7.4, to which was added 20 ng/ml histamine. Compared to the standard curve of histamine assayed in 0.1 *M* phosphate buffer, pH 7.4, the assay again underestimated the 20 ng/ml histamine present in each of the 1:20 diluted urine samples by as much as 35—70% (Fig. 1). The other important information that was obtained from this experiment was that diluting the urine by as much as 1:20 prior to analysis did not successfully remove the interference of urine with the assay.

The interference of urine with the radioenzymatic determination of histamine over a range of known histamine concentrations in urine was then evaluated. Urine from a normal individual was initially diluted 1:20 with 0.1 *M* phosphate buffer, pH 7.4, to which was then added histamine to give concentrations of 20, 50, and 100 ng/ml prior to analysis. The analysis of these urine samples was then compared to the analysis of samples of 20, 50, and 100 ng/ml histamine in 0.1 *M* phosphate buffer, pH 7.4, alone (Fig. 2). The curve relating [^{3}H]methylhistamine recovered to histamine concentrations in the urine samples was linear over this concentration range but the slope was considerably



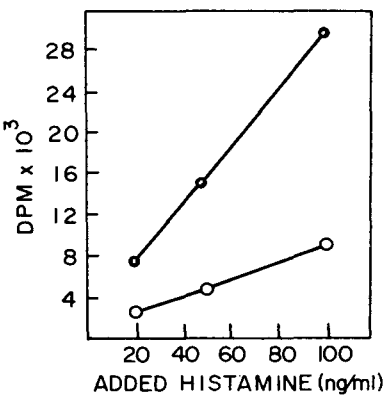


Fig. 1. Urine was obtained from five normal volunteers and diluted 1:20 with 0.1 *M* phosphate buffer, pH 7.4, to which was subsequently added 20 ng/ml histamine. The histamine concentration was then determined in the urine samples by single isotope radioenzymatic assay by comparison to a standard curve of histamine concentrations prepared in 0.1 *M* phosphate buffer, pH 7.4. Plotted is the percentage of radiolabeled methylhistamine recovered in the analyses of the urine samples compared to that recovered in the standard curve analysis of 20 ng/ml histamine in phosphate buffer.

Fig. 2. Comparison of the analysis by single isotope radioenzymatic assay of 20, 50, and 100 ng/ml of histamine in 0.1 *M* phosphate buffer, pH 7.4, and in urine. The urine was initially diluted 1:20 with 0.1 *M* phosphate buffer prior to addition of 20, 50, and 100 ng/ml histamine before analysis. Plotted are the curves relating recovered [^{3}H]methylhistamine dpm to histamine concentration present in phosphate buffer (•) and urine (○). Each data point represents the mean of duplicate or triplicate determinations.

more flat than the curve obtained from the analysis of histamine in phosphate buffer alone. These data again illustrated the inhibitory influence of urine with the radioenzymatic determination of histamine and again confirmed the failure of diluting the urine by as much as 1:20 before analysis to successfully remove this interference of urine with the assay.

Because diluting urine by as much as 1:20 prior to analysis did not successfully eliminate the interference of urine with the assay, a few additional simple maneuvers were examined in an attempt to eliminate this interference including briefly boiling the urine before analysis, using increased enzyme concentrations, and altered incubation times and temperature. However, none of these procedures successfully removed the interference of the urine with the radioenzymatic assay. It was also confirmed that the apparent interference could not be attributed to differences in the extraction recoveries of radiolabeled methylhistamine from buffer and urine (data not shown).

Because of these problems with the radioenzymatic determintion of urinary histamine which could not be easily rectified, efforts were directed towards the development of a reliable method for the quantification of urinary histamine by stable isotope dilution assay using GC—MS [19]. To further evaluate and elucidate the problems associated with the radioenzymatic assay of urinary histamine, levels of urinary histamine measured by the two assays were compared in a series of urines obtained from patients with mastocytosis. Urines were selected for analysis by GC—MS in which the levels of urinary histamine measured by the radioenzymatic assay ranged from normal to markedly increased. This was done to assess whether the ability of the radioenzymatic assay to measure increased concentrations of histamine in some urines could be attributed to less interference of these particular urines with the assay. The results of these determinations of urinary histamine by the two methods are shown in Table II. In urine obtained from two patients (2 and 4), the levels

TABLE II

HISTAMINE LEVEL MEASURED

Comparison of the levels of urinary excretion of histamine (μg per 24 h) determined by both single isotope radioenzymatic assay and stable isotope dilution assay with GC negative-ion chemical-ionization MS in 24-h urine collections obtained from ten patients with mastocytosis.

Patient	Radioenzymatic assay	GC—MS assay
1	20	272
2	11	11
3	13	67
4	15	8
5	49	335
6	44	65
7	83	231
8	563	867
9	526	1123
10	519	1882

of urinary histamine quantified by the two assays were in close agreement. However, in urine from all of the other patients, the GC—MS assay consistently measured levels of histamine that were higher than the levels quantified by the radioenzymatic assay. The magnitude of the differences measured by the two methods was quite remarkable. The overall mean level measured by the GC—MS assay in these urines was 4.6-fold greater than that obtained with the radioenzymatic assay with a range of 1.5-fold (patients 6 and 8) to as high as 13.6-fold (patient 1). In addition, it is apparent that the interference of the urine matrix with the radioenzymatic determination of histamine was not limited to the urines in which the radioenzymatic assay measured low levels of histamine but also occurred in urines in which the radioenzymatic assay measured very high levels.

Perhaps an even more straightforward demonstration of the disparity between these two methods for the analysis of urinary histamine is illustrated in Fig. 3. In this figure is shown the urinary excretion of histamine as measured by the two assays in a patient with mastocytosis in whom severe episodes of flushing were provoked by the ingestion of aspirin. Although we have found that to ameliorate the symptoms of mastocytosis requires treatment with high doses of aspirin to inhibit the release of prostaglandin D_2 from mast cells [16, 17], analogous to the asthmatic population, a small subset of patients with mastocytosis exists in whom attacks of flushing are initially triggered by the ingestion of small doses of aspirin or other non-steroidal antiinflammatory drugs. In this patient repeated doses of 10 mg of aspirin were administered over

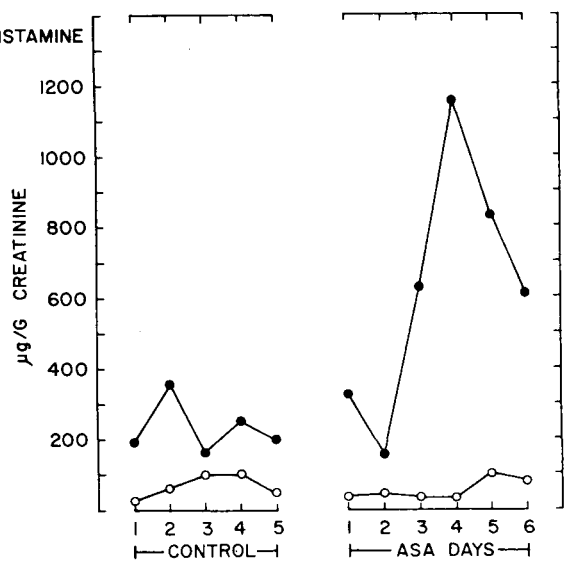


Fig. 3. Levels of urinary excretion of histamine (µg/g creatinine) determined by both single isotope radioenzymatic assay (○) and stable isotope dilution assay with GC negative-ion chemical-ionization MS (●) in a patient with mastocytosis and aspirin hypersensitivity. On the left are levels determined during control periods in the absence of aspirin ingestion and episodes of flushing. On the right are levels of urinary excretion of histamine determined when repeated doses of 10 mg of aspirin (ASA) were administered at varying intervals over six days during which time repeated episodes of severe flushing occurred.

varying time intervals for six days. This provoked recurrent episodes of severe flushing which, as determined by the GC—MS assay, were accompanied by a marked elevation in the urinary excretion of histamine compared to control days in the absence of aspirin and flushing reaching a maximum level of 1158 μg/g creatinine on day 4. However, using the radioenzymatic assay, no appreciable increase in the urinary excretion of histamine during these episodes of severe flushing provoked by aspirin could be detected. Although the interference of urine with the radioenzymatic assay during control quiescent days was of similar magnitude to that found in urine from the patients listed in Table II, the interference in urine collected from this patient during recurrent episodes of severe flushing was remarkably greater. On day 4 of aspirin administration when the urinary excretion of histamine determined by the GC—MS assay was 1158 μg/g creatinine, the radioenzymatic assay detected only 34 μg/g creatinine. This was a 34-fold difference in the levels of histamine measured in this urine by the two assay methods or expressed differently, the radioenzymatic assay only measured 2.9% of the urinary histamine actually present as determined by the GC—MS assay.

The data obtained in this patient indicate that greater amounts of substances which can interfere with the radioenzymatic determination of histamine were excreted at times when the patient was experiencing severe attacks of flushing and hypotension compared to quiescent days. Whether this same phenomenon can be generalized to other patients with mastocytosis remains to be determined.

DISCUSSION

The results of these experiments demonstrate that urine frequently interferes with the radioenzymatic determination of histamine. The magnitude of interference in most instances was quite substantial, although variable between urine samples analyzed. The interference appears to result from inhibitory substances in urine which prevent complete methylation of the histamine present by the histamine N-methyltransferase in the assay. Thus, the radioenzymatic assay frequently underestimated the concentration of histamine that was actually present in urine. At present the nature of these inhibitory urinary substances is not known. However, we could not satisfactorily eliminate the interference of urine with the assay by a few simple procedures, including a brief boiling of the urine prior to analysis, diluting the urine by as much as 1:20 prior to analysis, increasing the amount of enzyme in the assay, prolonging the incubation time, and altering the incubation temperature.

A recent report suggests that extraction of urinary histamine, in addition to analyzing diamine oxidase treated and untreated urine, does improve the accuracy of the double isotope radioenzymatic assay to an extent, but some interference and unreliability still remains [15]. Whether additional chromatographic purification following extraction of urinary histamine prior to analysis would satisfactorily remove interfering urinary substances remains to be determined but deserves investigation. Although we chose to circumvent these problems by developing the GC—MS method of analysis, these studies should help investigations into procedures which can potentially improve the radio-

enzymatic assay as mass spectrometer instrumentation may not be universally available to all laboratories interested in the quantification of urinary histamine.

It also seems important to mention that although the single isotope radioenzymatic assay was used in these studies to investigate the inhibitory influences of urine with the determination of histamine, it would not seem that simply using a double isotope radioenzymatic assay would successfully overcome the problems of inaccuracy with the method. Although the double isotope radioenzymatic assays incorporate a control for incomplete methylation of histamine in the assay, the accuracy of this control becomes substantially reduced with increasing degrees of interference of urine with the assay. With increasing interference, the slope of the curve relating histamine concentrations to recovered methylhistamine radioactivity becomes very flat. This phenomenon is illustrated in Fig. 2. An even flatter curve than depicted in Fig. 2 would be obtained with urines in which there was an even greater degree of interference such as the urine on day 4 of aspirin administration in Fig. 3. In other words, in the presence of substantial interference, a large increase in histamine concentration only produces a very small absolute increment in radiolabeled methylhistamine recovered. Because the accuracy of scintillation counting is proportional to the number of disintegrations per minute detected, the assay would be insensitive to small but meaningful differences in the concentration of histamine present in such urines and such differences could not be accurately and reliably measured. The correction factor that is incorporated into the calculations in the double isotope assay to control for incomplete methylation of histamine would, in this situation, only multiply the error associated with the scintillation counting. For analogous reasons, running a standard curve of histamine concentration in each individual urine sample analyzed, in addition to being very time-consuming, would also not overcome the inaccuracy of the radioenzymatic determination of histamine in urines which profoundly interfere with the assay.

In summary, these studies have elucidated substantial inaccuracy and unreliability with the radioenzymatic determination of histamine in urine. In laboratories where mass spectrometer instrumentation is available, the described stable isotope dilution GC—MS method enables quantification of urinary histamine that is not only accurate but also very efficient. However, for laboratories without access to mass spectrometer instrumentation, it seems likely that with further investigation, procedures can be found and incorporated which will improve the radioenzymatic methods for determination of urinary histamine to an acceptable degree. Until such procedures are determined, validated, and incorporated, however, one is left with substantial skepticism as to validity of levels of urinary histamine reported using radioenzymatic methods.

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CHROMBIO. 2414

AVAILABILITY OF 6-HYDROXYNICOTINIC ACID FOR RAPID IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* AND *SERRATIA MARCESCENS*

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SUMMARY

Gas chromatography—mass spectrometry has been used to identify specific metabolites produced by Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Escherichia coli* in a defined medium. 6-Hydroxynicotinic acid was detected in spent culture media of *Pseudomonas aeruginosa* and *Serratia marcescens*, but could not be detected in those of *Klebsiella pneumoniae* and *Escherichia coli*. The production of 6-hydroxynicotinic acid was recognized by the addition of nicotinic acid in urine with *Pseudomonas aeruginosa* or *Serratia marcescens*, but not without the addition of nicotinic acid.

Among 10^5 *Pseudomonas aeruginosa* per 1 ml of urine (criteria for the diagnosis of urinary tract infection), 0.15 μg of 6-hydroxynicotinic acid was detected in urine at 4 h incubation with nicotinic acid at the optimum pH of 6.9, 38°C. The production of 6-hydroxynicotinic acid was proportional to the number of the bacteria and displayed a time dependency. These results suggest that the availability of 6-hydroxynicotinic acid might make for more rapid identification of bacteria than current methods.

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INTRODUCTION

Serious infection by pathogenic microorganisms has been diminished by newly developed antibiotics, but the significance of Gram-negative microorganisms such as *Pseudomonas aeruginosa* has increased from the viewpoint of attributable microorganisms of opportunistic infection. Many studies have been carried out for rapid identification of such bacteria using gas chromatography [1—3] or other methods [4, 5], since the present routine diagnostic methods are time-consuming. At present the results of culture take one day and, in the case of growth, information about the identity is given on the second day. It would be desirable to reduce the long time interval and to provide the answer within one working day.

In the present study, the specific metabolites of *P. aeruginosa* and *Serratia marcescens* are identified, and the possibility of clinical diagnosis within 6 h using gas chromatography—mass spectrometry (GC—MS) is discussed.

MATERIALS AND METHODS

Bacteria

The bacteria used in this study, *P. aeruginosa* strain P101, strain P102, *S. marcescens* strain S108, *Klebsiella pneumoniae* strain K105, and *Escherichia coli* strain E101, were isolated from urine samples of patients suffering from urinary tract infections. The identity of the culture specimens was confirmed by conventional and serological methods [6, 7].

Reagents

6-Hydroxynicotinic acid (6HN) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Gasukuro Kogyo (Tokyo, Japan). Nicotinic acid and *p-n*-amylbenzoic acid (ABA) were the products of Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents were of the highest purity available commercially.

Identification of 6HN in spent culture media

Organisms, $1 \cdot 10^8$—10^9, of *P. aeruginosa* strain P101, *S. marcescens* strain S108, *K. pneumoniae* strain K105, or *E. coli* strain E101 were inoculated into Mølar-Hinton broth (Eiken, Tokyo, Japan) and incubated at 38°C for 20 h. After sterilization by filtration, 3 ml of the medium to which 20 μg of ABA (internal standard) had been added were acidified to pH 1 with 6 *M* hydrochloric acid and saturated with sodium chloride. Organic acids were extracted twice with 9 ml of ethyl acetate for 10 min, dehydrated over anhydrous sodium sulphate, and evaporated to dryness with a rotary evaporator and a stream of nitrogen. Organic acids were derivatized with 200 μl of BSTFA at 65°C for 30 min, and 2 μl of the sample were subjected to GC—MS.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a 5710A gas chromatograph (Hewlett-Packard) equipped with a 30-m OV-101 capillary column, a JMS D-300 mass spectrometer (JEOL) and a JMA 2000 data processing

system (JEOL). The carrier gas was helium. Electron-impact ionization (EI) mass spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 300 μA, ion source temperature 200°C, and accelerating voltage 3 kV. Chemical ionization mass spectra were recorded using methane as a reactant gas, and with an ionizing energy of 200 eV. The other conditions were the same as for EI.

Detection of 6HN in urine

Nicotinic acid solution was prepared by dissolving 100 mg of nicotinic acid in 100 ml of 0.2 *M* potassium dihydrogen phosphate—disodium hydrogen phosphate buffer at pH 7.0 and sterilized by filtration. Urine excreted from a healthy man was immediately sterilized by filtration. A 1-ml aliquot of the sterilized urine was incubated with *P. aeruginosa* strain P101 ($1 \cdot 10^7$) and/or 2 ml of nicotinic acid solution (1 mg/ml) at 38°C for 3 h. After centrifugation, 1 ml of the supernatant to which 10 μg of ABA had been added was acidified to pH 1 and saturated with sodium chloride. The organic acids were extracted twice with 3 vols. of ethyl acetate, dehydrated, dried and trimethylsilylated using the same procedure as with the culture media.

Quantitative determination of 6HN

The calibration curve for 6HN in urine was obtained by adding a known amount of standard to 1 ml of water. After addition of 10 μg of ABA (internal standard), 6HN was extracted using the sampe procedure as with the urine sample. Calibration curves relating the concentration of 6HN were obtained from the mass chromatogram. Ion *m/z* 268 was used for the quantitation of 6HN, and ion *m/z* 264 for the monitoring of an internal standard, ABA.

Recovery of 6HN

Quintuplicate estimations of recovery were carried out using 10 μg of 6HN, which were added to 1 ml of phosphate buffer. 6HN was extracted using the same procedure as with the urine sample and quantitated by mass chromatography. Recovery of 6HN was 61.6 ± 7.0% (mean ± S.D., $n = 5$).

pH Dependency of 6HN production

Each pH solution of nicotinic acid was prepared using 0.2 *M* potassium dihydrogen phosphate—disodium hydrogen phosphate and sterilized by filtration. A 1-ml volume of sterilized urine was added to 2 ml of the phosphate buffer containing nicotinic acid and incubated with *P. aeruginosa* strain P101 ($1.1 \cdot 10^7$) at 38°C for 3 h. 6HN was extracted twice with 3 ml of ethyl acetate from 1 ml of medium (see *Detection of 6HN in urine*) and analysed. The ratios of peak heights of 6HN to ABA were obtained on the mass chromatogram of each pH.

Production rate of 6HN

Experiment 1. A 1-ml volume of the sterilized urine to which 2 ml of the phosphate buffer containing nicotinic acid had been added, was incubated with *P. aeruginosa* strain P101, $1.0 \cdot 10^7$, $1.0 \cdot 10^6$, $1.0 \cdot 10^5$, $1.0 \cdot 10^4$, or $1.0 \cdot 10^3$, for 1, 2, 4, or 6 h at 38°C.

The ratios of peak heights of 6HN and ABA on the mass chromatogram of each case were obtained, and the production of 6HN in each case was quantitated using the standard curve.

Experiment 2. Production rate of 6HN by *P. aeruginosa* strain P102 was estimated using the same procedure as for Experiment 1. A 1-ml volume of the sterilized urine to which 2 ml of the phosphate buffer containing nicotinic acid had been added, was incubated with $1.2 \cdot 10^8$, $1.2 \cdot 10^7$, $1.2 \cdot 10^6$, or $1.2 \cdot 10^5$ *P. aeruginosa* strain P102 for 1, 2, 4, or 6 h at 38°C.

RESULTS

Fig. 1a—d shows the gas chromatograms of trimethylsilyl (TMS) derivatives of organic acids in media incubated with (a) *P. aeruginosa* strain P101, (b) *S. marcescens* strain S108, (c) *K. pneumoniae* strain K105, and (d) *E. coli* strain E101; Fig. 1e shows a control medium. The peaks were identified by comparing their mass spectra with those of the trimethylsilylated authentic compounds or the mass spectra in the literature.

Peak 63 was detected in spent culture media of *P. aeruginosa* and *S. marcescens*, but could not be detected in those of *K. pneumoniae* and *E. coli*. The EI mass spectrum of peak 63 is shown in Fig. 2 (upper spectrum). The chemical ionization mass spectrum using methane as a reactant gas showed that the molecular ion of the compound was 283. High-resolution mass spectrometry of the *m/z* 283 ion showed an exact mass of 283.1005, an error of 0.0056 and a probable composition of $C_{12}H_{21}NO_3Si_2$. These data revealed the original composition of $C_6H_5NO_3$ and the structure of hydroxynicotinic acid. The EI mass spectrum of the TMS derivative of 6HN is shown in Fig. 2 (lower spectrum). Peak 63 was identified as trimethylsilylated 6HN, because peak 63 and trimethylsilylated 6HN showed identical retention times and identical mass spectra.

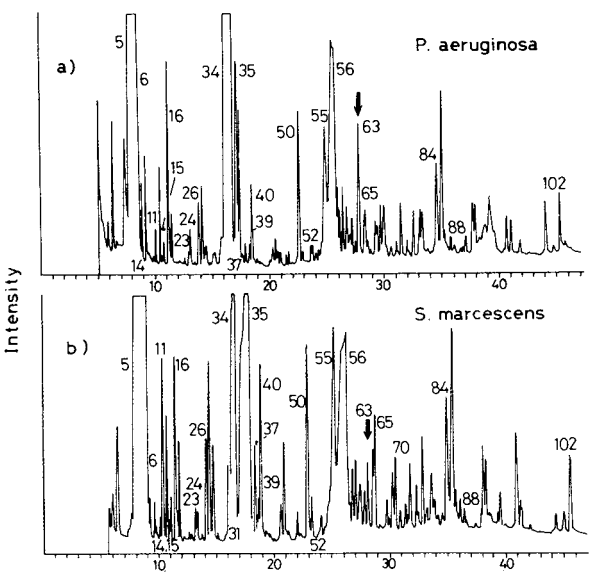

Fig. 1.

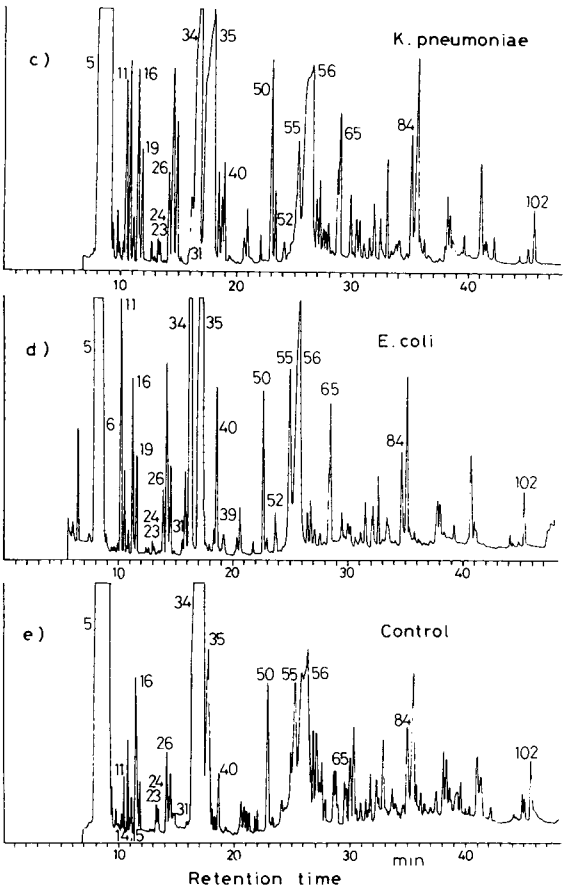

Fig. 1. Gas chromatogram of TMS derivatives of ethyl acetate extracts of: (a) *P. aeruginosa* strain P101 spent culture medium; (b) *S. marcescens* strain S108 spent culture medium; (c) *K. pneumoniae* strain K105 spent culture medium; (d) *E. coli* strain E101 spent culture medium; (e) control medium. Peak identification: 5 = lactic acid; 6 = glycolic acid; 11 = 2-hydroxybutyric acid; 14 = *p*-cresol; 15 = ethyleneglycol; 16 = 3-hydroxybutyric acid; 19 = 2-hydroxyisovaleric acid; 24 = 3-hydroxyvaleric acid; 26 = benzoic acid; 31 = nicotinic acid (minor of two components); 34 = glycerol; 35 = succinic acid; 39 = glyceric acid; 40 = fumaric acid; 50 = 2-deoxytetronic acid; 56 = pyroglutamic acid; 63 = 6-hydroxynicotinic acid; 84 = internal standard (ABA); 88 = 4-hydroxymandelic acid; 102 = palmitic acid. The column temperature was programmed from 100°C to 250°C at 3°C/min.

Fig. 3 gives the gas chromatogram of TMS derivatives in urine incubated with nicotinic acid and/or *P. aeruginosa* strain P101. 6HN could not be detected in urine incubated with only *P. aeruginosa* (b), but was found in urine incubated with both *P. aeruginosa* and nicotinic acid (a). These results indicate that *P. aeruginosa* metabolized nicotinic acid to 6HN within 3 h. (As shown in Fig. 1, the Mølar-Hinton broth contains nicotinic acid.)

Fig. 4 shows the pH dependency of the 6HN production. The pH of the urine to which 2 ml of each of the buffer containing nicotinic acid were added, was not changed before or after incubation. The optimum pH of 6HN production was at 6.9.

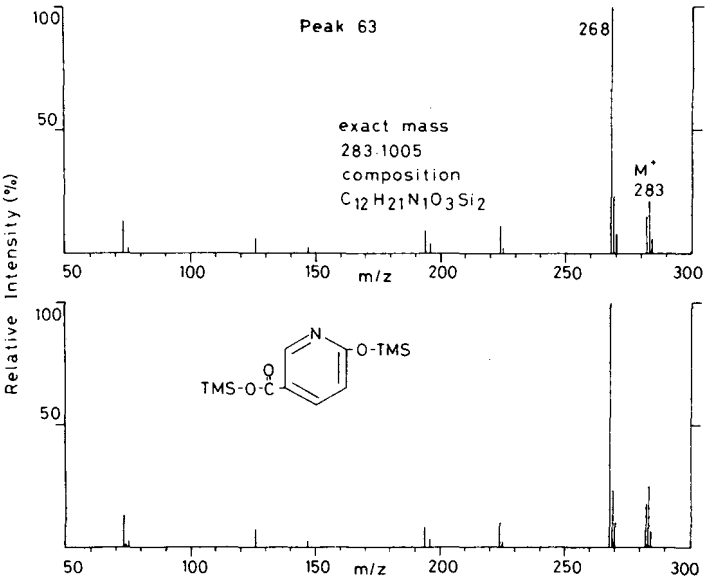

Fig. 2. EI mass spectra of trimethylsilylated 6-hydroxynicotinic acid (lower spectrum) and of peak 63 in Fig. 1a (upper spectrum).

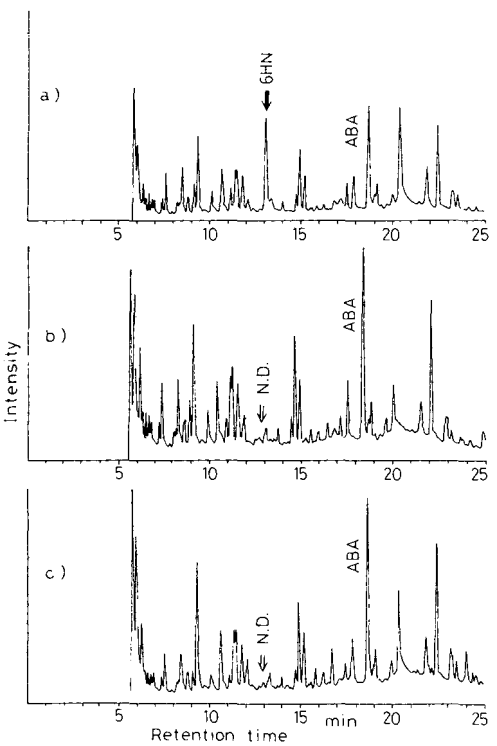

Fig. 3. Gas chromatogram of TMS derivatives of organic acids in urine incubated with (a) *P. aeruginosa* strain P101 and nicotinic acid, (b) *P. aeruginosa* strain P101, (c) nicotinic acid, at 38°C for 3 h. The column temperature was programmed from 150°C to 230°C at 3°C/min. 6HN = 6-hydroxynicotinic acid, ABA = *p-n*-amylbenzoic acid (internal standard), N.D. = not detected.

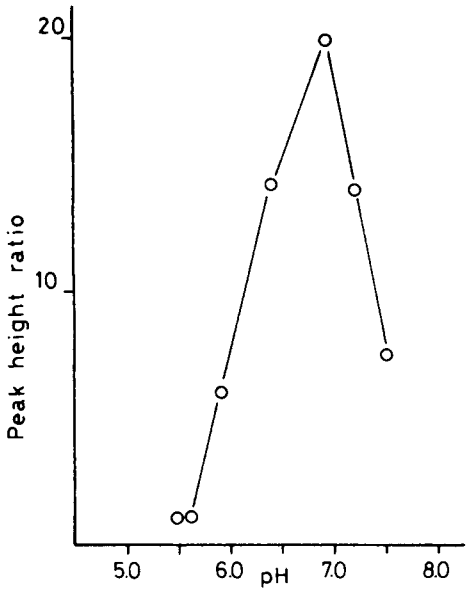

Fig. 4. pH Dependency of 6HN production. A 1-ml volume of urine was incubated with $1.1 \cdot 10^7$ *P. aeruginosa* strain P101 at 38° C for 3 h.

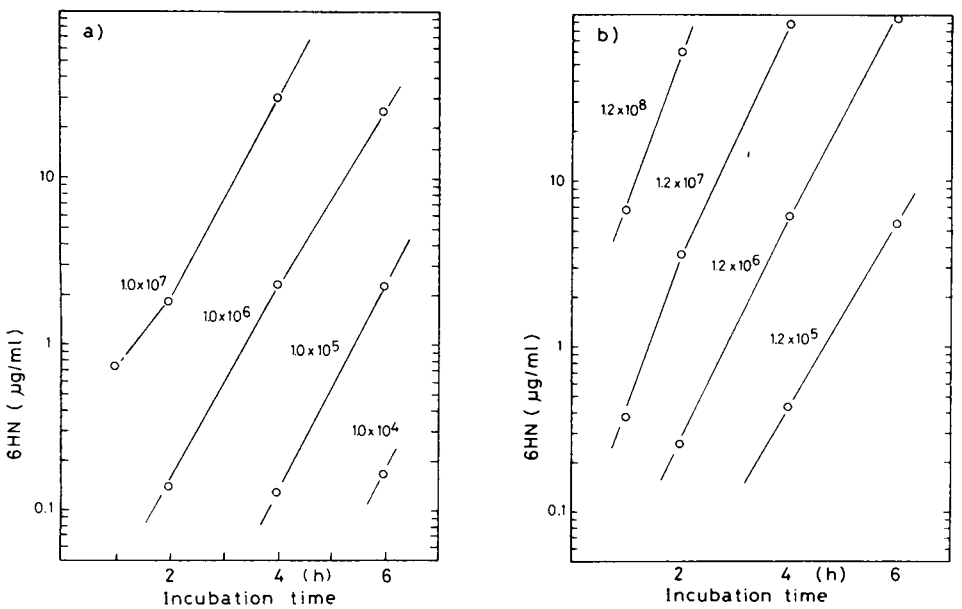

Fig. 5. Production rate of 6HN (a) by *P. aeruginosa* strain P101, (b) by *P. aeruginosa* strain P102. A 1-ml volume of urine to which 2 ml of 1 mg/ml phosphate buffer pH 7.0 containing nicotinic acid had been added, was incubated with *P. aeruginosa*.

Fig. 5a shows the production rate of 6HN by *P. aeruginosa* strain P101. In $1.0 \cdot 10^5$ bacteria, 0.15 µg of 6HN could be detected at 4 h incubation and 2.2 µg at 6 h incubation. In $1.0 \cdot 10^7$ organisms, 0.75 µg of 6HN could be detected

at 1 h incubation. The production of 6HN was proportional to the number of strain P101 and evidenced a time dependency.

Fig. 5b shows another set of experiments of the production rate of 6HN by *P. aeruginosa* strain P102. In $1.2 \cdot 10^5$ organisms, 0.43 μg of 6HN could be detected at 4 h incubation and 5.5 μg at 6 h incubation. In $1.2 \cdot 10^8$ bacteria, 6.7 μg of 6HN could be detected at 1 h incubation and 59.5 μg at 2 h incubation.

DISCUSSION

In this study 6HN was first discovered to be produced by *P. aeruginosa* or *S. marcescens* by means of GC—MS. 6HN has been known to be an intermediate in oxidative degradation of nicotinic acid by some bacteria such as *Clostridium* species [8, 9], *Bacillus* species [10], and *Pseudomonas* fluorescens [11]. There has, however, been no report about the production of 6HN concerning the clinically important microorganisms.

Although 6HN could not be detected in urine incubated with *P. aeruginosa* or *S. marcescens* because of the very low concentration of nicotinic acid, the substrate, the production of 6HN was recognized by the addition of nicotinic acid in urine, proportional to the number of bacteria, and time-dependent. *P. aeruginosa* strain P102 was found to possess the ability to produce 6HN as well as strain P101. These results suggest the availability of 6HN for rapid identification of bacteria.

A typical urinary tract infection reveals the presence of 10^5 or more microorganisms per ml of urine. Our results show that, in the presence of $1 \cdot 10^5$ *P. aeruginosa*, it was necessary to detect urinary 6HN at 4 h incubation, and 0.15—0.43 μg of 6HN could be detected.

Hayward [12] applied head-space gas—liquid chromatography (HS-GLC) to rapid diagnosis of urinary tract infection. The advantages of HS-GLC are that the sample preparation is more quick and simple to perform than our GC—MS method. The adavantage of our method is that specificity of 6HN is excellent, which is important to characterize the bacteria. Although 6HN was found to be helpful for the detection of *P. aeruginosa* or *S. marcescens*, other factors are necessary for the identification of the bacteria. Further characterization of the bacteria, including 6HN-negative bacteria such as *E. coli* or *K. pneumoniae*, is now in progress in our laboratory.

CONCLUSIONS

(1) 6HN was detected in spent culture media of *P. aeruginosa* and *S. marcescens*, but could not be detected in those of *K. pneumoniae* and *E. coli*.

(2) *P. aeruginosa* or *S. marcescens* synthesized 6HN from nicotinic acid.

(3) Detection of 6HN, infected urine or blood incubated with nicotinic acid may be clinically useful for the rapid identification of bacteria.

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF MOLECULAR SPECIES OF ALKYL ETHER, VINYL ETHER, AND MONOACYL LYSOPHOSPHOLIPIDS

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SUMMARY

An isocratic reversed-phase high-performance liquid chromatographic method was developed which resolved individual molecular species of choline and ethanolamine lysophospholipids utilizing a C_{18} bonded porous silica stationary phase with a mobile phase comprised of methanol—water—acetonitrile (57:23:20) containing 20 m*M* choline chloride. Solute retention was primarily determined by hydrophobic interactions with the stationary phase permitting separation of individual molecular species of lysophospholipids according to the composition of the aliphatic chain and the nature of its covalent attachment to the *sn*-1 hydroxyl group. The elution profile of unsaturated monoacyllysophospholipid or lysoplasmalogen molecular species was readily obtained by measuring UV absorbance at 203 nm. Identification of column eluates containing saturated monoacyl and alkyl ether lysophospholipids was possible utilizing relative retention factors that were obtained for the majority of molecular species present in animal tissues.

INTRODUCTION

Conventional approaches for preparing labeled homogeneous phospholipid probes required for studies of the metabolism and physicochemical properties of plasmalogens are based on the acylation of vinyl ether lysophospholipids. The total synthesis of homogeneous lysoplasmalogens with the appropriate *sn*-1 side-chain is, however, a laborious process accompanied by poor yields. Accordingly, the acylation reaction is routinely performed on mixtures of lysoplasmalogen molecular species obtained from biologic sources (typically alkaline hydrolysates of beef heart choline phosphoglycerides). This is an inefficient approach for the preparation of homogeneous plasmalogens or alkyl ethers and impractical when the desired species is a minor component of the

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mixture (e.g., synthesis of phospholipid probes with an octadecyl side-chain at the *sn*-1 position).

To overcome these limitations, we developed a high-performance liquid chromatographic (HPLC) method for separating individual molecular species of lysophospholipids to provide milligram quantities of starting material for subsequent use in the synthesis of homogeneous diradylphospholipid probes. To achieve high-efficiency separation based on differences in the composition of the *sn*-1 side-chain, a reversed-phase approach was employed using octadecylsilane-derivatized porous silica as the stationary phase. Elution of individual components is accomplished isocratically with a solvent system that allows the measurement of UV absorbance to monitor the separation. The chromatographic procedure was evaluated using synthetic homogeneous lysophospholipid standards and mixtures of monoacyl, alkyl ether, and vinyl ether lysophospholipids derived from biologic sources.

EXPERIMENTAL

High-performance liquid chromatography

HPLC was performed using a liquid chromatographic system from Waters Assoc. consisting of a Model 720 system controller, a U6K injector, a Model 6000A pump, a Model 450 variable-wavelength UV detector with an 8-μl flow cell and a Model 730 printer—plotter integrator. Samples were injected in 10—200 μl vols. of chloroform—methanol and UV absorbance was monitored at a wavelength of 203 nm. All separations were performed at room temperature using a stainless-steel column (25 cm × 4.6 mm I.D.) packed with Ultrasphere ODS (C_{18} bonded-phase on 5-μm porous silica microparticles, Altex) with a pre-column (8 cm × 3 mm I.D.) containing Co:Pell ODS pellicular packing (C_{18} bonded-phase on 30-μm glass beads, Whatman). Sample components were eluted isocratically at a flow-rate of 2 ml/min with a mobile phase of methanol—water—acetonitrile (57:23:20, v/v/v) containing 20 m*M* choline chloride.

Solvent reagents and phosphate analysis

Methanol and acetonitrile were obtained from Burdick & Jackson Labs. and doubly distilled water was obtained from J.T. Baker. Choline chloride was obtained from Sigma (St. Louis, MO, U.S.A.). All solvents had a UV cutoff below 210 nm and were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed under vacuum prior to use. The phosphate content of column eluates was determined by the microphosphate assay method of Chen et al. [1].

Lipids

Lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) containing 14:0, 16:0, 18:0, and 18:1 fatty acids and beef heart lecithin were purchased from Avanti (Birmingham, AL, U.S.A.). 1-O-[Alkyl-1′,2′-^{3}H]glyceryl-3-phosphorylcholine was obtained from New England Nuclear (Boston, MA, U.S.A.). Bovine brain LPE, and soybean LPC were obtained from Sigma.

Purity of each of the lipids was verified by the presence of a single spot after thin-layer chromatographic (TLC) separation and iodine staining and scintillation spectrometry of the radiolabeled compounds.

Choline lysoplasmalogen was prepared as previously described [2]. The 1-alkenyl groups in the final product were 14:0 (6%), 15:0 (7%), 16:0 (68%), 17:0 (4%), 18:0 (10%) and 18:1 (4%) as determined by fast atom bombardment mass spectrometry (MS) and gas chromatography (GC) of the dimethylacetal derivatives.

Derivatization, GC and GC—MS

Column fractions were obtained from individual peaks in the UV absorbance profile. To each ml of effluent collected, 0.3 ml of water and 0.6 ml of chloroform were added to form a biphasic extraction mixture. Lysophospholipids were recovered in the lower chloroform layer following two successive extractions, dried under nitrogen, and derivatized by acid methanolysis as previously described [3]. The fatty acid methyl ester (FAME) and/or dimethylacetal (DMA) products were injected onto a 30 m × 0.25 mm fused-silica capillary GC column coated with SP 2330 (Supelco) utilizing a split ratio of 5 to 10:1 and eluted isothermally at 190°C utilizing a Varian Model 3700 chromatographic system (Varian, Walnut Creek, CA, U.S.A.) with helium as a carrier gas at a flow-rate of 2 ml/min. Components eluting from the column were detected and quantified with a flame-ionization detector and Varian Model 4270 integrator. Mass spectra were obtained by electron impact (70 eV) at a source temperature of 150°C following GC separation on a 1-m glass column packed with 10% SP 2330 on 100—120 mesh Chromosorb W AW (Supelco) using a Finnigan Model 3200 GC—MS system. Identification of lysoplasmalogen molecular species was established by comparison of the GC retention times of their DMA derivatives with those derived from myocardial sarcolemma (containing approximately 40% plasmalogens [3]) and by GC—MS of the DMA derivatives prepared from column eluates corresponding to each peak. The assignment of molecular species with methyl branched-chain substituents was based on comparison of the relative GC retention times of the branched-chain and straight-chain DMA derivatives containing equal numbers of carbon atoms [4], by comparison of the mass spectra of both isomers demonstrating parent ions of identical mass, and based on the previous demonstration of branched-chain isomers in choline plasmalogen from bovine heart in the same relative proportions that we observed [5]. Unsaturated monoacyllysophospholipids were identified by comparing GC retention times of their FAME derivatives with commercially available standards and by GC—MS of the FAME derivatives prepared from column eluates.

Computation of relative retention time

Relative retention time (RRT) values for individual molecular species were adjusted to account for the time required to displace the void volume using the following formula: RRT = (retention time of component species $- t_0$)/(retention time of 18:1 monoacyllysophosphatidylcholine $- t_0$), where t_0 = time to reach solvent front peak.

RESULTS

For isocratic elution of lysophospholipid standards, a ternary mobile phase containing methanol, water and acetonitrile was found to provide greater separation selectivity than binary solvent mixtures of methanol—water or acetonitrile—water. An example of the separation of individual species of choline lysoplasmalogens and alkylglycerylphosphorylcholines is shown in Fig. 1. With the exception of fractions 3p and 8p, all other fractions were homogeneous as determined by the presence of a single component on GC analysis of acid methanolysates prepared from column eluates. As suggested by the HPLC chromatogram, there were three components in fraction 3p and two components in fraction 8p. In both cases, the predominant species were methyl branched-chain isomers. The identity of the minor constituents was not established.

For the alkyl ether choline lysophospholipids (which do not have appreciable UV absorbance), ^{3}H-labeled 1-O-alkyl-2-lysophosphatidylcholine [lyso-platelet activating factor (lyso-PAF)] was used to establish the retention time of individual molecular species. Since lyso-PAF was prepared by hydrogenation of choline lysoplasmalogen with tritium gas the distribution of saturated ^{3}H-labeled product species reflects that of the choline lysoplasmalogen starting material. Accordingly, the identification of lyso-PAF species was made by comparing the retention times and relative peak areas from the elution profile of the ^{3}H-labeled components to that of their choline lysoplasmalogen precursors (Fig. 1).

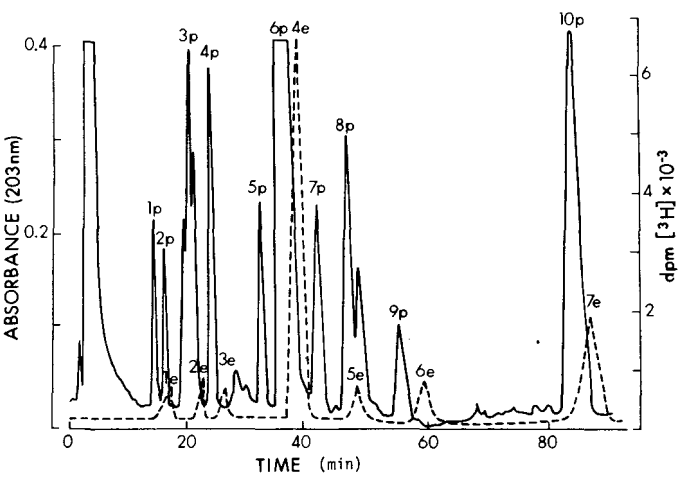

Fig. 1. Separation of molecular species of vinyl ether and ^{3}H-labeled alkyl ether lysophosphatidylcholine. Bovine heart choline lysoplasmalogen (1.4 μmol) and 1-O-[alkyl-1',2'-^{3}H]-glyceryl-3-phosphorylcholine (4.5 nCi, specific activity = 45 mCi/μmol) dissolved in 150 μl of chloroform—methanol (2:1, v/v) were injected onto a 5-μm Ultrasphere ODS C_{18} reversed-phase column. Lysophospholipids were eluted isocratically at room temperature (20—24° C) with a flow-rate of 2 ml/min using a solvent mixture of methanol—water—acetonitrile (57:23:20, v/v/v) containing 20 m*M* choline chloride. Individual molecular species were identified as described in the text. The numbers above each peak correspond to those in Table I. ——, UV absorbance at 203 nm; - - - -, ^{3}H radioactivity.

The separation of monoacyllysophospholipids is illustrated in Fig. 2. For saturated species, detection was accomplished by measuring the phosphate content of column eluates collected at 2-min intervals following the injection of synthetic homogeneous lysophospholipid standards. The retention times of the 14:0, 16:0 and 18:0 monoacyl species were 13, 28, and 59 min, respectively. The 18:2 FAME derivatives prepared from column eluates corresponding to peaks 2a and 3a were indistinguishable by comparison of GC retention times or electron-impact mass spectra. Peak 3a represents 1-linoleoyl-*sn*-glycero-3-phosphocholine and peak 2a was tentatively identified as a positional isomer (i.e., differing with respect to location of the acyl chain or position of the double bonds) and was not characterized further.

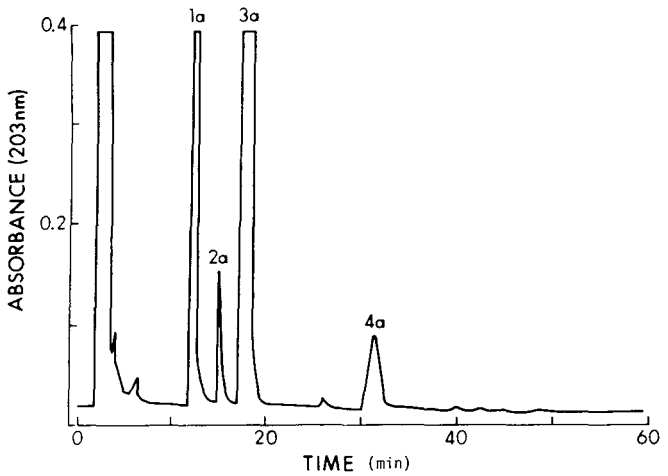

Fig. 2. Separation of molecular species of monoacyllysophosphatidylcholine. Soybean LPC (1.4 μmol, prepared by phospholipase A_2 catalyzed hydrolysis of soybean phosphatidylcholine) was dissolved in 50 μl of chloroform—methanol (2:1, v/v) and injected. Conditions for separation and peak detection and methods used for identification of individual molecular species are the same as those for the vinyl ether species given in Fig. 1. The numbers above each peak correspond to those in Table I.

Fig. 3 represents an example of the separation of bovine brain LPE. The UV absorbing fractions were homogeneous, however, the sample also contained saturated monoacyl LPE species that were not detected by measurement of UV absorbance. Accordingly, equal amounts of 14:0, 16:0 and 18:0 monoacyl LPE standards were injected separately and the phosphate content of fractions collected at 2-min intervals was determined to establish retention times of saturated monoacyl LPE species. The 14:0, 16:0 and 18:0 LPE molecular species eluted at 13, 28 and 58 min, respectively.

In order to compare the retention times of molecular species in different samples, relative retention factors were obtained in a separate series of injections by adding 18:1 monoacyl LPC as an internal reference standard to each sample. The relative retention time of individual molecular species was then calculated (Table I). Comparison of the relative retention factors demonstrated that substitution of ethanolamine for choline in the polar head group had no appreciable effect on the retention time of vinyl ether and monoacyllyso-

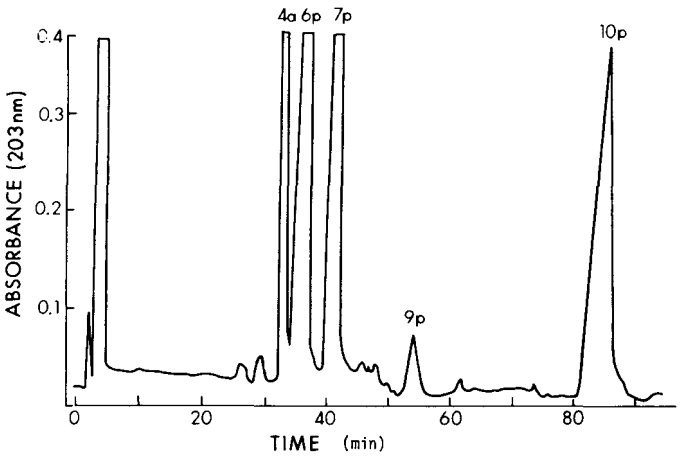

Fig. 3. Separation of molecular species of lysophosphatidylethanolamine from bovine brain. LPE (1.5 μmol, prepared by phospholipase A_2 catalyzed hydrolysis of bovine brain phosphatidylethanolamine) was dissolved in 50 μl of chloroform—methanol (1:1, v/v) prior to injection. Methods for separation, peak detection, and identification of individual molecular species are identical to those described for the choline lysoplasmalogen species in Fig. 1. The numbers above each peak correspond to those in Table I.

TABLE I

RELATIVE RETENTION TIMES FOR INDIVIDUAL LYSOPHOSPHOLIPID MOLECULAR SPECIES

RRT values were computed as described in the experimental section. Numbers in parentheses identify individual peaks in the elution profiles of Figs. 1—3.

Species*	Alkyl ether	Vinyl ether	Monoacyl ester
14:0 br	—	0.478 (1p)	—
14:0	0.533 (1e)	0.532 (2p)	0.366
18:3	—	—	0.371 (1a)
18:2	—	—	0.559** (isomer 1) (2a)
	—	—	0.589** (isomer 2) (3a)
15:0 br	0.733 (2e)	0.682 (3p)	—
15:0	0.867 (3e)	0.799 (4p)	—
16:0 br	—	1.087 (5p)	—
16:0	1.267 (4e)	1.184 (6p)	0.805
18:1	—	1.411 (7p)	1.000*** (4a)
17:0 br	1.687 (5e)	1.622 (8p)	—
17:0	1.967 (6e)	1.886 (9p)	—
18:0	2.800 (7e)	2.863 (10p)	1.885

*Listed in order of elution from the column. Individual molecular species are designated in the form *a*:*b* where *a* is the number of carbon atoms and *b* is the number of double bonds in the aliphatic chain (br. indicates a methyl branched-chain isomer).
** Isomer 1 and 2 were tentatively identified as positional isomers (see text).
***Used as the internal standard.

phospholipids. Thus, under the conditions employed, separation selectivity is determined primarily on the basis of interactions of the stationary phase with the hydrophobic portion of the lysophospholipid molecule.

For all samples, optimal separation efficiency was achieved when the mobile phase contained 20—25% (v/v) water with typical plate counts of 20 000 to 35 000 plates per m for monooleoyllysophosphatidylcholine at a flow-rate of 2 ml/min (mobile phase velocity of 0.2 cm/sec). Varying the flow-rate from 1.0 to 2.5 ml/min resulted in a two-fold decrease in retention time but had no significant effect on the plate count. Operating pressures required to maintain a 2 ml/min flow-rate ranged from 170 to 310 bars. Routine use of a guard column reduced the rate at which operating pressure increased with age of the column without adversely affecting separation efficiency.

In order to reduce peak tailing that results from interaction of the polar head group with highly adsorptive sites on the porous silica, it was necessary to add an ionic modifying agent to the mobile phase. From previous reports of the reversed-phase separation of diacylphospholipid species, such mixed-mode retention was prevented by adding phosphoric acid [6] or choline chloride [7] to the mobile phase. Since plasmalogens are acid-labile, choline chloride was used as the modifying agent. With 20 m*M* choline chloride interactions of the polar head group with the stationary phase were effectively suppressed. This was demonstrated by an improvement in peak symmetry and by the identical retention times obtained for monooleoyl LPC and LPE standards. With an ionic modifier in the mobile phase, it was possible to inject up to 2 mg of lysophospholipid without overloading the column. When it was necessary to remove the excess choline from column eluates (e.g., prior to acylation of the lysophospholipid), a two-phase extraction was performed by adding 0.6 ml chloroform and 0.3 ml water to each ml of mobile phase, mixing, centrifuging, and finally collecting the lower chloroform layer to recover the lysophospholipids. Using this extraction procedure, recovery of lysophospholipids exceeded 93% based on results obtained with ^{3}H-labeled alkyl ether lysophospholipids and phosphate measurements following separation of monoacyllysophospholipid standards.

DISCUSSION

Reversed-phase chromatographic methods have recently been successfully applied to the separation of diacylphospholipid molecular species using a stationary phase of octadecylsilyl groups covalently bound to porous silica microparticles [6, 7]. In those studies, it was necessary to add an ionic modifying agent to the mobile phase to mask highly adsorptive sites that remained after derivatization in order to prevent the loss of resolution that arises from mixed-mode retention. Utilizing this information we developed the present method which, for the first time, permits separation of lysophospholipids into homogeneous fractions containing a single molecular species.

For the separation of a homologous series of compounds with long-chain aliphatic groups the step-wise addition of methylene ($-CH_2-$) units leads to a step-wise increase in the logarithm of the distribution constant, K_D [8]. Since the relative retention time is directly proportional to K_D, by placing individual lysophospholipid species into classes based on similarities in the covalent attachment of the aliphatic chain to the polar head group, the degree of unsaturation, and the presence (or absence) of methyl branching, a plot of

log (RRT) versus chain length for each class should produce a series of parallel straight lines with different intercepts [9]. This is indeed the case as shown in Fig. 4. As expected, the values for the intercept increase in the observed order of elution: O-acyl esters < vinyl ethers < alkyl ethers since the polar character of these functional groups decreases accordingly. In addition to changes in solute polarity, retention time also appears to be dependent on steric interactions. Thus, while the introduction of methyl branching or carbon—carbon double bonds would not have a marked effect on solute polarity, these structural modifications which occur near the methyl end of the aliphatic chain also resulted in a significant decrease in the value of the intercept.

Since we have found that measurements of the retention time relative to the 18:1 monoacyl species are reproducible within ± 10% (n = 10), it would be possible to make a tentative identification of an unknown lysophospholipid species based on measurement of the RRT value and using the information given in Fig. 4. This is illustrated by point A in Fig. 4 where if an unknown lysophospholipid detected by the UV monitor had an RRT value of 0.447 [log (10 × RRT) = 0.65] it would tentatively be identified as a 16:1 monoacyllysophospholipid.

By monitoring UV absorbance, the present method is ideally suited for separating mixtures of lysoplasmalogens since each individual species is readily detected by the UV absorbance which arises from the π—π^* transition of electrons forming the double bond between the C-1 and C-2 carbon atoms of the vinyl ether group. For vinyl ethers with saturated aliphatic chains, the extinction coefficient is not affected by changes in chain length or methyl branching and therefore comparison of the peak areas provides a direct assessment of the

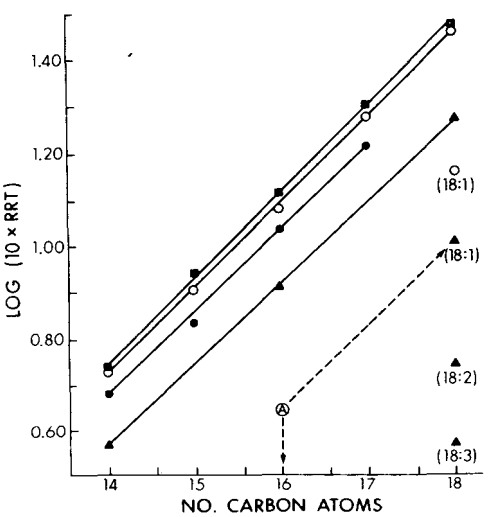

Fig. 4. Relationship between the relative retention time (RRT) and the number of carbon atoms in the acyl, alkyl, or alkenyl *sn*-1 side chain. Data for this figure were obtained from Table I. Individual molecular species were placed into one of the following groups: straight-chain 1-O-alkylglyceryllysophospholipids (■), straight-chain 1-O-alk-1′-enyllysophospholipids (○), branched-chain 1-O-alk-1′-enyllysophospholipids (●), and 1-O-acyllysophospholipids (▲). The linear regression correlation coefficient (r) had an average value of 0.9988 for the four groups shown.

relative amounts of each lysoplasmalogen present. For species with additional olefin groups, a better estimate of the relative amount of lysophospholipid contained in the corresponding fraction is obtained by dividing the area under each peak by the total number of unsaturated centers per molecule. This approach provides only an approximation, however, since the contribution of the vinyl ether double bond to the measured UV absorbance was substantially greater than that due to olefin groups.

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CHROMBIO. 2417

NEW APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR ANALYSIS OF URINARY C-PEPTIDE

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SUMMARY

A successful application of high-performance liquid chromatography for analysis of urinary C-peptide is described. Samples (1.0 ml of human urine) were first subjected to gel chromatography to remove interfering substances, and then applied to a reversed-phase column (LiChrosorb RP-18, 7 μm). The detection of C-peptide was performed using a highly specific radioimmunoassay.

With the newly developed techniques, at least four forms of immunoreactive C-peptide were detected in human urine. One of these peptides was indistinguishable from authentic C-peptide. The present study has clearly demonstrated the heterogeneity of urinary C-peptide.

INTRODUCTION

It is now well known that insulin is synthesized as a precursor peptide, proinsulin, which is converted within the pancreatic β-cell to insulin and C-peptide [1]. C-peptide (31 amino acids, molecular weight approximately 3000) is subsequently secreted in equimolar amounts with insulin into the circulation [2], and is largely metabolized by the kidney [3]. Since a fraction of the total C-peptide produced is excreted in the urine, the measurement of urinary C-peptide immunoreactivity (CPR) is useful in assessing β-cell secretory capacity [4, 5]. However, very little information is available at present on the nature of urinary CPR.

High-performance liquid chromatography (HPLC) coupled with radioimmunoassay (RIA) has recently emerged as an excellent analytical tool for many bioactive peptides. This is the first study reporting the successful application of the HPLC—RIA technique for the analysis of urinary C-peptide.

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EXPERIMENTAL

Reagents and standard

All reagents were of analytical grade purity. Acetonitrile (E. Merck, Darmstadt, F.R.G.) was chromatographic quality; water was deionized and double-distilled.

Synthetic human C-peptide (position 33—63 of proinsulin) was kindly provided by Dr. Inoue (Shionogi Research Labs., Fukushima-Ku, Osaka, Japan) [6].

Sample collection

Urine samples were collected for 2 h after meals from healthy volunteers ranging in age from 22 to 72 years. The urine was stored at −20° C; samples were thawed and centrifuged before analysis.

High-performance liquid chromatography

HPLC was performed using a Waters HPLC apparatus (Waters Assoc., Milford, MA, U.S.A.) consisting of two pumps (Model 6000 A and Model M-45), a Model 660 solvent flow programmer, a Model U6K injector, a Model 441 UV detector operating at 214 nm, and a Unicord dual-channel chart recorder (Nippon Denshi Kagaku, Kyoto, Japan). The column (250 × 4 mm) consisted of reversed-phase LiChrosorb RP-18 (7 μm) packing supplied by Kanto Kagaku (Tokyo, Japan). The method of separation is based on the earlier report of Igano et al. [6]. The HPLC mobile phase, consisting of 0.1 *M* sodium phosphate pH 7.0—acetonitrile (81.6:18.4), was eluted isocratically for 20 min followed by a linear gradient of acetonitrile from 18.4% to 80% in 15 min at a flow-rate of 1.0 ml/min.

Radioimmunoassay of human C-peptide

The RIA of human C-peptide was performed using a kit provided by Daiichi Radioisotope Labs. (Tokyo, Japan). The detection limit of this kit is 0.1 ng of C-peptide. This assay system is highly specific for human C-peptide (and its derivatives), and, except for human proinsulin, no other cross-reactive materials are known in human urine [7, 8].

Major CPR peptides (a, b, c and d in Fig. 2) after HPLC were serially diluted and measured by RIA, and the dilution slopes were compared with that of synthetic human C-peptide.

Procedure

All urine samples were subjected to gel chromatography first and then to HPLC. A 1-ml volume of clarified urine was applied to a Bio-Gel P-10 column (100—200 mesh, 40 × 1.5 cm) and eluted with 1 *M* acetic acid at a flow-rate of 0.4 ml/min. Aliquots of each 1.0 ml fraction were lyophilized, reconstituted in water, and assayed for C-peptide. The fractions containing CPR were pooled and lyophilized. The residues were reconstituted in 120 μl of the mobile phase and filtered under centrifugal force through a 0.45-μm filter (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A 90-μl aliquot of the solution was injected into the HPLC column and 1-min fractions (1.0 ml) were collected. A

0.1-ml aliquot of each fraction was lyophilized, reconstituted in water, and assayed for C-peptide.

Recovery

Urinary CPR peptides (a, b, c and d in Fig. 2) after HPLC were desalted separately by a Bio-Gel P-2 column (22 × 1.0 cm, 1 *M* acetic acid), lyophilized, and then known quantities (4—10 ng) of these peptides were subjected to HPLC in the same way as described above. A minimum of three chromatographic runs was performed for each peptide and their recoveries were determined by measuring the corresponding peak areas of CPR.

The significance of differences between means was assessed by Student's *t* test.

RESULTS

A representative gel chromatography pattern of immunoreactive C-peptide from urine is shown in Fig. 1. In all cases investigated, CPR in the eluate was demonstrated in a single peak; the apparent molecular weight for this peak was 3000. The mean recovery of CPR from the gel chromatography was 87.6 ± 4.2% (mean ± S.D., $n = 8$).

After partial purification by gel chromatography, the samples were analysed by HPLC. The results of a typical HPLC run are shown in Fig. 2. Four peaks of CPR were identified with retention times of (a) 4.0 min, (b) 9.0 min, (c) 17.5 min, and (d) 28.0 min. Peak c co-eluted with human C-peptide standard. The levels of CPR present in each peak area were determined and are summarized in Table I together with those of an additional seven samples. The relative amounts of the four peaks were 19.1 ± 1.5% (mean ± S.D.) for peak a, 9.7 ± 1.7% for peak b, 52.4 ± 4.2% for peak c and 14.1 ± 3.7% for peak d. The zones between the peaks contained only trace amounts (0.8—3.7%) of activity. The total recovery of CPR from HPLC was 88.5 ± 4.5% (mean ±

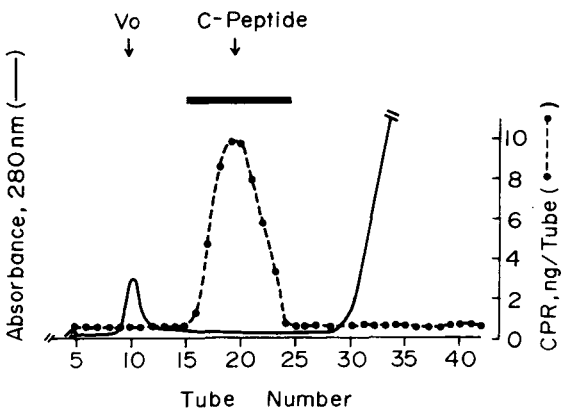

Fig. 1. Representative elution profile of urinary CPR, V_0 = void volume. A 1-ml volume of urine was applied to a Bio-Gel P-10 column (40 × 1.5 cm) and eluted with 1 *M* acetic acid. Aliquots of the fractions were assayed for CPR. The fractions containing CPR were pooled (as marked), lyophilized, and then subjected to HPLC. The column was calibrated with C-peptide standard (molecular weight 3000) as indicated by the arrow.

	(ng)									(%)
		a*	ab*	b*	bc*	c*	cd*	d*	Total	
1	24.3	4.1	0.4	2.3	0	11.4	0	2.6	20.8	85.6
		19.7	1.9	11.1	0	54.8	0	12.5	100	
2	15.7	3.2	0.2	1.5	0	8.7	0	1.9	15.5	98.7
		20.7	1.3	9.7	0	56.1	0	12.3	100	
3	22.6	3.6	1.1	2.1	0.2	11.3	0.1	2.5	20.9	92.5
		17.2	5.3	10.1	1.0	54.1	0.5	12.0	100	
4	21.5	3.3	1.1	1.9	0	10.8	0.1	1.5	18.7	87.0
		17.6	5.9	10.2	0	57.8	0.5	8.0	100	
5	29.4	5.0	0.9	2.5	0.5	12.4	0	3.7	25.0	85.0
		20.0	3.6	10.0	2.0	49.6	0	14.8	100	
6	42.7	7.1	1.6	4.6	0.9	17.5	0.5	5.6	37.8	88.5
		18.8	4.2	12.2	2.4	46.3	1.3	14.8	100	
7	15.7	2.8	0.5	1.0	0.1	6.1	0	2.8	13.3	84.7
		21.1	3.8	7.5	0.8	45.9	0	21.1	100	
8	16.1	2.4	0.5	0.9	0	7.6	0.1	2.4	13.9	86.3
		17.3	3.6	6.5	0	54.7	0.7	17.3	100	
Mean ± S.D. (%)		19.1 ± 1.5	3.7 ± 1.4	9.7 ± 1.7	1.6 ± 0.7	52.4 ± 4.2	0.8 ± 0.3	14.1 ± 3.7		88.5 ± 4.5

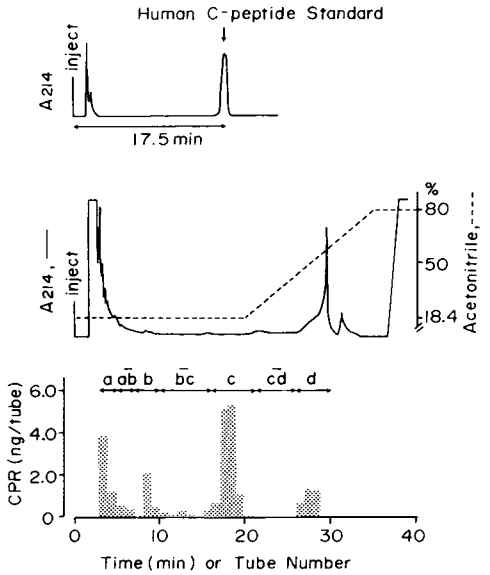

Fig. 2. Representative HPLC of the CPR peptides purified by gel chromatography. Chromatographic conditions: column, LiChrosorb RP-18, 250 × 4 mm; eluents, 18.4% acetonitrile in 0.1 *M* sodium dihydrogen phosphate pH 7.0, isocratic for initial 20 min followed by a linear gradient of acetonitrile from 18.4% to 80% in 15 min and maintained at 80% for an additional 5 min; flow-rate 1.0 ml/min; room temperature. Fractions of 1.0 ml were collected and aliquots used for RIA. The fractions containing CPR (fractions 4—30) were divided into seven zones as indicated: four peak zones (a, b, c and d) and three zones between the peaks ($\overline{ab}$, $\overline{bc}$ and $\overline{cd}$). For comparison, a chromatogram of C-peptide standard is shown in the upper panel.

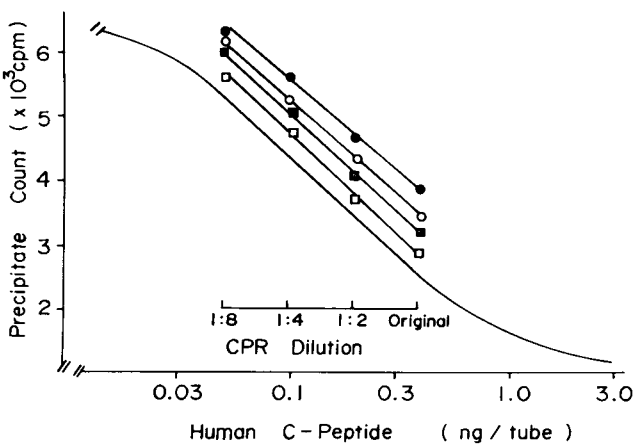

Fig. 3. Standard curves of human C-peptide (—) and dilution slopes of urinary CPR peptides. (○) peak a; (●) peak b; (□) peak c; (■) peak d.

S.D.), and there were no significant differences ($p > 0.05$) in the recoveries of the four CPR peptides from HPLC: 88.4 ± 4.1% (mean ± S.D.), 89.9 ± 2.9%, 86.8 ± 5.6% and 91.7 ± 7.6% for peaks a, b, c and d, respectively.

Serial dilutions of the four CPR peptides of urine were parallel to the displacement curve of synthetic human C-peptide in the RIA (Fig. 3).

TABLE II

REPRODUCIBILITY

	Urinary CPR peptides			
	a*	b*	c*	d*
Intra-assay (n = 4)				
Mean (ng)	5.1	2.7	12.9	4.1
S.D. (ng)	±0.43	±0.25	±0.89	±0.45
C.V. (%)	8.4	9.3	6.9	11.0
Inter-assay (n = 4)				
Mean (ng)	6.8	3.8	18.4	4.1
S.D. (ng)	±0.37	±0.51	±2.4	±0.53
C.V. (%)	5.5	13.5	12.8	13.0

*a, b, c, d are shown in Fig. 2.

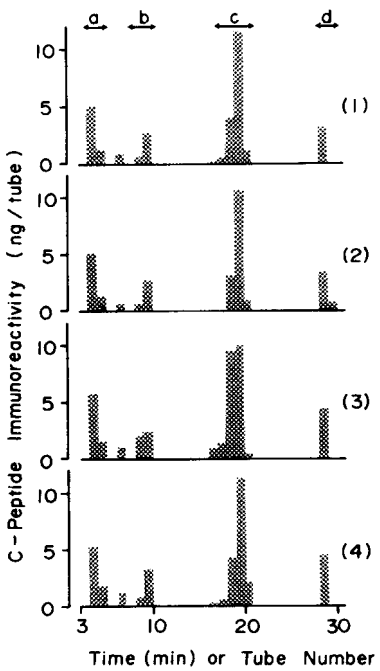

Fig. 4. Effect of sample storage on HPLC pattern of urinary CPR peptides. One urine sample was divided into four equal volumes and analysed on four different occasions: before storage (1) and after storage at −20°C for two days (2), seven days (3) and fourteen days (4). a, b, c, d = peak zones of CPR.

The stability of the HPLC system from day to day was confirmed by checking the retention time of C-peptide standard; the average time was 17.5 ± 0.38 min (mean ± S.D., n = 8). Within-day reproducibility of the method was estimated by carrying out four determinations of one urine sample containing CPR at a concentration of 29.4 ng/ml. Between-day reproducibility was tested by measuring 1.0-ml aliquots of the same sample on four different days over a period of two weeks. These results are shown in Table II and Fig. 4.

DISCUSSION

In the present study, we have described a new application of HPLC coupled with RIA detection for the analysis of urinary C-peptide. Igano et al. [6] reported the successful application of HPLC for the purification of synthetic C-peptide. In their experiment, the retention time of C-peptide standard was about 4 min. Under these conditions, however, the ability of the method to differentiate between authentic C-peptide and potential analogues in urine is questionable. We therefore designed the HPLC conditions to increase the retention time of C-peptide, employing another type of column and lower concentrations of acetonitrile. In this study, the retention time of C-peptide standard was 17.5 min (on average), and the stability of the chromatographic system was extremely high.

For sample purification prior to HPLC analysis, we used gel chromatography. As reported previously by other investigators [4, 9], urinary CPR eluted as a single peak from Bio-Gel P-10 columns in the region corresponding to the C-peptide marker. No immunoreactivity was found in the proinsulin (molecular weight approximately 9000) region of the column. This procedure is simple and rapid in removing proinsulin, if any, and major interfering substances. An additional advantage is the higher recovery of CPR from gel chromatography.

The HPLC system described has demonstrated the excellent separation of urinary CPR peptides. The intra- and inter-assay precision studies have indicated the method to be reliable and reproducible. As shown in Fig. 4, a higher stability for each CPR peptide in urine on storage at -20° C is indicated. Kuzuya et al. [7] reported that there were no changes of CPR concentrations for up to one year.

With the newly developed HPLC method, we have clearly demonstrated the presence of the heterogeneity of urinary C-peptide. In all cases tested, at least four forms of CPR were detected. It was suggested that these four peptides have similar antigenic determinants, but that they are probably not structurally identical because of their different behaviours on HPLC. One of the peptides (peak c in Fig. 2) was chromatographically indistinguishable from authentic C-peptide; the contribution of this peptide to the total CPR was 52.4 ± 4.2%. There were no considerable differences in the recoveries of the CPR peptides from HPLC, suggesting that the relative abundance of the four forms of CPR is independent of the chromatographic procedure.

Gaynor et al. [10] have recently reported the presence of the heterogeneity of urinary C-peptide using polyacrylamide gel electrophoresis. Moreover, Kuzuya and co-workers [11, 12] have indicated heterogeneity of circulating and pancreatic C-peptide. The pathophysiological importance of the heterogeneity of C-peptide remains to be elucidated.

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QUANTITATIVE ANALYSIS OF VERALIPRIDE IN PLASMA AND URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY AND GAS CHROMATOGRAPHY WITH FLAME-IONIZATION DETECTION

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SUMMARY

A highly sensitive and selective quantitative assay for unchanged veralipride has been developed. The compound is extracted from alkalized samples (plasma or urine) with dichloromethane and converted to its trimethylated derivative by reaction with trimethylanilinium hydroxide. The reaction mixture is then chromatographed on a 3% OV-1 column. Trimethylated derivatives of plasma samples were assayed by selected-ion monitoring in the chemical-ionization mode and quantified by comparing the intensity of the quasi-molecular ion *m/z* 426 (M + H) with the intensity of the corresponding ion from trideuterated internal standard, *m/z* 429 (M + H). Flame-ionization detection was used for the assay of urine samples. The peak height ratio of trimethylated veralipride over trimethylated sulpiride, the internal standard, was used for quantitation of urine samples. A relative standard deviation of less than 10% was found when quantifying 10 ng/ml veralipride in plasma or 1 μg/ml in urine.

INTRODUCTION

Veralipride is a new non-hormonal non-steroidal agent displaying antidopaminergic and antigonadotropic acitivity [1—3]. It is used as regulator of sudden elevations in body temperature and other physiological disturbances associated with menopause. Veralipride is classified in the *o*-methoxybenzamide family because of its structural analogy.

The present paper deals with the quantitative analysis of veralipride in human biological fluids, plasma and urine, after oral or intravenous administration. Gas chromatography (GC)—mass spectrometry (MS) and GC with flame-ionization detection (FID) were used for the quantitative analysis of veralipride

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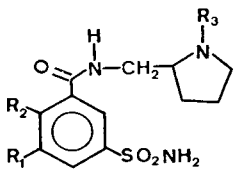
in plasma and urine, respectively. Internal standards were veralipride-d_3 for plasma samples and sulpiride for urine samples.

EXPERIMENTAL

Chemicals

Veralipride, [N-(1-allyl-2-pyrrolidinyl-methyl)] 2,3-dimethoxy-5-sulfamoyl benzamide, and sulpiride (Fig. 1) were supplied by Delagrange (Paris, France). Veralipride-d_3 [4, 5] was synthesized in our laboratory by a slight modification of the manufacturer's method [4]. The C^2H_3 was introduced on the methoxy group in the 2 position (Fig. 1).



	R_1	R_2	R_3
Veralipride	OCH_3	OCH_3	CH_2—CH=CH_2
Sulpiride	H	OCH_3	CH_2—CH_3
Veralipride-d_3	OCH_3	OC^2H_3	CH_2—CH=CH_2

Fig. 1. Chemical structures of veralipride, sulpiride and veralipride-OC^2H_3.

Trimethylanilinium hydroxide [6, 7] (Methelute) was obtained from Pierce (Spiral, Dijon, France). All solvents and chemicals were of analytical grade and supplied by Merck (Darmstadt, F.R.G.).

Instrumentation

The gas chromatograph—mass spectrometer was a Ribermag R10-10 (Delsi, Rueil-Malmaison, France) equipped with a 2.1-m glass column (2 mm I.D.) packed with 3% OV-1 on Chromosorb W AW DMCS 80—100 mesh (Spiral). Helium was used as carrier gas at a flow-rate of 20 ml/min. Chromatograph oven, injector block and interface temperatures were set at 290°C. The ion source temperature was about 100°C. Mass spectra were recorded at an electron energy of 70 eV in both electron-impact (EI) and chemical-ionization (CI) modes. Data acquisition and reduction were done with the Nermag Sidar computing system. Quantitative analyses of plasma samples were performed on the system described above by selected-ion monitoring at *m/z* 426 and *m/z* 429 in ammonia-CI-MS, including peak area calculations.

A Girdel Series 30 gas chromatograph (Suresnes, France) equipped with a flame-ionization detector was used for urine samples. For the chromatographic separations, a 2.7-m glass column (2 mm I.D.) packed with 3% OV-1 on Chromosorb W AW DMCS, 80—100 mesh, was used. Nitrogen was used as a carrier gas at a flow-rate of 30 ml/min. The column oven temperature was 270°C; injector block and detector temperatures were 300°C and 290°C, respectively. Flow-rates of hydrogen and oxygen used for the detector were 25 and 370 ml/min, respectively.

Glassware

Extraction and derivatization tubes were washed with RBS R25 (Fluka, Basle, Switzerland), rinsed sufficiently with deionized water and dried at 100°C. Pipettes, micropipettes and other labware were of disposable glassware.

Internal standards and standard solutions

Veralipride-d_3, 10 μg/ml in methanol, was used as internal standard for the plasma sample; 50 μl were mixed with plasma to obtain a final concentration of 500 ng/ml of plasma. For urine analyses, sulpiride was used as the internal standard at a concentration of 200 μg/ml in dichloromethane and 50 μl of this solution were mixed with each urine sample to give a final concentration of 10 μg/ml. Standard calibration curves were obtained by enrichment of blank plasma with 10, 20, 50, 100, 250, 500, 1000 and 1500 ng/ml veralipride and 500 ng/ml veralipride-d_3. For urine analyses blank urine was enriched with 1, 2.5, 5, 7.5, 10, 25, 50, 75 and 100 μg/ml veralipride and 10 μg/ml sulpiride as internal standard.

Analytical procedure

A 50-μl volume of internal standard solution was introduced into a 10-ml tube fitted with a PTFE-lined screw cap; after evaporation of the organic solvent, 1 ml of plasma or urine was first added and then 0.5 ml of sodium carbonate buffer (0.5 *M*, pH 9.5) for alkalization. The mixture was extracted with 4 ml of dichloromethane. After shaking for 3 min and centrifugation, the organic phase was transferred to a 4-ml tube (fitted with a PTFE-lined open screw cap). The extraction of the alkaline aqueous phase was repeated with 4 ml of dichloromethane. Organic phases were combined and evaporated to dryness under a flow of nitrogen. After addition of 50 μl of trimethylanilinium hydroxide the reaction mixture was allowed to stand for 5—10 min at room temperature; 1—3 μl of the mixture were injected into the gas chromatograph.

RESULTS AND DISCUSSION

Plasma analysis and quantitation

The pK_a of veralipride, determined by potentiometric titration, was 7.9 ± 0.0076. The solubilities of veralipride in some common solvents are listed in Table I. The percentage of veralipride base extracted by chloroform as a function of pH is given in Table II. The extracted veralipride was measured by spectrophotometry at 291.7 nm for 10^{-3} *M* solutions. It can be seen that veralipride is quantitatively extracted at pH > 9.

TABLE I

SOLUBILITY OF VERALIPRIDE BASE IN VARIOUS SOLVENTS

Solvent	Solubility (g per 100 ml at 20°C)
Acetone	18.6
Chloroform	18.9
Cyclohexane	0.009
Dichloromethane	36.3
Ethanol	7.3
Methanol	18.7
Toluene	0.05

TABLE II

PERCENTAGE OF VERALIPRIDE BASE EXTRACTED BY CHLOROFORM AT DIFFERENT pH VALUES

pH	Veralipride base extracted (%)	pH	Veralipride base extracted (%)
7.0	28.5	9.6	100.9
8.0	48.7	10.0	99.3
8.4	79.3	10.4	98.2
8.8	92.4	10.8	100.4
9.0	98.7	11.0	97.3
9.2	98.6		

A study of the stability and reproducibility of the overall GC—MS method was made. Three samples of different concentration were extracted and analysed in a series of multiple consecutive injections. The results obtained, 2146.8 (± 25.7) ng/ml, coefficient of variation (C.V.) 1.2% (n = 5), 433.8 (± 4.5) ng/ml, C.V. 1.0% (n = 10) and 41.4 (± 0.5) ng/ml, C.V. 1% (n = 5), showed good reproducibility for the GC—MS method and good stability of the trimethylated derivatives of veralipride.

The integrated analytical procedure with extraction and derivatization was controlled using two blank plasmas spiked with veralipride and the internal standard; ten aliquots were extracted. After derivatization the samples were analysed by GC—MS. Results obtained gave 7% variation for 50 ng/ml samples and 3.9% for 1000 ng/ml samples.

The isotopic purity of veralipride-d_3 was verified to be 95.5%. Thus 4.5% of unlabelled compound remained (Table III). This quantity corresponded to 22.5 ng of veralipride for 500 ng of internal standard used per ml of plasma sample.

This result permitted us to calculate the extraction yield of veralipride from plasma using dichloromethane. This was determined by spiking 1-ml plasma samples with four different concentrations of veralipride (Table IV). After the extraction 500 ng/ml internal standard was added and the samples were

TABLE III

DETERMINATION OF THE QUANTITY OF VERALIPRIDE CONTAINED IN THE VERALIPRIDE-d_3

Injection	Quantity of veralipride in the veralipride-d_3, 500 ng/ml	
	Peak area ratio	%
1	0.044	4.4
2	0.045	4.5
3	0.046	4.6
4	0.046	4.6
5	0.045	4.5
Mean	0.045	4.5
S.D.	0.0008	0.08
C.V. (%)	1.85	1.85

TABLE IV

RECOVERY OF VERALIPRIDE EXTRACTED FROM PLASMA BY DICHLOROMETHANE

Quantity (ng/ml)	Measured quantity (mean ± S.D.)	C.V. (%) (n = 5)	Theoretically expected quantity	Extraction yield (%)
10	17.05 ± 2.45	70.5	32.5	50.4
25	28.75 ± 4.17	12.0	47.5	60.4
50	66.6 ± 6.5	13.2	72.5	91.9
1000	1032.9 ± 35.1	3.5	1022.5	101.0

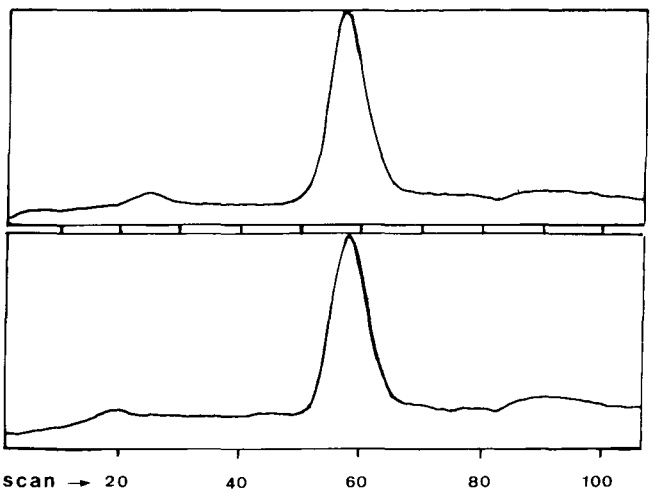


Fig. 2. Reconstructed mass chromatogram of ions m/z 426.3 (bottom) and 429.3 (top) of trimethylated veralipride (retention time 2 min 9 sec) and veralipride-d_3 (internal standard, retention time 2 min 8 sec), respectively, using a 3% OV-1 packed column.

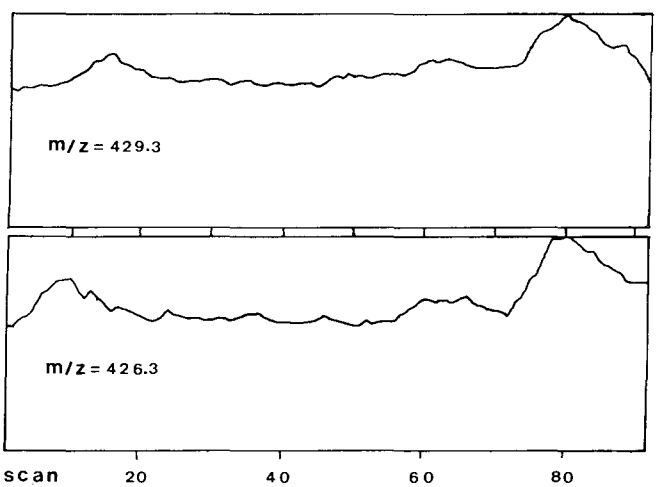


Fig. 3. Reconstructed mass chromatogram of ions m/z 426.3 and 429.3 of a blank plasma without internal standard because of the residual veralipride contained in the veralipride-d_3. No contamination from plasma or reagents interfered in the analysis.

analysed. The difference between the measured quantities and those theoretically expected permitted calculation of the extraction efficiency (Table IV). The apparent over-estimation of the low-concentration samples was compensated by the calibration curves established daily in the same conditions as the unknown samples. In other words, a standard small quantity of veralipride was systematically introduced in all samples and standards. A good linearity was observed in the range 10—1500 ng/ml of plasma. The equation was $y = ax + b$ where $a = 1$ (± 0.03) and $b = 2.97$ (± 1.7) for $n = 30$ with $r > 0.999$.

Fig. 2 shows a reconstructed ion chromatograph of veralipride and veralipride-d_3. This method allowed the quantification of about 10 ng/ml veralipride in plasma with a precision better than 10%. No contamination interfered with the analysis, as is shown by a blank obtained without veralipride and internal standard (Fig. 3).

Urine analysis and quantitation

Urine samples were analysed and quantified by GC—FID using sulpiride as the internal standard. The reproducibility tested by repeated injections of samples of two different concentrations (2.7 ± 0.18 μg/ml, C.V. 6.9%, $n = 10$, and 23.75 ± 0.15 μg/ml, C.V. 0.6%, $n = 10$) was satisfactory. Repeated extractions and analyses demonstrated good stability of the overall process (Table V). Extraction yield was about 50% for low-concentration samples and rapidly increased to ± 80% (Table VI) at around 20 μg/ml. Therefore no attempt was made to improve the extraction yields because large quantities of veralipride were found in urine and good signals were obtained. For quantitation peak height ratios of trimethylated veralipride over trimethylated sulpiride have been used instead of peak area measurement since the results were found to be

TABLE V

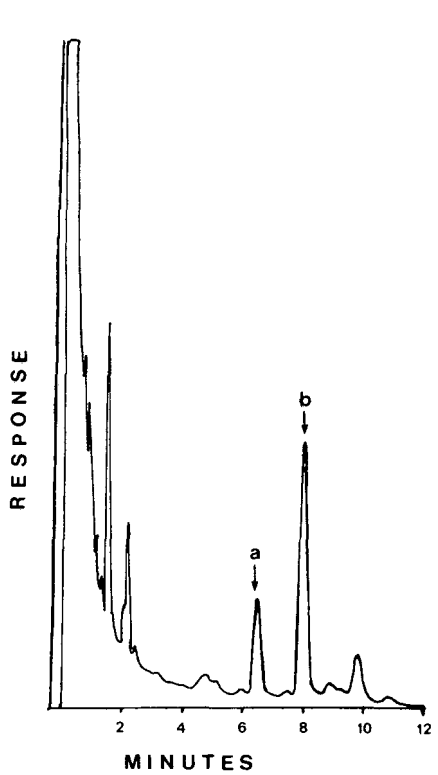
REPRODUCIBILITY OF THE EXTRACTION AND DERIVATIZATION PROCEDURE AND THE GC—FID QUANTITATION OF VERALIPRIDE IN URINE

Quantity (μg/ml)	Recovered (mean ± S.D.)	C.V. (%) ($n = 10$)
2.6	2.35 ± 0.08	3.6
7.78	7.43 ± 0.33	3.0
25.95	23.95 ± 0.55	2.3

TABLE VI

RECOVERY OF VERALIPRIDE EXTRACTED FROM URINE BY DICHLOROMETHANE USING SULPIRIDE AS EXTERNAL STANDARD

Quantity (μg/ml)	Measured (mean ± S.D.)	C.V. (%) ($n = 5$)	Extraction yield (%)
2.59	1.29 ± 0.069	5.3	50
7.78	5.17 ± 0.34	6.5	66.5
25.9	21.70 ± 2.91	13.3	83.8



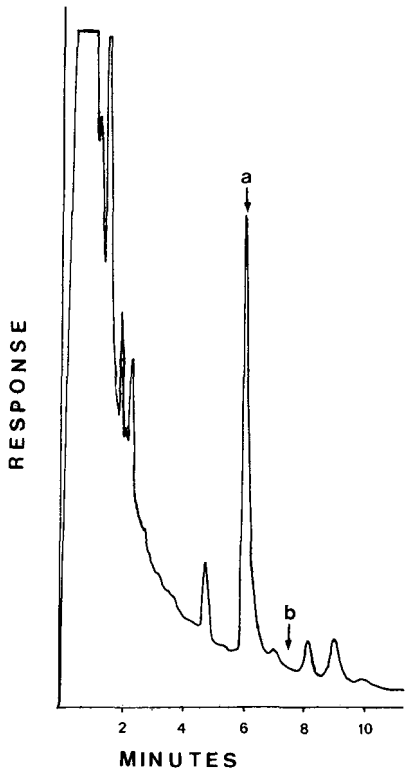


Fig. 4. Chromatogram obtained after GC—FID analysis, on a 3% OV-1 column, of urinary veralipride (b) using sulpiride (a) as internal standard.

Fig. 5. Chromatogram of a blank urine sample. Same experimental conditions as described in Fig. 4. a = Sulpiride; b = veralipride.

identical. Good chromatographic separation has been obtained in these conditions (Fig. 4) without any interferences (Fig. 5).

Calibration curves were established periodically ($y = 1.08x - 0.95$, $r = 0.998$) and gave good linearity in the range 1—100 μg/ml of urine. Standards with known quantities were injected daily to check the accuracy of the method.

Blood and urinary levels

The methods discussed above were used to determine blood and urine concentrations of unchanged veralipride in man after a single intravenous infusion or single oral administration. Two different oral dosage forms were administered: hard gelatine capsules and an oral solution. This paper reports typical biological data which illustrate the analytical method. Plasma concentrations obtained after a 30-min, 100-mg intravenous infusion of veralipride are shown in Fig. 6. The theoretical curves given by computerized calculation were close to the experimental points. Similar results have been obtained for all twelve volunteers participating in this study. Fig. 7 shows a typical curve obtained after analysis of unchanged veralipride excreted in urine after intravenous or oral administration. Different plasma concentration curves were

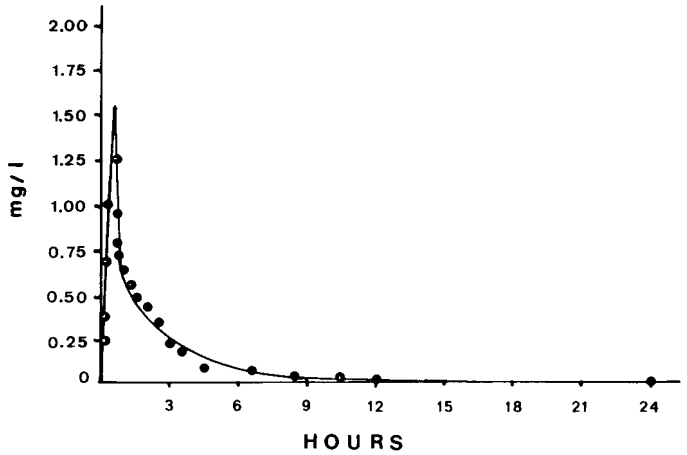


Fig. 6. Veralipride plasma levels after a 100-mg, 30-min intravenous infusion.

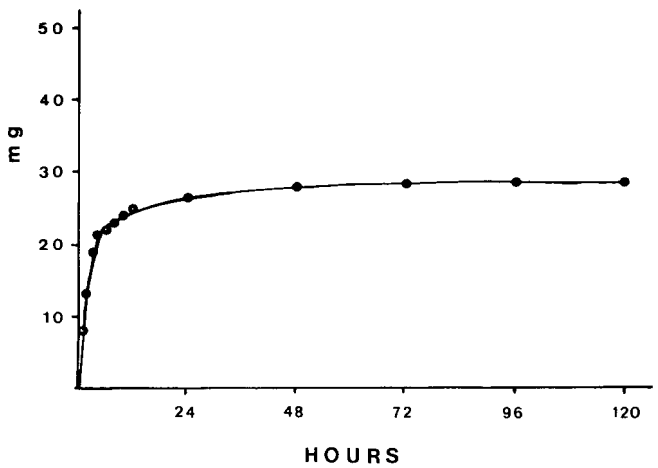


Fig. 7. Cumulated amounts of unchanged veralipride after a 100-mg, 30-min intravenous infusion. The general aspect of the curves after oral administration was similar except that amounts of eliminated veralipride were different.

obtained after oral administration. Fig. 8 shows curves obtained from one volunteer after administration of four single doses of veralipride as an oral solution. Two peaks of maximum concentration appear for the three doses. This phenomenon was observed regularly when this dosage was administered. It was proved to be due to distribution of veralipride in plasma and not to analytical error since it was constantly observed when repeating the same analysis at different dates and with different operators. Samples were injected into the GC—MS system in various working conditions. The same results were always obtained with less than 10% standard error. On the other hand, no double maximum concentration peaks were observed when veralipride was administered in 100-mg hard gelatine capsules (Fig. 9). Only two volunteers among twelve presented a slight rebound effect.

The analytical assays described above permitted successful analysis of

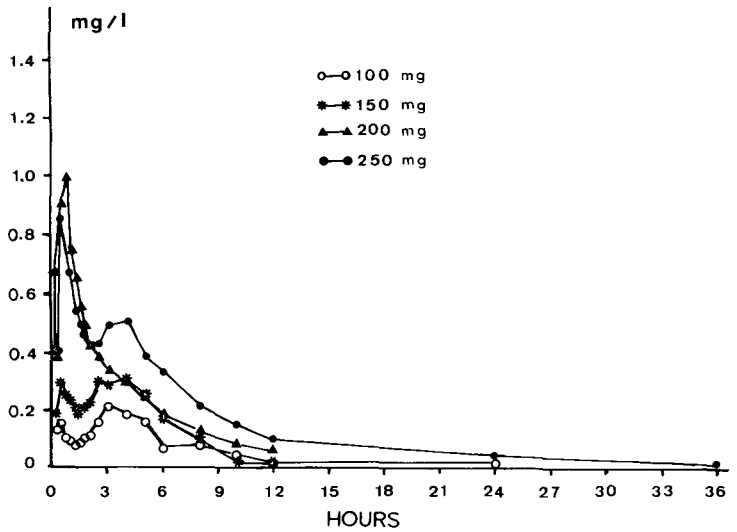


Fig. 8. Plasma levels after single oral administration of various doses of veralipride to one volunteer.

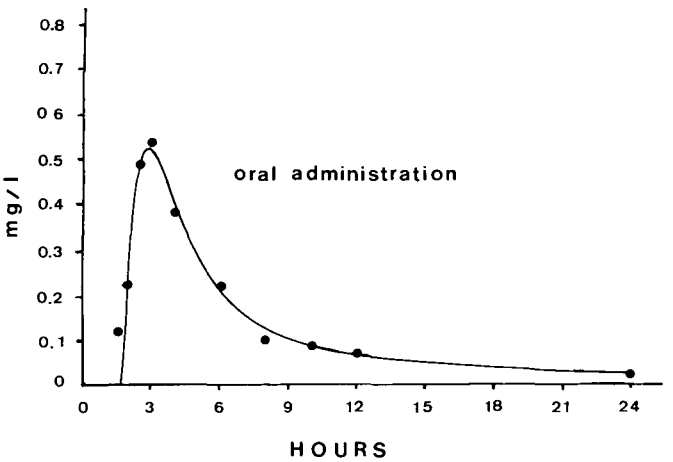


Fig. 9. Plasma levels after oral administration of hard gelatine capsules containing 100 mg of veralipride.

unchanged veralipride in human plasma and urine. This method has been used to establish the pharmacokinetic parameters of veralipride in man and its bioavailability from two oral dosage forms [8, 9].

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ANALYSIS OF BUPRENORPHINE AND ITS N-DEALKYLATED METABOLITE IN PLASMA AND URINE BY SELECTED-ION MONITORING

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SUMMARY

A selected-ion monitoring method was developed for determination of buprenorphine and its N-dealkylated metabolite (norbuprenorphine) in human plasma and urine. N-Propylnorbuprenorphine was added as internal standard to 2—3 ml of sample and the alkaloids were extracted with toluene—2-butanol at pH 9.4. After back-extraction in dilute sulphuric acid, the compounds were heated at 110°C. This procedure led to quantitative loss of methanol followed by ring formation between the 6-methoxy group and the branched side-chain of all compounds. The derivatives were extracted into dichloromethane—2-butanol and treated with pentafluoropropionic anhydride. The resulting derivatives were suitable for selected-ion monitoring analysis. The coefficient of variation was found to be 4.5% at 5 ng/ml and 8.9% at 50 ng/ml in urine. The corresponding values for plasma were 6.2% and 5.3%, respectively.

The lower limit of detection in plasma was 150 pg/ml, permitting analysis of plasma levels of buprenorphine for 24 h and urine levels of buprenorphine and norbuprenorphine for more than seven days after a therapeutic dose of buprenorphine. This method is the first with sufficient specificity and sensitivity for characterization of the clinical pharmacokinetics of buprenorphine.

INTRODUCTION

Buprenorphine, N-cyclopropylmethyl-7α-[1-(*S*)-hydroxy-1,2,2-trimethyl-

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propyl]-6,14-*endo*-ethano-6,7,8,14-tetrahydronororipavine, is a long-acting opiate analgesic of the oripavine series [1]. It is highly potent with a typical dose range of 0.3—0.6 mg (0.005—0.01 mg/kg) for parenteral administration. Studies on its pharmacokinetics have been rendered difficult by the low blood concentrations attained and lack of sufficiently specific and sensitive methods. Lloyd-Jones et al. [2] used a selected-ion monitoring method and were able to measure levels above 20 ng/ml attained by the injection of 5 mg/kg in baboons. For use in humans a sensitive radioimmunoassay has been developed [3]. Using this technique Bullingham and co-workers [4—7] have studied the single-dose kinetics and bioavailability of intravenous, intramuscular and sublingual buprenorphine. The sensitivity of the method was high, down to 50 pg/ml, but with possibilities of cross-reaction either with the glucuronide conjugate (serum L30) or with the N-dealkylated metabolite (serum L31). It is therefore uncertain to what extent metabolites of buprenorphine can have contributed to the measured blood levels.

The present communication describes a new selected-ion monitoring procedure for the determination of both buprenorphine and its N-dealkylated metabolite. The method which is based on a crucial chemical degradation step has a sensitivity with a lower limit in plasma and urine of about 150 pg/ml.

EXPERIMENTAL

Materials

Buprenorphine hydrochloride, norbuprenorphine hydrochloride, N-propyl-norbuprenorphine hydrochloride (internal standard) and the acid rearrangement product, RX 2001 M, were gifts from Reckitt and Colman, Pharmaceutical Division, Hull, U.K., through the courtesy of Dr. G. Lloyd-Jones. Pentafluoropropionic anhydride (PFPA) was obtained from Reagenta, Uppsala, Sweden. All other chemicals and solvents were of analytical grade, available through ordinary commercial channels.

Carbonate buffer (pH 9.4) was prepared by titrating a saturated solution of sodium bicarbonate with a saturated solution of sodium carbonate to pH 9.4.

Thin-layer chromatography (TLC) was conducted on silica gel F_{254} plates (E. Merck, Darmstadt, F.R.G.). The solvent system used was chloroform—diethyl ether—ammonia (75:25:1).

Analysis in plasma

The internal standard (20 or 40 ng) and 1 ml of carbonate buffer (pH 9.4) were added to 2.0 ml of plasma in a 15-ml test tube. After addition of 6 ml of toluene containing 20% (v/v) of 2-butanol, the tube was extracted for 15 min using a mechanical shaker. The two phases were separated by centrifugation; the upper organic layer was transferred to a new tube containing 1 ml of 0.05 *M* sulphuric acid. After extraction and centrifugation the organic phase was discarded and the aqueous phase was heated in a 110°C oil-bath for 1 h. The aqueous phase was then made alkaline with 0.5 ml of carbonate buffer, and extracted with 4 ml of dichloromethane containing 20% (v/v) of 2-butanol. The sample was centrifuged, the aqueous phase discarded and the organic phase

poured into a clean tube and evaporated to dryness under a stream of nitrogen at 60° C.

The pentafluoropropionyl (PFP) derivatives were prepared by addition of 100 μl of PFPA. The tubes were then allowed to stand for 15 min at 70° C before the excess PFPA was evaporated by a stream of nitrogen at 60° C. The residue was dissolved in 50 μl of chloroform and the contents of the tube were thoroughly mixed. Then 1—5 μl of this mixture was injected for gas chromatography—mass spectrometry (GC—MS).

The concentrations of buprenorphine and norbuprenorphine were evaluated using a calibration graph established by the use of five standard samples carried through the same procedure.

Analysis in urine

For the determination of free buprenorphine and norbuprenorphine, 20 or 40 ng of the internal standard were added to 3.0 ml of urine, and the sample was alkalinized by the addition of 1 ml of carbonate buffer (pH 9.4) and extracted with 8 ml of toluene—2-butanol (8:2). The organic layer was transferred to a tube containing 1 ml of sulphuric acid (0.05 *M*) and the subsequent extraction steps were then carried out as described above for plasma.

When the total (free and conjugated) amount of buprenorphine and norbuprenorphine was determined, the internal standard was added to 3.0 ml of urine, followed by addition of concentrated hydrochloric acid to a final concentration of 2 *M*. The tube was then heated to 110° C for 1 h, alkalinized with 300 μl of ammonia and 1 ml of carbonate buffer (pH 9.4) and the drugs were extracted with 8 ml of the toluene—2-butanol mixture. The same extraction steps were then performed as described above with the exception that the heating was omitted after the back-extraction step.

Mass spectrometric conditions

The GC—MS analysis was performed on a Finnigan 4000 gas chromatograph—mass spectrometer equipped with a fused-silica OV-1701 capillary column (Orion, 25 m × 0.3 mm I.D.). Helium was used as carrier gas, and column temperature was 280° C.

The injection system used was of the solventless type. A droplet (1—5 μl) of the sample was transferred to the tip of a movable glass needle, and after evaporation of the solvent the needle with the sample was moved down to the column.

The mass spectrometer was operated in the electron-impact mode with the electron emission current at 0.3 A and the energy of the electrons at 40 eV. The electron multiplier voltage was set at 2.5 kV. All GC—MS data were collected, stored and processed using an Incos 2300 Data System.

RESULTS AND DISCUSSION

Recovery

The recovery of buprenorphine was determined using ^{3}H-labelled buprenorphine. The yield of this compound through the extraction procedure was 30%. Silanization of all glassware did not improve the recovery.

Chemical degradation

When we first considered the development of a gas chromatographic assay we decided that a chemical modification of the molecule would be desirable to reduce problems with adsorption and resultant low sensitivity. A mild acid hydrolysis of buprenorphine, norbuprenorphine and N-propylnorbuprenorphine gave a quantitative loss of methanol followed by ring formation between the side-chain and the methoxy group as shown for buprenorphine in Fig. 1. That the degradation gave a single product was shown by TLC (Fig. 2).

Gas chromatography of pentafluoroacylated derivatives of the degradation products gave for each compound a single peak. The structure of these compounds could be deduced from the mass spectra (Fig. 3A—C). For the buprenorphine derivative, the molecular ion at *m/z* 581 indicates that loss of 32 corresponding to methanol had occurred from the parent compound. Major fragments of *m/z* 540 (loss of cyclopropyl) and 497 (loss of part of the furano ring) supported the structure shown in Fig. 3A.

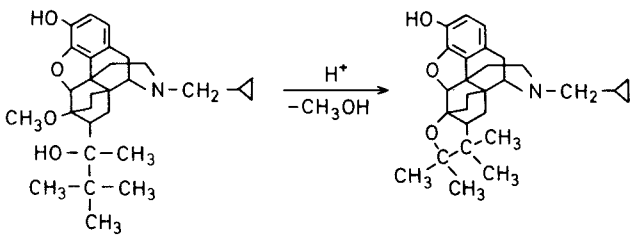

Fig. 1. Chemical degradation of the buprenorphine molecule following mild acid hydrolysis. The result is quantitative loss of methanol and ring formation between the 6-methoxy group and the side-chain. The same degradation also occurs for norbuprenorphine and N-propylnorbuprenorphine upon treatment with dilute acid.

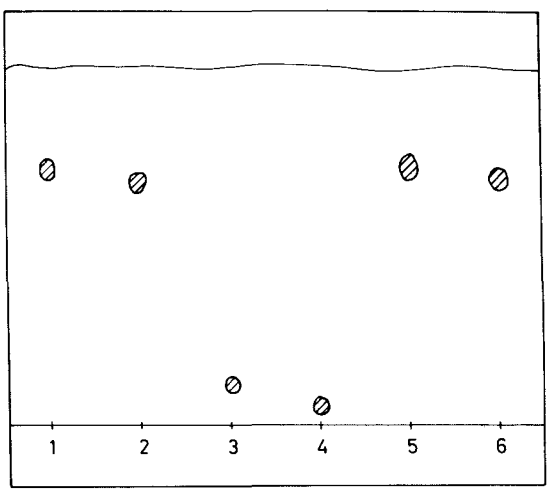

Fig. 2. Thin-layer chromatogram showing (1) buprenorphine, (2) degradation product of buprenorphine, (3) norbuprenorphine, (4) degradation product of norbuprenorphine, (5) internal standard, and (6) degradation product of internal standard. Each compound gives a single spot, indicating that the degradation step is quantitative.

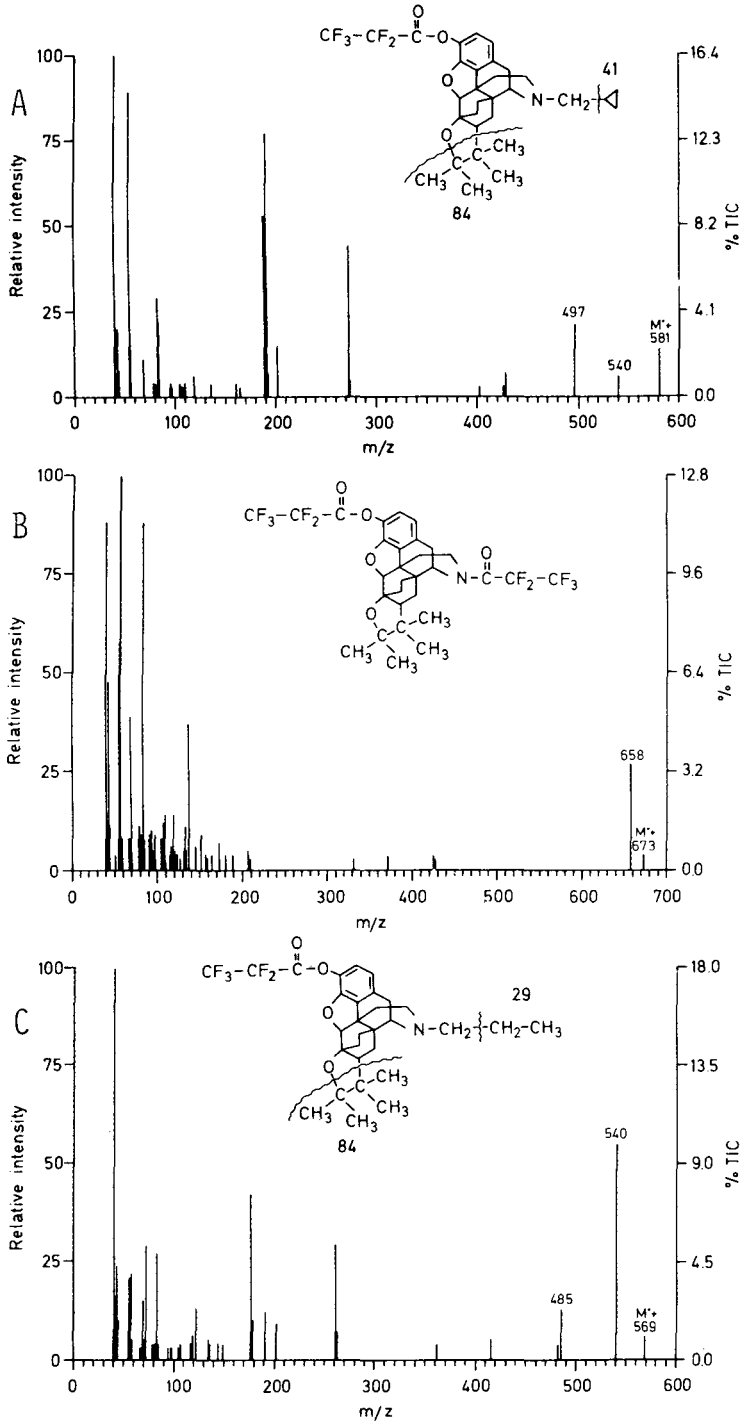

Fig. 3. Mass spectra of the pentafluoroacylated derivatives of the degradation products of (A) buprenorphine, (B) norbuprenorphine and (C) N-propylnorbuprenorphine (internal standard).

Comparison between the buprenorphine acid rearrangement product, RX 2001 M (supplied by Reckitt and Colman), and the degradation product obtained in our laboratory upon treatment of buprenorphine with dilute acid, showed coincident retention times for the two peaks with gas chromatography, as well as identical mass spectra and identical R_F values upon TLC analysis. Thus the structure of the degradation products of buprenorphine was identical to that of RX 2001 M.

For norbuprenorphine a similar structure could be proposed (Fig. 3B). Usefully a considerable portion of the ion yield was found at *m/z* 658, presumably appearing through loss of one methyl group from the molecular ion.

N-Propylnorbuprenorphine was converted to a similar derivative by the acid treatment. The mass spectrum of the PFP derivative is shown in Fig. 3C. The base peak at *m/z* 540 is presumably formed by loss of ethyl (29) by cleavage β to the nitrogen.

These mass spectra provide strong evidence for quantitative removal of methanol in all three compounds with the formation of a furano ring between the oxygen function at carbon-6 and the side-chain. Furthermore, the mass spectra of the PFP derivatives revealed strong peaks in the high mass region suitable for focusing with selected-ion monitoring (mass fragmentography). Accordingly, the fragments chosen for the further analytical work were *m/z* 581 for buprenorphine, *m/z* 658 for norbuprenorphine and *m/z* 569 for N-propyl-norbuprenorphine.

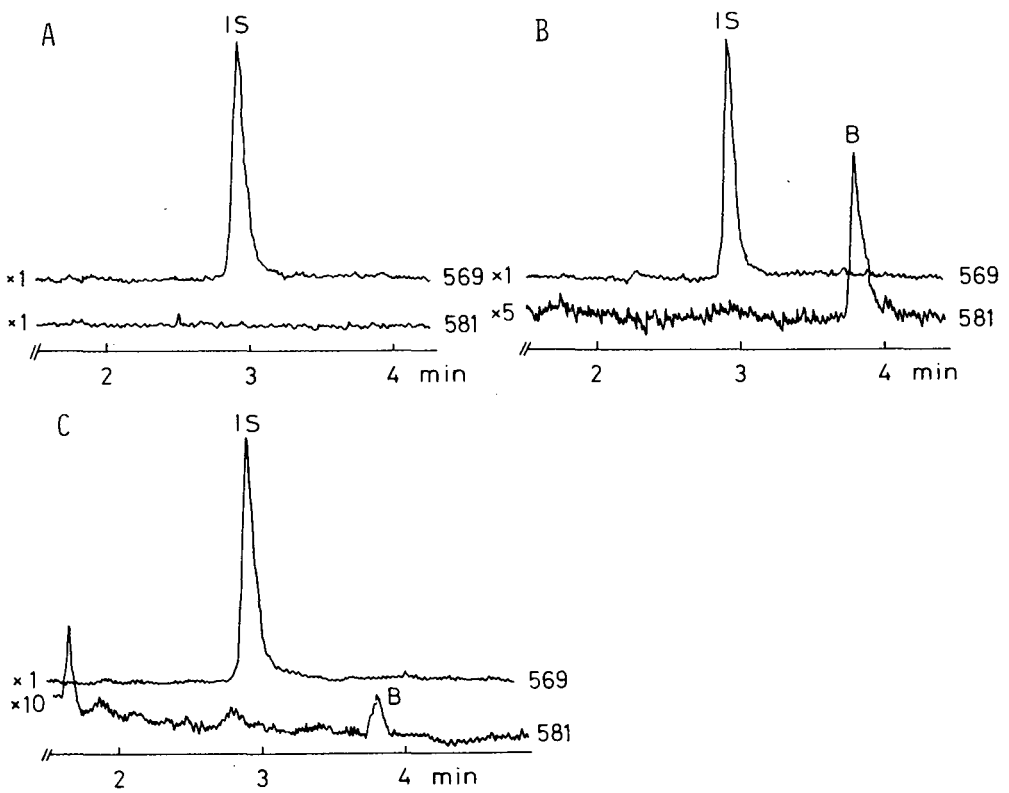

Fig. 4.

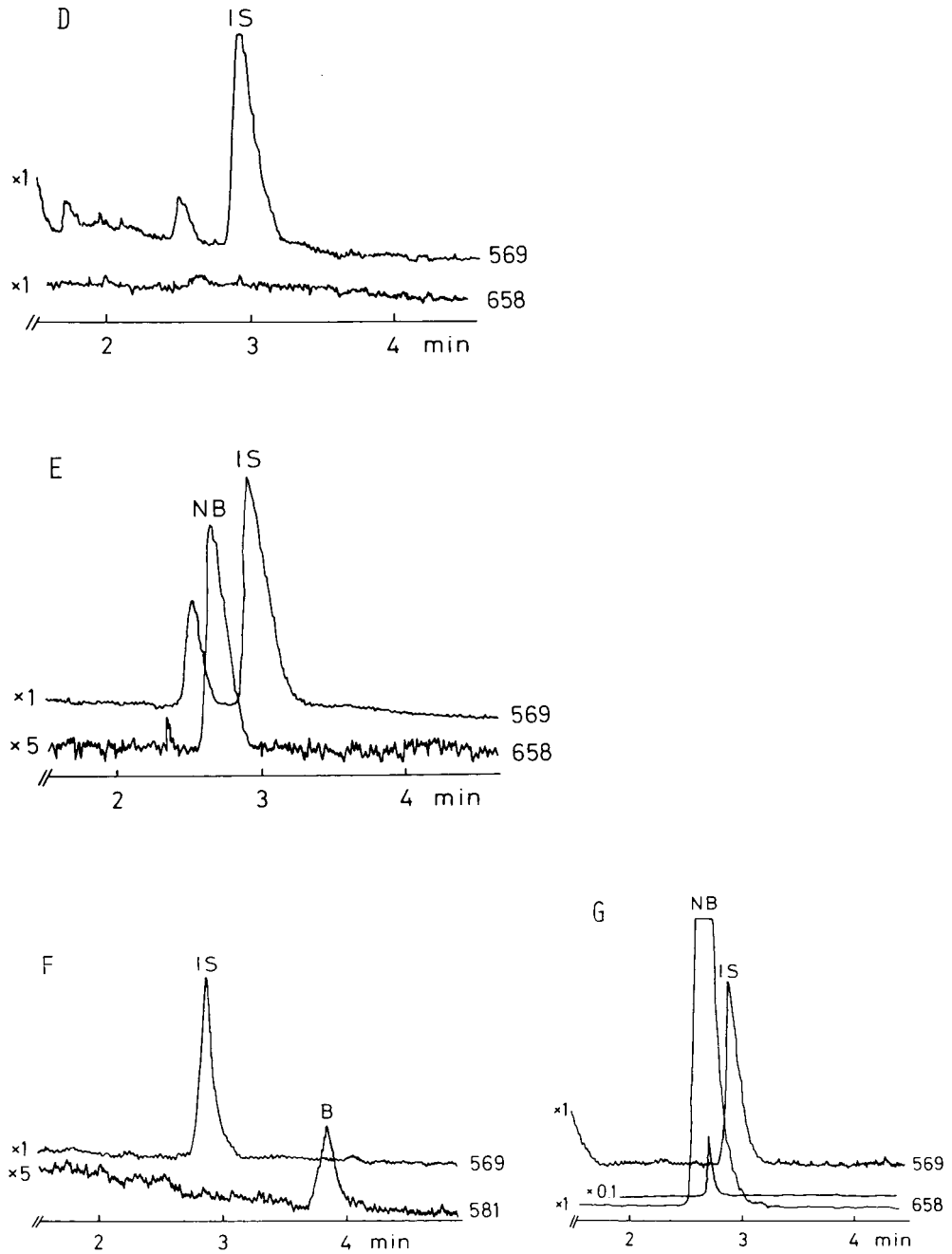

Fig. 4. Selected-ion monitoring profiles obtained from the assay of buprenorphine (B) and norbuprenorphine (NB) after intravenous administration of 0.6 mg of buprenorphine to a healthy volunteer. IS = internal standard. (A) Plasma blank, buprenorphine; (B) buprenorphine in plasma, 1 h; (C) buprenorphine in plasma, 10 h; (D) plasma blank, norbuprenorphine; (E) norbuprenorphine in plasma, 1 h; (F) buprenorphine in urine, four days; (G) norbuprenorphine in urine, four days.

Analysis in plasma

The mass fragmentograms of blank plasma revealed no major background at *m/z* 581 (Fig. 4A). One hour after intravenous injection of 0.6 mg of buprenorphine to a healthy volunteer, a strong peak was observed with a retention time corresponding to buprenorphine (Fig. 4B). Significant peaks of buprenorphine were observed even after 10 h (Fig. 4C) and 24 h. A plasma elimination curve for buprenorphine is shown in Fig. 5.

The buprenorphine derivative gave a very sensitive signal permitting analysis with a lower limit of 150 pg/ml. Norbuprenorphine was found to be present in plasma for a few hours after the injection of buprenorphine (Fig. 4E).

To determine the precision of the assay, buprenorphine was added to normal plasma, which was then divided into six equal samples and analysed separately. When the added buprenorphine was 50 ng/ml, the coefficient of variation was 5.3%, while at the 5 ng/ml level it was 6.2%.

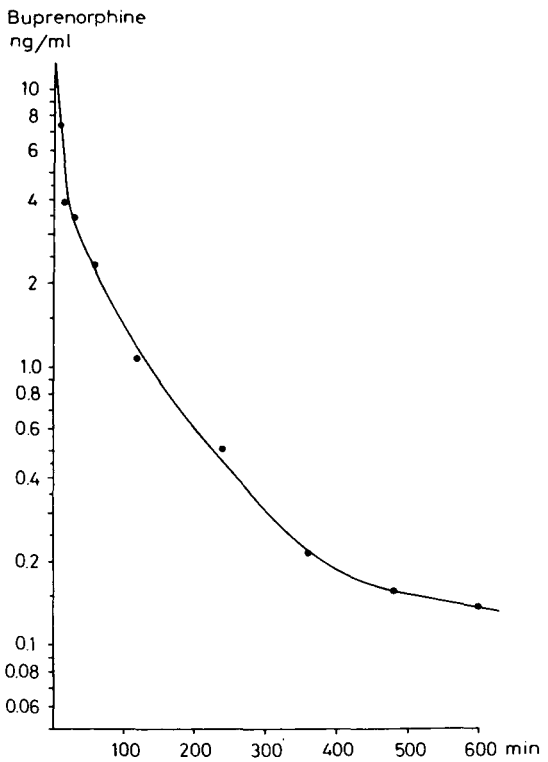

Fig. 5. Plasma elimination curve for buprenorphine after administration of 0.6 mg intravenously to a healthy volunteer.

Analysis in urine

In previous studies in rats, the urinary excretion products of buprenorphine were dominated by conjugated material, mainly the glucuronide conjugate [8]. We therefore decided to analyse both free and conjugated drug in urine. The total amount of buprenorphine in urine was determined after hydrolysis of the conjugates with hydrochloric acid, while the amount of free drug was

determined without prior treatment with the acid. The amount of conjugated buprenorphine in urine was calculated as the difference between total and free drug.

After injection of 0.6 mg of buprenorphine, both norbuprenorphine and buprenorphine were excreted in urine for at least seven days (data not shown). The selected-ion monitoring profiles of buprenorphine and norbuprenorphine four days after injection are shown in Fig. 4F and G, respectively.

The cumulative amounts of both buprenorphine and norbuprenorphine excreted over four days in one subject are shown in Fig. 6.

The precision of the assay was determined by adding buprenorphine to normal human urine, which was divided into six equal samples and analysed separately. The concentration of buprenorphine in each sample was 50 or 5 ng/ml. The coefficients of variation at these levels were 8.9% and 4.5%, respectively.

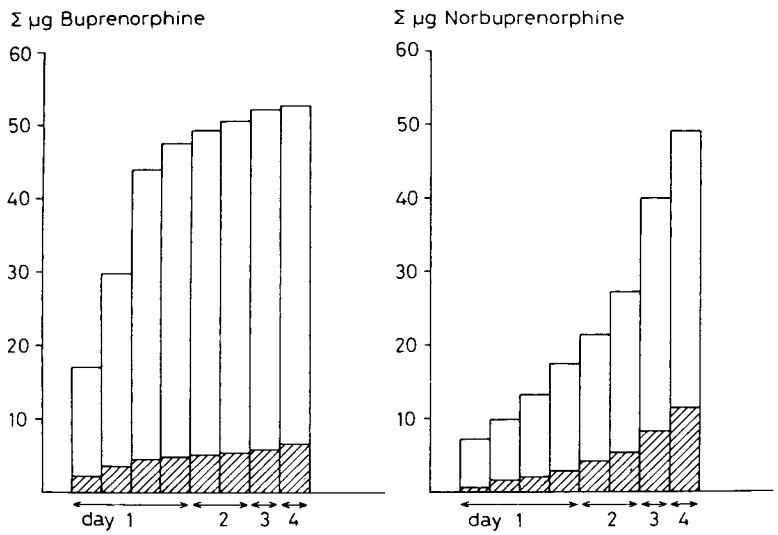

Fig. 6. Cumulative amount of buprenorphine and norbuprenorphine excreted in urine after intravenous administration of 0.6 mg of buprenorphine. □, Conjugated drug or metabolite; ▨, free drug or metabolite.

Comments on usefulness

Buprenorphine is one of the more interesting newer opiate drugs. It combines a high degree of analgesic efficacy with a low degree of opiate-like side-effects such as respiratory depression and physical dependence [1]. Its development in clinical medicine has, however, been hampered by lack of knowledge of its pharmacokinetics in man. Thus neither the bioavailability nor the single- or multiple-dose pharmacokinetics have been adequately determined. The present analytical method can hopefully help rectify the situation.

The analytical method is based on a crucial chemical degradation step leading to a loss of the secondary alcohol group in the form of methanol and the formation of a furane ring. The product has as the PFP ester improved

properties for GC—MS analysis in comparison to the parent compound due to decreased adsorption and the presence of a suitable ion for focusing in the high mass region (*m/z* 581). The same degradation patterns were found for norbuprenorphine and N-propylnorbuprenorphine used as internal standard. Thus the major metabolite of buprenorphine together with buprenorphine itself could be determined simultaneously by the basic procedure. The same chemical degradation was recently reported by Cone et al. [9] but was not used for analytical purposes. The application of the present method in the characterization of the clinical pharmacokinetics of buprenorphine is currently under way.

ACKNOWLEDGEMENT

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DETERMINATION OF DEXTROMETHORPHAN AND METABOLITES IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

Sensitive methods were developed for the analysis of dextromethorphan (I) and two metabolites, (+)-17-methyl-morphinan-3-ol (II) and (+)-morphinan-3-ol (III), in plasma as well as dextromethorphan and three metabolites II, III and (+)-3-methoxymorphinan (IV) in urine using high-performance liquid chromatography followed by detection with a fluorometer. Dextromethorphan and its metabolites were extracted from plasma and urine and separated in the reversed-phase mode. The practical lower limits of determination for I, II, and III in plasma were 0.5, 5, and 5 ng/ml, respectively; for I, II, III, and IV in urine, the limits were 20 ng/ml, 0.6 μg/ml, 0.5 μg/ml, and 15 ng/ml, respectively. The linearity of the calibration graphs was excellent (r varied from 0.9994 to 0.9999) over concentration ranges of two orders of magnitude.

INTRODUCTION

Dextromethorphan (I), (+)-3-methoxy-17-methylmorphinan, is a widely used synthetic antitussive agent. Analytical methods for its determination in plasma include radioimmunoassay [1], gas—liquid chromatography (GLC) following extraction and conversion to an electron-capturing derivative with trichloroethyl chloroformate [2], and GLC following extraction using a nitrogen-selective detector [3, 4]. Two of these methods have been used to determine it in urine [2, 4]. It has also been determined in urine using high-performance liquid chromatography (HPLC) with ultraviolet detection [5].

Methods for the determination of dextrorphan (II), (+)-17-methylmorphinan-3-ol, the O-demethylated metabolite of dextromethorphan, and its glucuronide and sulfate ester conjugates in plasma and urine have been reported. After enzymatic hydrolysis of the esters and extraction, total

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dextrorphan has been determined in plasma by direct fluorescence [6] and HPLC with fluorescence detection [7], in plasma and urine by GLC using a nitrogen-selective detector [4], and in urine by GLC using a flame-ionization detector [8], as well as with HPLC using ultraviolet detection [5].

Metabolites (+)-morphinan-3-ol (III), after either enzymatic or acid hydrolysis of its glucuronide and sulfate ester conjugates, and (+)-3-methoxymorphinan (IV) have been determined in human urine by GLC with a nitrogen-selective detector [4] and a flame-ionization detector [8], as well as by HPLC using ultraviolet detection [5].

This report describes HPLC methods with fluorescence detection for determination of I, II and III in plasma, as well as I, II, III, and IV in urine. Since II and III exist as glucuronide and sulfate ester conjugates in plasma and urine, samples were subjected to enzymatic hydrolysis prior to extraction so that total II and III could be determined. Plasma profiles and urinary excretion data following single doses of 60 mg dextromethorphan hydrobromide are also presented.

EXPERIMENTAL

Apparatus

The HPLC system was assembled from a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system, a Waters Assoc. Model 710B WISP automatic sample processor, a 25 cm × 4.6 mm I.D. stainless-steel column packed with RP-18 (5-μm Ultrasphere; Altex, Berkeley, CA, U.S.A.) for analysis of II and III in plasma, RP-18 (5-μm Hypersil; Chromanetics, Baltimore, MD, U.S.A.) for analysis of I in plasma, or RP-Phenyl (5-μm Spherisorb; Chromanetics) for analysis of I, II, III, and IV in urine, a 6.0 cm × 4.6 mm I.D. stainless-steel pre-column packed with RP-18 (10-μm Bondapak C_{18}; Waters Assoc.) for analysis of I, II, and III in plasma, or a pre-column packed with RP-Phenyl (37—50 μm Bondapak; Waters Assoc.) for analysis of I—IV in urine, a Schoeffel Instrument (Westwood, NJ, U.S.A.) Model FS970 spectrofluorometer operated at λ_{ex} of 200 nm and no emission cut-off filter for analysis of I, II, and III in plasma as well as I and IV in urine and λ_{ex} of 200 nm with a Corning (Medfield, MA, U.S.A.) Model 7-54 220—400 nm band-pass emission filter for analysis of II and III in urine. The liquid chromatograph was connected to a Spectra Physics (Santa Clara, CA, U.S.A.) Model 4100 integrator calculator.

Reagents

Dextromethorphan hydrobromide and levallorphan tartrate were obtained from the United States Pharmacopeial Convention, (Rockville, MD, U.S.A.); II · tartrate and III · HBr were supplied by Hoffmann-La Roche (Nutley, NJ, U.S.A.); IV · HCl was obtained from Pennwalt (Rochester, NY, U.S.A.); viloxizine base was supplied by the Stuart Pharmaceuticals Division of ICI Americas (Wilmington, DE, U.S.A.); β-glucuronidase type H-1 (containing 300 000—400 000 U β-glucuronidase and 15 000—40 000 μmol U of arylsulfatase activity per g) was obtained from Sigma (St. Louis, MO, U.S.A.); sequanol-grade triethylamine came from Pierce (Rockford, IL, U.S.A.); 1-octanesulfonate as the sodium salt was supplied by Regis (Morton Grove, IL, U.S.A.); *n*-nonylamine was from Aldrich (Milwaukee, WI, U.S.A.); acetonitrile was

analytical-reagent grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); all other reagents were reagent grade.

Solutions

A β-glucuronidase solution containing approximately 3000 U/ml β-glucuronidase and 230 μmol U of arylsulfatase activity was prepared by dissolving β-glucuronidase type H-1 in 0.1 *M* sodium citrate, pH 5.0.

The following mobile phases (as well as the stationary phases) were selected to give optimal resolution between the compounds of interest and matrix components.

The mobile phase for the determination of I in plasma was composed of 0.022 *M* sodium acetate—0.094 *M* perchloric acid—0.0196 *M* *n*-nonylamine, pH 4.3 (using sodium hydroxide)—40% acetonitrile. The volumetric flow-rate of the mobile phase was 0.7 ml/min.

For the determination of II and III in plasma, the mobile phase was composed of 0.022 *M* sodium acetate—0.094 *M* perchloric acid—0.0072 *M* triethylamine, pH 4.3 (using sodium hydroxide)—27% acetonitrile. The volumetric flow-rate of the mobile phase was 1.0 ml/min.

The mobile phase for the determination of I and IV in urine was composed of 0.01 *M* potassium dihydrogen phosphate, pH 3.0 (using phosphoric acid), and 30% acetonitrile. The volumetric flow-rate of the mobile phase was 0.7 ml/min.

For the determination of II and III in urine, the mobile phase was composed of 0.01 *M* potassium dihydrogen phosphate—0.01 *M* 1-octanesulfonate sodium salt, pH 3.0 (using phosphoric acid)—30% acetonitrile. The volumetric flow-rate of the mobile phase was 1.5 ml/min.

Stock solutions of I · HBr, II · tartrate, III · HBr, IV · HCl, and internal standards levallorphan tartrate and viloxizine base (0.1 mg/ml in water) were prepared and stored at 4° C. Standard curves were generated by spiking blank plasma or urine with varying amounts of I, II, III or IV and a constant amount of either internal standard. Microliter aliquots of the stock solutions or dilutions of the stock solutions in water were added to the plasma or urine. The internal standard concentrations in plasma were 15 ng/ml levallorphan for analysis of I and 200 ng/ml viloxizine for analysis of II and III. The internal standard concentrations in urine were 800 ng/ml levallorphan for analysis of I and IV and 17 μg/ml levallorphan for analysis of II and III.

For each compound, separate standard curves were prepared daily. Concentrations of the drug and metabolite standards in plasma and urine were as follows: I: 0.5—24 ng/ml in plasma, I: 20—4900 ng/ml in urine; II: 5—496 ng/ml in plasma, II: 0.6—56 μg/ml in urine; III: 5—495 ng/ml in plasma, III: 0.5—50 μg/ml in urine; and IV: 15—3590 ng/ml in urine. These samples were treated according to the extraction procedures described.

Enzymatic hydrolysis

The quantity of β-glucuronidase plus arylsulfatase and the incubation time required for complete hydrolysis of the conjugated metabolites were determined. Aliquots of plasma containing the equivalent of more than 496 ng/ml II and III, and urine containing 56 μg/ml of II and III were hydrolyzed

under a variety of conditions. An incubation time of 1.0 h at 37°C using 1 ml of enzyme solution per ml of plasma and 30 min at 37°C using 0.5 ml of enzyme solution per 0.1 ml of urine gave complete hydrolysis. To insure complete hydrolysis, a 2.0-h incubation time was used for plasma and a 1.0-h incubation time for urine.

Analytical procedure

The following procedures were selected because they were found to give optimal recoveries of I—IV while minimizing coextraction of sample matrix components.

Determination of I in plasma. Plasma samples (2 ml) were placed into 40-ml PTFE-stoppered silanized-glass centrifuge tubes, followed by the addition of internal standard, levallorphan tartrate (15 ng/ml of plasma). They were made alkaline by the addition of 0.5 ml of saturated sodium carbonate in water, followed by extraction with hexane (20 ml containing 0.1%, v/v, triethylamine). The tubes were gently shaken on a horizontal shaker for 20 min. After centrifugation ($\geqslant$ 1000 *g*) for 10 min, the upper organic layer was transferred to a second centrifuge tube followed by evaporation to dryness under nitrogen in a 50°C water bath. The residues were then dissolved in mobile phase (300 µl), and mixed in a Vortex mixer. Aliquots (150 µl) of these solutions were then injected into the HPLC system.

Determination of total II and III in plasma. Internal standard viloxizine (200 ng/ml plasma) was added to plasma samples (1 ml) in 15-ml PTFE-stoppered silanized-glass centrifuge tubes. β-Glucuronidase type H-1 (1.0 ml of 3000 U/ml solution in 0.1 *M* sodium citrate buffer, pH 5.0) was added, followed by incubation for 2 h at 37°C. Saturated sodium carbonate in water (0.5 ml) and 10% *n*-butanol in *n*-butyl chloride (5 ml) were then added and the tubes gently shaken on a horizontal shaker for 20 min. After centrifugation ($\geqslant$ 1000 *g*) for 5 min, the upper organic layer was transferred to a 40-ml PTFE-stoppered silanized-glass centrifuge tube. The remaining aqueous layer was re-extracted with 10% *n*-butanol in *n*-butyl chloride (5 ml) in the same manner. After centrifugation ($\geqslant$ 1000 *g*) for 5 min, the upper organic layer was combined with the first organic extracts. The combined organic layers were extracted with 1% acetic acid (500 µl) by vortex-mixing for 1 min. After centrifugation ($\geqslant$ 1000 *g*) for 5 min, aliquots (50 µl) of the lower aqueous phase were injected into the HPLC system.

Determination of I and IV in urine. Internal standard levallorphan tartrate (800 ng/ml of urine) was added to urine samples (1 ml) in 40-ml PTFE-stoppered silanized-glass centrifuge tubes. Saturated sodium carbonate in water (0.5 ml) and hexane (20 ml containing 0.1% triethylamine) were added to the samples, and the tubes gently shaken on a horizontal shaker for 20 min. After centrifugation ($\geqslant$ 1000 *g*) for 5 min, the upper organic layer was transferred to a second centrifuge tube followed by evaporation to dryness under nitrogen in a 50°C water bath. The residues were dissolved in acetonitrile—1% acetic acid (300 µl, 1:1, v/v), and mixed on a Vortex mixer. Aliquots (60 µl) of these solutions were injected into the HPLC system.

Determination of total II and III in urine. Internal standard levallorphan (17 µg/ml urine) was added to urine samples (100 µl) in 15-ml PTFE-stoppered

silanized-glass centrifuge tubes. β-Glucuronidase type H-1 (0.5 ml of a 1500 U per 0.5 ml solution in 0.1 *M* sodium citrate buffer, pH 5.0) was added, followed by incubation at 37°C for 1 h. Saturated sodium carbonate in water (0.5 ml) and 10% *n*-butanol in *n*-butyl chloride (10 ml) were then added and the tubes gently shaken on a horizontonal shaker for 20 min. After centrifugation ($\geqslant$ 1000 *g*) for 5 min, the upper organic layer was transferred to a second centrifuge tube after first freezing the aqueous layer in a dry ice—acetone bath, followed by evaporation to dryness under nitrogen in a 50°C water bath. The residues were then dissolved in acetonitrile—1% acetic acid (2 ml, 1:1, v/v) and mixed on a Vortex mixer. Aliquots (50 μl) of these solutions were injected into the HPLC system.

Extraction efficiency

The extraction efficiency of each compound from plasma and urine was determined. Plasma and urine samples were spiked with I—IV (except IV in

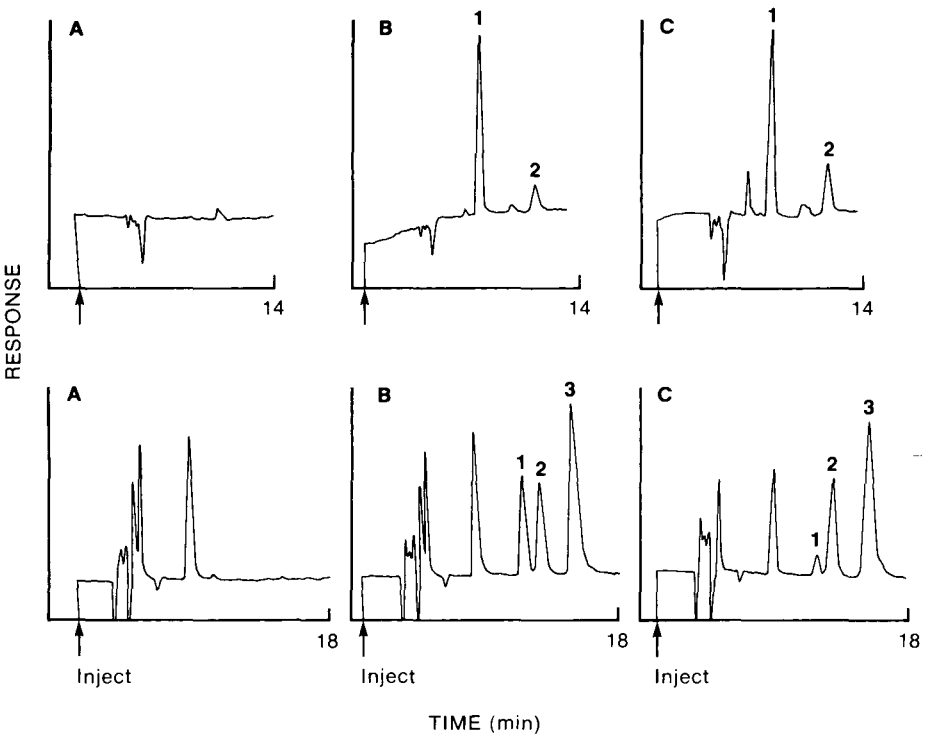

Fig. 1. (Top) Chromatograms of an analysis of (A) a plasma blank; (B) a plasma sample with added dextromethorphan (2.55 ng/ml) and internal standard, levallorphan (15 ng/ml); and (C) a plasma sample from a subject dosed with 60 mg dextromethorphan hydrobromide with added internal standard (4.69 and 15 ng/ml, respectively). Peaks: 1 = internal standard, levallorphan (retention time = 7.87 min); 2 = dextromethorphan (retention time = 11.77 min). (Bottom) Chromatograms of an analysis of (A) a plasma blank; (B) a plasma sample with added dextrorphan (48.5 ng/ml, (+)-morphinan-3-ol (47.3 ng/ml), and internal standard, viloxizine (200 ng/ml); and (C) a plasma sample from a subject dosed with 60 mg dextromethorphan hydrobromide with added internal standard (11.49, 10.86, and 200 ng/ml, respectively). Peaks: 1 = (+)-morphinan-3-ol (retention time = 11.3 min); 2 = dextrorphan (retention time = 12.49 min); 3 = internal standard viloxizine (retention time = 14.81 min).

plasma) and carried through the entire analysis procedure. The recovery of each compound was determined by comparing peak areas of extracted standards with those of unextracted standards. The unextracted standards were prepared by the addition of I—IV to water to give concentrations equivalent to those of the extracted standards.

RESULTS AND DISCUSSION

Figs. 1 and 2 show typical chromatograms for dextromethorphan and its metabolites in spiked human plasma and urine, respectively, as well as plasma

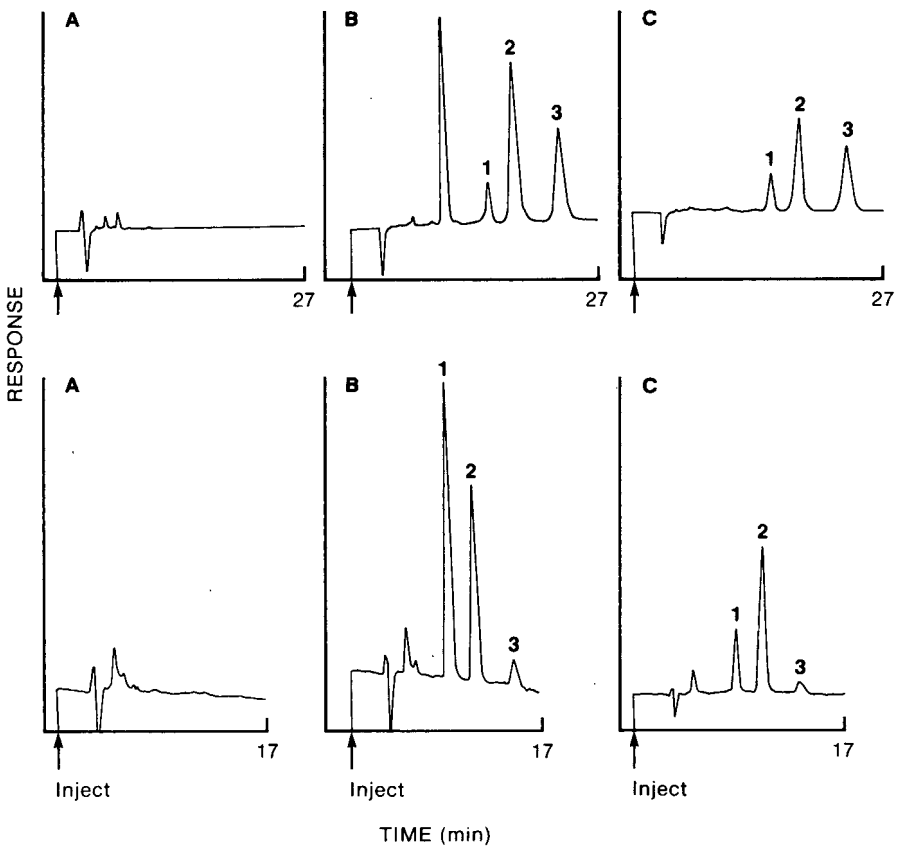

Fig. 2. (Top) Chromatograms for an analysis of (A) a urine blank; (B) a urine sample with added dextromethorphan (481.95 ng/ml), (+)-3-methoxymorphinan (473.2 ng/ml), and internal standard, levallorphan (800 ng/ml); and (C) a urine sample from a subject dosed with 60 mg dextromethorphan hydrobromide with added internal standard (312.5, 335.6 and 800 ng/ml, respectively). Peaks: 1 = internal standard, levallorphan (retention time = 14.89 min); 2 = (+)-3-methoxymorphinan (retention time = 17.84 min); 3 = dextromethorphan (retention time = 22.9 min). (Bottom) Chromatograms of an analysis of (A) a urine blank; (B) a urine sample with added dextrorphan (13.99 μg/ml), (+)-morphinan-3-ol (14.79 μg/ml), and internal standard, levallorphan (17 μg/ml); and (C) a urine sample from a subject dosed with 60 mg dextromethorphan hydrobromide with added internal standard (23.75, 6.95, and 17 μg/ml, respectively). Peaks: 1 = (+)-morphinan-3-ol (retention time = 8.16 min); 2 = dextrorphan (retention time = 10.17 min); 3 = internal standard levallorphan (retention time = 13.32 min).

TABLE I

ANALYSIS OF DEXTROMETHORPHAN IN PLASMA SAMPLES

Nominal value (ng/ml)	Determined value (ng/ml)	Mean	S.D.	R.S.D. (%)
0.48	0.57	0.48	0.08	16.8
	0.38			
	0.51			
	0.46			
2.40	2.51	2.40	0.12	4.8
	2.29			
	2.49			
	2.31			
4.8	5.23	4.80	0.3	6.2
	4.68			
	4.74			
	4.55			
24.0	24.96	24.0	0.87	3.6
	23.76			
	24.35			
	22.92			

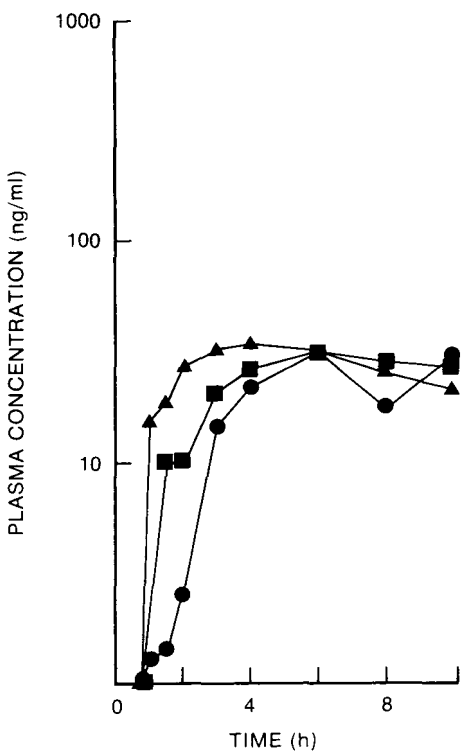

Fig. 3. Plasma concentrations of dextromethorphan (●), dextrorphan (▲), and (+)-morphinan-3-ol (■) in subject 1 following a single oral dose of 60 mg dextromethorphan hydrobromide.

and urine obtained from a human subject after oral ingestion of 60 mg dextromethorphan hydrobromide. Analysis of pre-dose plasma and urine samples from eighteen human subjects presented no chromatographic peaks that would interfere with either dextromethorphan and its metabolites or internal standards. Plots of peak area ratios (I, II, III or IV to internal standard) against I, II, III or IV concentrations were linear. Typical standard curves for plasma and urine had correlation coefficients of 0.9994—0.9999 over concentration ranges of two orders of magnitude.

The recoveries of I, II, and III from plasma were approximately 96%, 87%, and 90%, respectively. The recoveries from urine were approximately 90% for I, II, and III, and 67% for IV. Recoveries from plasma and urine of internal standards levallorphan and viloxizine were approximately 66% and 69%, respectively. These values for recoveries of I—IV agree with those recently reported [5].

TABLE II

ANALYSIS OF DEXTRORPHAN AND (+)-MORPHINAN-3-OL IN PLASMA SAMPLES

Dextrorphan					(+)-Morphinan-3-ol				
Nominal value (ng/ml)	Determined value (ng/ml)	Mean	S.D.	R.S.D. (%)	Nominal value (ng/ml)	Determined value (ng/ml)	Mean	S.D.	R.S.D. (%)
4.96	4.75	4.96	0.28	5.7	4.95	4.59	4.95	0.68	13.7
	5.30					5.93			
	4.88					4.90			
	5.21					5.21			
	4.66					4.13			
9.92	9.60	9.92	0.42	4.2	9.90	9.43	9.90	0.77	7.8
	9.97					9.96			
	9.72					11.11			
	9.68					9.09			
	10.63					9.93			
24.8	25.26	24.81	0.49	2.0	24.75	24.69	24.74	0.90	3.6
	25.14					25.38			
	24.39					25.22			
	24.18					23.20			
	25.07					25.22			
99.2	97.10	99.20	1.48	1.5	99.0	95.50	99.01	3.64	3.7
	99.98					103.23			
	100.37					101.21			
	100.36					100.16			
	98.19					94.93			
248	247.87	248.03	4.77	1.9	247.5	244.32	247.46	6.60	2.7
	254.22					256.47			
	251.11					251.43			
	242.59					239.46			
	244.35					245.63			
496	459.67	495.95	28.85	5.8	495.0	461.68	495.11	27.74	5.6
	487.34					488.70			
	518.74					514.29			
	482.77					531.30			
	531.25					479.57			

TABLE III

ANALYSIS OF DEXTROMETHORPHAN AND (+)-3-METHOXYMORPHINAN IN URINE SAMPLES

Dextromethorphan					(+)-3-Methoxymorphinan				
Nominal value (ng/ml)	Determined value (ng/ml)	Mean	S.D.	R.S.D. (%)	Nominal value (ng/ml)	Determined value (ng/ml)	Mean	S.D.	R.S.D. (%)
19.6	19.77	19.60	0.64	3.3	14.4	12.24	14.40	1.56	10.8
	18.55					15.81			
	19.57					14.36			
	19.86					15.19			
	20.26								
98.0	103.02	98.0	5.24	5.3	71.8	72.04	71.80	0.79	1.1
	99.96					71.17			
	92.81					73.07			
	102.22					71.51			
	91.98					71.21			
490.0	461.43	490.0	22.2	4.5	359.0	365.44	361.29	4.46	1.2
	523.08					356.57			
	494.33					357.37			
	487.13					364.23			
	484.25					362.85			
2450.0	2236.0	2449.6	191.6	7.8	1793.0	1817.0	1793.6	29.7	1.7
	2321.0					1769.0			
	2696.0					1819.0			
	2596.0					17.54.0			
	2399.0					1809.0			
4899.0	4634.0	4899.2	550.4	11.2	3588.0	3544.6	3588.6	154.6	4.3
	4480.0					3410.0			
	4470.0					3522.0			
	5172.0					3646.0			
	5740.0					3821.0			

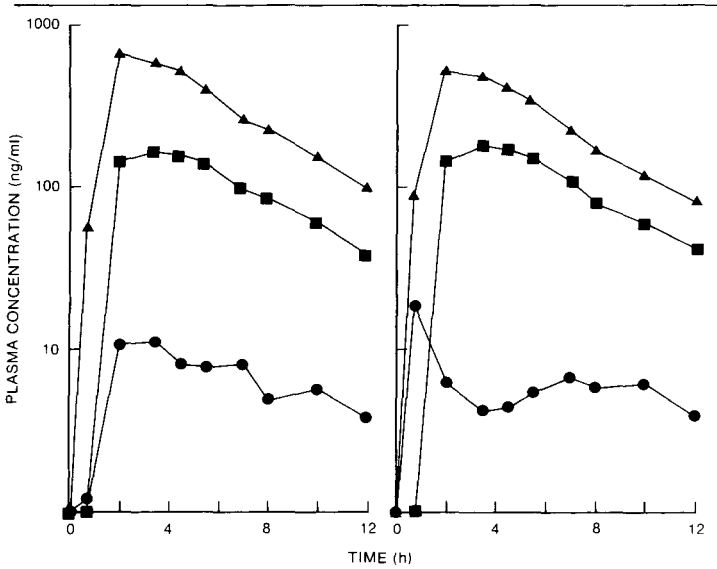

Fig. 4. Plasma concentrations of dextromethorphan (•), dextrorphan (▲), and (+)-morphinan-3-ol (■) in subject 2 (left) and 3 (right) following a single oral dose of 60 mg dextromethorphan hydrobromide.

TABLE IV

ANALYSIS OF DEXTRORPHAN AND (+)-MORPHINAN-3-OL IN URINE SAMPLES

Dextrorphan					(+)-Morphinan-3-ol				
Nominal value (μg/ml)	Determined value (μg/ml)	Mean	S.D.	R.S.D. (%)	Nominal value (μg/ml)	Determined value (μg/ml)	Mean	S.D.	R.S.D. (%)
0.58	0.569	0.58	0.009	1.6	0.45	0.479	0.446	0.06	13.1
	0.593					0.346			
	0.585					0.479			
	0.576					0.484			
	0.577					0.440			
1.45	1.448	1.43	0.03	2.1	1.25	1.252	1.251	0.05	4.1
	1.442					1.294			
	1.435					1.163			
	1.437					1.275			
	1.371					1.272			
5.64	5.674	5.64	0.12	2.2	4.42	4.445	4.340	0.10	2.3
	5.663					4.429			
	5.450					4.233			
	5.786					4.500			
	5.626					4.391			
28.2	26.94	28.20	1.39	4.9	24.6	23.42	24.60	1.03	4.2
	26.65					23.95			
	28.29					25.68			
	29.49					24.29			
	29.61					25.68			
56.4	53.16	56.40	2.00	3.5	49.2	46.59	49.21	3.81	7.7
	56.47					54.40			
	58.66					44.51			
	56.69					50.26			
	57.01					50.27			

The precision of the assay methods, expressed as the relative standard deviation, ranged from 16.8% to 3.6%, 5.8% to 1.5%, and 13.7% to 2.7% for I, II, and III in plasma, respectively. Precision ranged from 11.2% to 3.3%, 4.9% to 2.1%, 13.1% to 2.3%, and 10.8% to 1.1% for I, II, III, and IV in urine, respectively (Tables I—IV).

The practical lower limits of determination (where chromatographic peaks have a signal-to-noise ratio of $\geqslant$ 5:1) for I, II, and III in plasma were 0.5, 5, and 5 ng/ml, respectively. The practical lower limits of determination for I, II, III, and IV in urine were 20 ng/ml, 0.6 μg/ml, 0.5 μg/ml, and 15 ng/ml, respectively.

To demonstrate the usefulness of these methods, results from the analysis of plasma samples obtained from four healthy subjects who had ingested 60 mg dextromethorphan hydrobromide in water are shown in Figs. 3—5. Cumulative urinary excretion of dextromethorphan and its metabolites are shown in Figs. 6—8. Urinary recovery of dextromethorphan and its metabolites, expressed as dextromethorphan base, was 20.8%, 61.9%, 48.0%, and 64.5% for subjects 1—4, respectively.

Earlier reports [1—11] have suggested that dextromethorphan is rapidly and extensively metabolized via first-pass hepatic metabolism, and that metab-

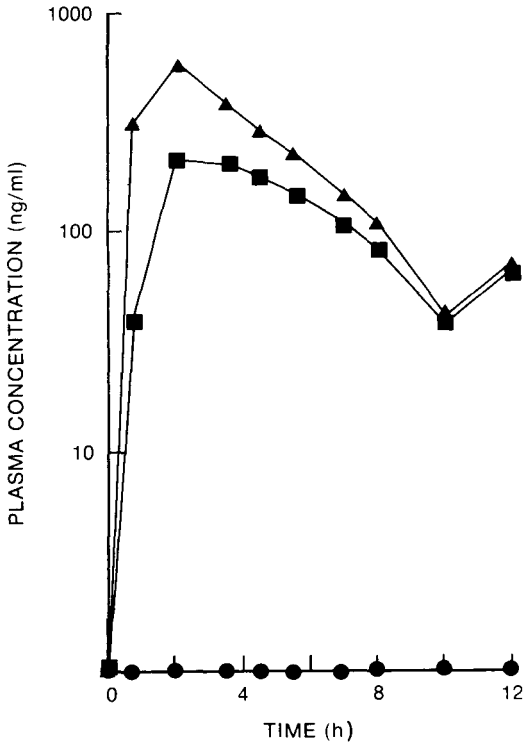

Fig. 5. Plasma concentrations of dextromethorphan (•), dextrorphan (▲), and (+)-morphinan-3-ol (■) in subject 4 following a single oral dose of 60 mg dextromethorphan.

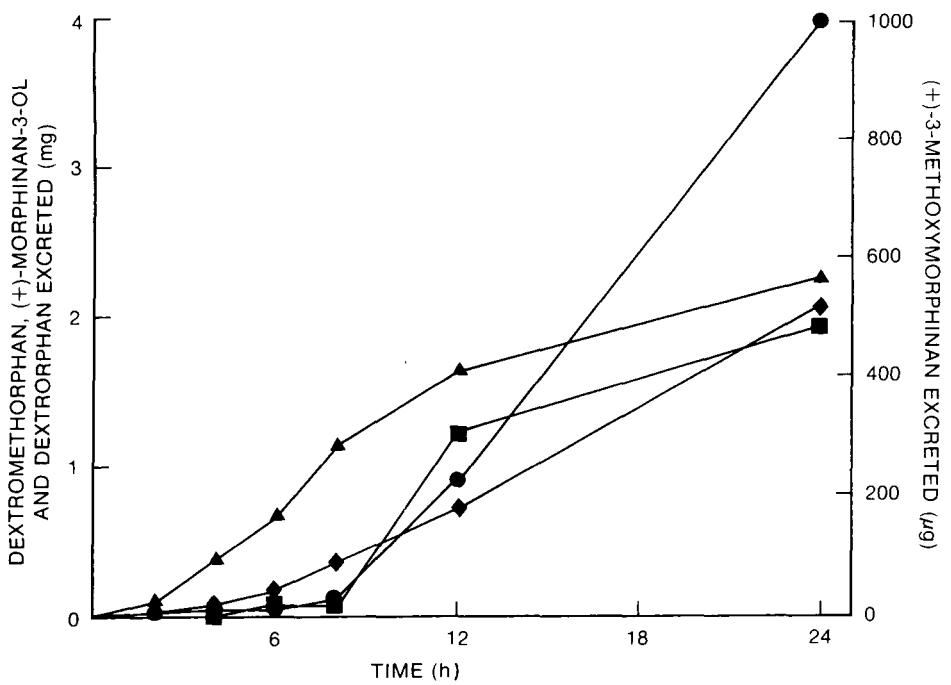

Fig. 6. Cumulative urinary excretion in subject 1 of dextromethorphan (•), dextrorphan (▲), and (+)-morphinan-3-ol (■) (left scale), and (+)-3-methoxymorphinan (♦) (right scale).

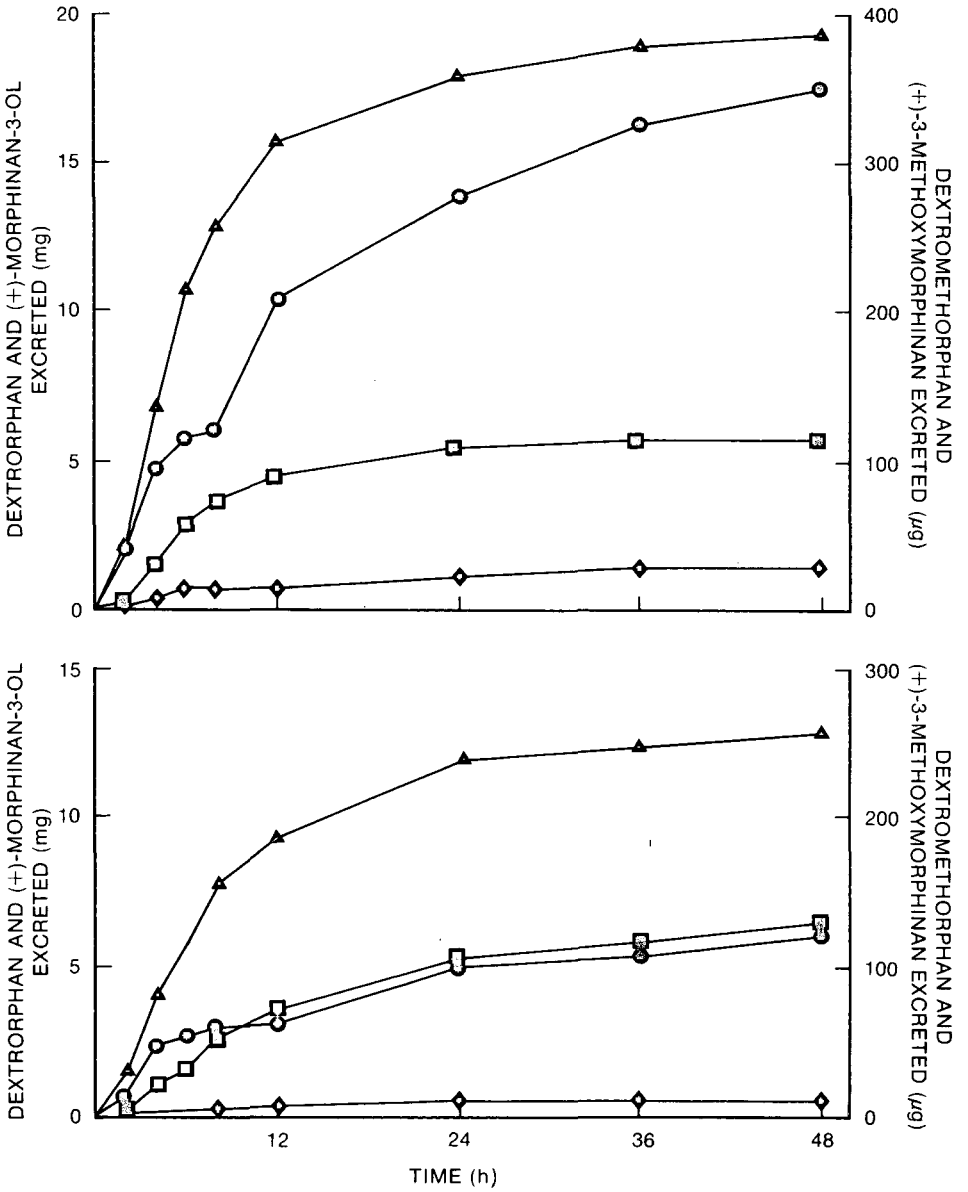

Fig. 7. Cumulative urinary excretion in subject 2 (top) and 3 (bottom) of dextrorphan (▲) and (+)-morphinan-3-ol (■) (left scale), and dextromethorphan (●), and (+)-3-methoxymorphinan (♦) (right scale).

olism is highly variable between subjects [2, 4, 8]. The antitussive activity of oral doses of I were attributed to the possible pharmacological activity of one or more of the metabolites [1, 2, 11]. One investigative group reported that subjects could be divided into three groups [4]. The plasma of one group contained primarily conjugated II, with no detectable I. Another group with plasma containing primarily conjugated II also contained levels of I that were

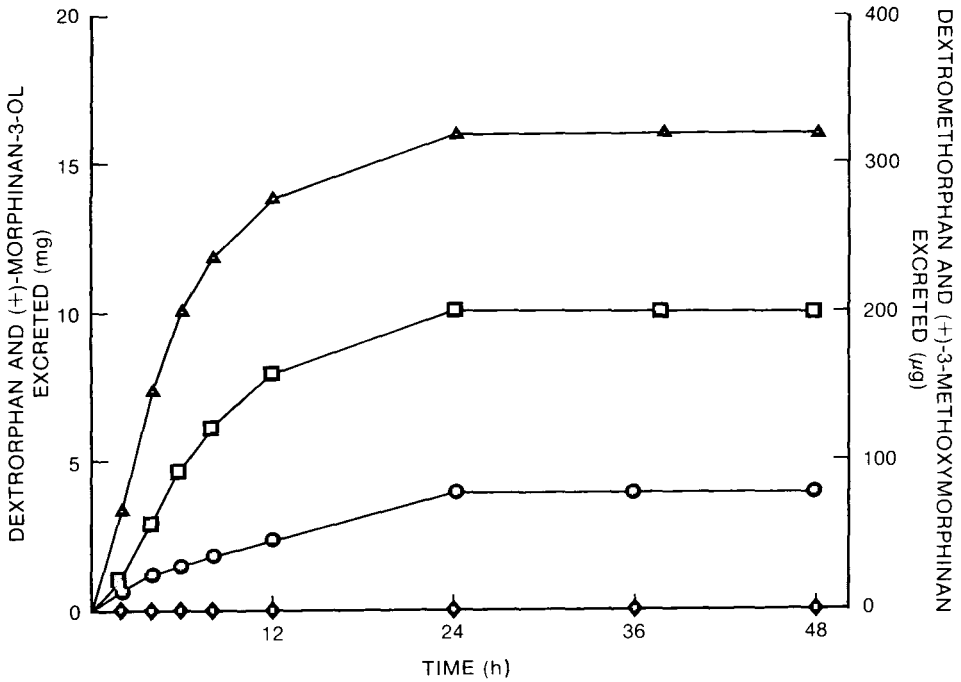

Fig. 8. Cumulative urinary excretion in subject 4 of dextrorphan (▲) and (+)-morphinan-3-ol (■) (left scale), and dextromethorphan (●) and (+)-3-methoxymorphinan (◆) (right scale).

approximately twenty times higher than those of the first group. Plasma from the third group contained only I. They determined that average urinary recoveries within 48 h were 86%, 74%, and 20% for the three groups, respectively, after a 25-mg oral dose of dextromethorphan hydrobromide. The plasma profiles and urinary recoveries seen in our four subjects, as well as those seen in subsequent studies with other subjects, support their findings.

An earlier report [7] noted the appearance of a chromatographic peak following hydrolysis of post-dose plasma samples. This peak did not appear when the hydrolysis step was omitted, and was thought to be another conjugated metabolite of dextromethorphan. Our findings suggest that this metabolite was (+)-morphinan-3-ol (III). Without prior enzymatic hydrolysis, no unconjugated III was detected in plasma using the method described.

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DETERMINATION OF ISONIAZID, ACETYLISONIAZID, ACETYLHYDRAZINE AND DIACETYLHYDRAZINE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic assay for the determination of isoniazid, acetylisoniazid, acetylhydrazine and diacetylhydrazine (plasma and urine) was developed. The *m*-chlorobenzoyl derivatives of isoniazid, acetylhydrazine and the internal standard propionylhydrazine were prepared, separated on a RP-18 column and detected at 220 nm. Acetylisoniazid, diacetylhydrazine and the internal standard dipropionylhydrazine were converted to isoniazid, acetylhydrazine, and propionylhydrazine by acidic hydrolysis and subsequently derivatized with *m*-fluorobenzoyl chloride, separated on a RP-18 column and detected at 220 nm. The lower limits of detection in plasma are acetylhydrazine 0.5 nmol/ml, isoniazid 1.0 nmol/ml, diacetylhydrazine 1.0 nmol/ml and acetylisoniazid 2.0 nmol/ml, and in urine, acetylhydrazine 10 nmol/ml, isoniazid 15 nmol/ml, diacetylhydrazine 20 nmol/ml and acetylisoniazid 40 nmol/ml. This method is sensitive, reproducible, accurate and precise; therefore, it is well suited for detailed pharmacokinetic studies.

INTRODUCTION

Isoniazid (INH, isonicotinic acid hydrazide) is a major drug used in the treatment of tuberculosis. A major drawback to its use is the frequent occurrence of hepatotoxicity [1, 2]. Animal studies have demonstrated that the hepatotoxicity of isoniazid is caused by acetylhydrazine which is formed by hydrolysis of the major INH metabolite acetylisoniazid. Apart from the N-acetylation of acetylhydrazine to diacetylhydrazine, it is further metabolized by a cytochrome P-450 dependent reaction. In this reaction a reactive acyl radical or acyl cation is formed which binds covalently to liver macromolecules, an event that may cause liver cell necrosis [2—4]. The higher incidence of INH-related hepatotoxicity in rapid acetylators has been attributed to their higher

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rate of formation of acetylhydrazine from acetylisoniazid [1, 2]. Recent studies have definitely identified acetylhydrazine as a metabolite of INH in man [5]. However, no detailed studies on the pharmacokinetics of INH and its hydrazine metabolites have been performed in patients to determine if formation and metabolism of acetylhydrazine might be correlated with isoniazid hepatotoxicity in man. Such studies are difficult to perform because methods with high sensitivity and specificity were not available for quantification of INH and its hydrazine metabolites in plasma and urine. Older methods employed the formation of the corresponding Schiff base and required the separate derivatization and determination of each compound [6]. As stated by the authors, this method was only applicable for the measurement of INH and hydrazine metabolites after single-dose administration. A specific gas—liquid chromatographic method for the determination of INH and hydrazine metabolites in urine has been reported [7]. This method, however, requires large volumes of organic solvents (600 ml), is time-consuming and the internal standard (the *p*-fluorobenzaldehyde derivative) is added after derivatization. Recently, a specific and sensitive gas chromatographic—mass spectrometric (GC—MS) method using single-ion monitoring (SIM) has been described [8]. Although SIM is, at present, the most specific method available, it requires access to a GC—MS system, which is not always readily available.

We describe a high-performance liquid chromatographic (HPLC) method which allows for the simultaneous determination of INH and its hydrazine metabolites in plasma and urine after formation of the corresponding hydrazides with halogen-substituted benzoyl chlorides, followed by separation on a reversed-phase column and UV detection. This method has been satisfactorily used by us during the last two years to measure INH and its hydrazine metabolites with good specificity, sensitivity, accuracy and precision.

MATERIALS AND METHODS

Synthesis of reference compounds and internal standards

INH was obtained from Bayer (Leverkusen, F.R.G.) and was recrystallized in methanol—diethyl ether (1:4). The melting point was 171—173°C [9].

Acetylhydrazine (AH) was synthesized by reacting a four-fold excess of hydrazine hydrate with the methyl ester of acetic acid. The reaction was allowed to proceed for 12 h at room temperature with continuous stirring. The excess hydrazine was removed by vacuum distillation. Acetylhydrazine was recrystallized in dichloromethane—*n*-hexane (2:1). The melting point of the synthesized product was 68—70°C (lit. [10]: 67°C).

Diacetylhydrazine (DAH) was synthesized by reacting hydrazine hydrate with a four-fold excess of the methyl ester of acetic acid at room temperature with continuous stirring for 12 h. Diacetylhydrazine was recrystallized in dichloromethane—*n*-hexane (1:4). The melting point of the synthesized product was 133—136°C (lit. [11]: 138—143°C).

Acetylisoniazid (AINH) was synthesized by reacting isoniazid with a four-fold excess of acetic acid anhydride at room temperature for 1.5 h with continuous stirring. The synthesized product was recrystallized in methanol—diethyl ether (1:4). The melting point of the final product was 157—160°C (lit. [12]: 162—163°C).

Propionylhydrazine (PH) was synthesized by reacting a four-fold excess of hydrazine hydrate with the methyl ester of propionic acid. The reaction conditions were the same as described for the synthesis of acetylhydrazine. The final product was distilled under vacuum. The melting point was 37°C (lit. [13]: 40°C).

Dipropionylhydrazine (DPH) was synthesized in the same way as described for the synthesis of diacetylhydrazine using the methyl ester of propionic acid instead of the methyl ester of acetic acid. The melting point of the synthesized product was 137—139°C (lit. [14]: 136°C).

3-Chlorobenzoyl chloride and 3-fluorobenzoyl chloride were purchased from EGA Chemie and purified by distillation under vacuum.

Apparatus

The liquid chromatographic system consisted of a Spectra-Physics 3500 B liquid chromatograph coupled to a Spectra-Physics Model 770 spectrophotometer. Detection was at 220 nm. A 300 mm (6 mm O.D. and 4.5 mm I.D.) stainless-steel column packed with 10-μm Nucleosil RP-18 (Macherey, Nagel & Co., Düren, F.R.G.) was used.

HPLC system A. The 3-chlorobenzoyl derivatives were separated with the following mobile phase: 10 m*M* phosphate buffer pH 5.5—methanol—acetonitrile—dichloromethane (73:17:9:1) at a flow-rate of 2 ml/min at 50°C.

HPLC system B. For the separation of 3-fluorobenzoyl derivatives (after hydrolysis) the mobile phase consisted of 10 m*M* phosphate buffer pH 5.1—acetonitrile—dichloromethane (84.2:15:0.8). The flow-rate was 2 ml/min at 50°C.

A LKB 2091 mass spectrometer (direct inlet system) was used to record the mass spectra.

The melting points were measured with a heating microscope (Zeiss/Reichert).

Analysis of INH and AH in plasma

The internal standards PH and DPH (20 nmol and 50 nmol, respectively, in 10 μl of acetonitrile) were added to 2 ml of plasma. Trichloroacetic acid (1 ml 20%) was added to precipitate plasma proteins. The sample was then centrifuged at 3000 *g* for 10 min. The supernatant was transferred to a test tube to which 20 μl of 0.1 *M* dipotassium hydrogen phosphate and 50 μl of 11 *M* sodium hydroxide were added to adjust the pH to 5—6. After 5 min of standing, interfering substances were extracted into 4 ml of dichloromethane—acetonitrile (9:1). Following centrifugation the aqueous phase (2 ml) was transferred to another test tube. Phosphate buffer (200 μl, 0.5 *M*, pH 4) was added and the pH adjusted to 2 with 11 *M* hydrochloric acid. Next, 3-chlorobenzoyl chloride (40 μmol; 200 μl of 0.2 *M* solution in acetonitrile) was added and the sample was allowed to react for 30 min at room temperature. The sample was then saturated with 1.4 g of sodium chloride and mixed with 4 ml of *n*-hexane for 30 sec on a vortex mixer. Following centrifugation at 3000 *g*, the organic phase was discarded and the sample was adjusted to pH 6—6.5 by addition of 0.45 ml of 1 *M* sodium bicarbonate and extracted into 4 ml of dichloromethane—acetonitrile (9:1) by mixing the sample for 30 sec on a vortex mixer.

After centrifugation, 3 ml of the organic phase were transferred to a tapered test tube and evaporated under a nitrogen stream. The remaining aqueous phase containing AINH, DAH and DPH was transferred to another test tube and used for the determination of AINH and DAH. The residue of the organic phase was dissolved in the mobile phase (0.2 ml; system A) and a 0.1-ml aliquot was injected onto the HPLC column. Mobile phase system A was used for the determination of INH and AH.

Analysis of AINH and DAH in plasma

The aqueous phase that had been used for the determination of INH and AH was extracted with 4 ml of dichloromethane—acetonitrile (9:1) to remove any remaining 3-chlorobenzoyl derivatives of isoniazid and acetylhydrazine. After centrifugation the organic phase was aspirated. To 1.5 ml of the aqueous phase 0.5 ml of 5 *M* hydrochloric acid was added and the sample was hydrolysed for 90 min at 50°C to transform AINH, DAH and DPH into INH, AH and PH. After hydrolysis phosphate buffer (0.2 ml; 0.5 *M*; pH 4) was added and the sample was adjusted to pH 2 with sodium hydroxide (450 µl; 2 *M*). 3-Fluorobenzoyl chloride (30 µmol; 0.10 ml of a 0.30 *M* solution in acetonitrile) was added and the sample was allowed to react for 30 min at room temperature. The sample was then saturated with sodium chloride (1.4 g), extracted with *n*-hexane (4 ml) for 30 sec on a vortex mixer, and centrifuged for 5 min at 3000 *g*. The aqueous phase containing the fluorobenzoyl derivatives formed from AINH, DAH and DPH was adjusted to pH 6—6.5 with 0.45 ml of 1 *M* sodium bicarbonate. After 5 min the derivatives were extracted into 4 ml of dichloromethane—acetonitrile (9:1). Following centrifugation the organic phase (3 ml) was transferred to a tapered test tube and evaporated to dryness under nitrogen. The residue was dissolved in mobile phase (0.2 ml; system B) and an aliquot (0.1 ml) was injected onto the column for determination of AINH and DAH.

Urine samples

For the determination of INH and the hydrazino metabolites in urine, the following procedure was used: internal standards PH and DPH (3 µmol; dissolved in 10 µl of acetonitrile) were added to urine (3 ml), then hydrochloric acid (30 µl; 11 *M*) was added. After 5 min the sample was extracted with dichloromethane—acetonitrile (9:1; 4 ml), centrifuged, the organic phase aspirated and the aqueous phase (2 ml) was transferred to another test tube. Subsequently the procedure was used as described for the plasma samples except for the following: (1) To each sample 60 µmol of 3-chlorobenzoyl chloride were added instead of 40 µmol. (2) The residue was dissolved in 2 ml of mobile phase (system A) and a 0.1-ml aliquot was injected onto the column. (3) For the determination of AINH and DAH 1 ml of the aqueous phase was hydrolysed with 1 ml of hydrochloric acid (2.5 *M*) under conditions described for plasma. (4) The residue was dissolved in 1 ml of mobile phase (system B) and a 0.1-ml aliquot was injected onto the column.

Standard curves

Quantification was done by use of the peak height ratio of 3-chlorobenzoyl-

isoniazid or 3-chlorobenzoyl-acetylhydrazine and 3-fluorobenzoyl-isoniazid or 3-fluorobenzoyl-acetylhydrazine to the internal standards 3-chlorobenzoyl-propionylhydrazine and 3-fluorobenzoyl-propionylhydrazine. A linear relation was obtained for the range tested. (Plasma: from the lower limit of detection (see Table I) to 50, 500, 100, and 500 nmol/ml for AH, INH, DAH, and AINH. Urine: from the lower limit of detection (see Table I) to 0.5, 5.0, 1.0, and 5.0 μmol/ml for AH, INH, DAH, and AINH.)

RESULTS

Figs. 1 and 2 show representative chromatograms of plasma and urine samples obtained from patients after the administration of INH. The compounds are well separated and no interfering peaks could be observed in blank plasma and urine samples. Retention times were 5.4, 6.9 and 9.1 min for AH, PH and INH, and 4.7, 6.2 and 9.2 min for DAH, DPH and AINH. Figs. 3 and 4 show the concentration—time curves of INH and its metabolites in plasma and urine.

Hydrolysis

Since the kinetics of acidic hydrolysis of AINH and DAH are different (Fig. 5), it was necessary to choose conditions such as temperature and time of the hydrolysis so that DPH could be used as a standard for both metabolites. Under the aforementioned conditions 60% AH and 40% INH were hydrolysed from DAH and AINH, respectively.

Fig. 1. Chromatograms of a plasma sample before (A) and after (B) acidic hydrolysis from a patient 3 h after intravenous administration of 10 mg/kg isoniazid. AINH = acetylisoniazid; AH = acetylhydrazine; DAH = diacetylhydrazine; DPH = dipropionylhydrazine; INH = isoniazid; PH = propionylhydrazine.

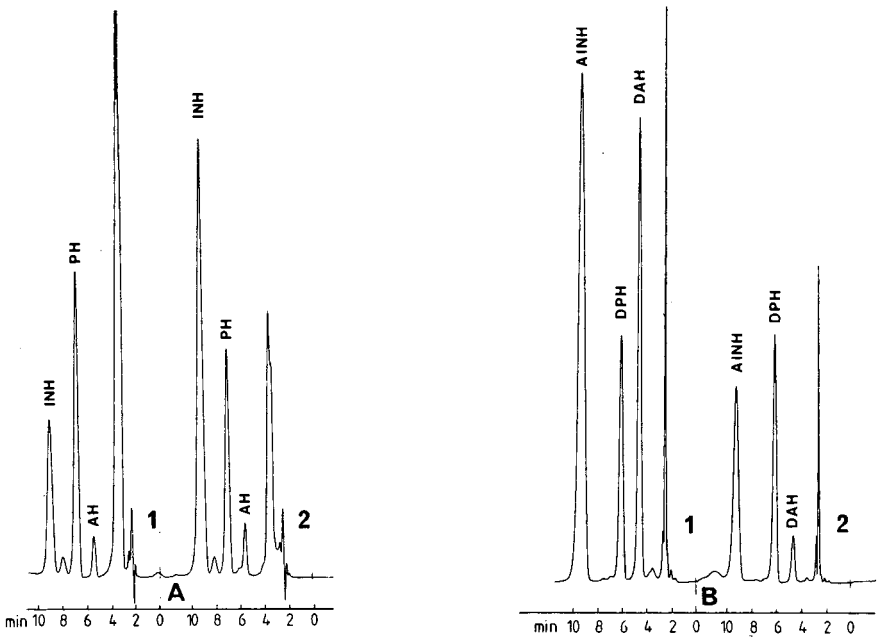

Fig. 2. Chromatograms of urine samples before (A) and after (B) acidic hydrolysis from a rapid (1) and a slow (2) acetylator after intravenous administration of 10 mg/kg isoniazid. Abbreviations as in Fig. 1.

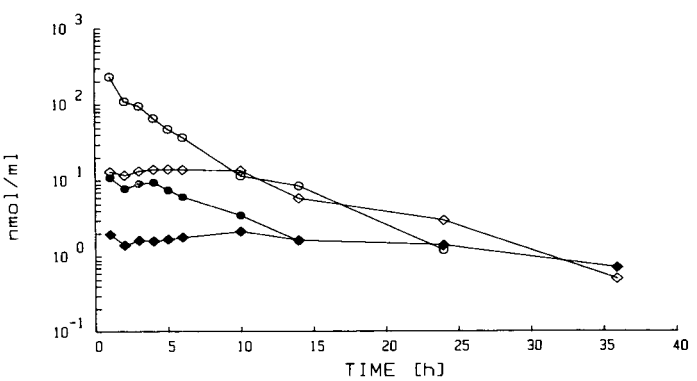

Fig. 3. Plasma level—time course of isoniazid (○), acetylhydrazine (◇), acetylisoniazid (●) and diacetylhydrazine (◆) following intravenous administration of 10 mg/kg isoniazid to a slow acetylator patient.

Sensitivity

The lower limits of detection in plasma and urine at a detector attenuation of 0.04 a.u.f.s. (signal-to-noise ratio of 5:1) are listed in Table I.

Precision

The precision for the determination of INH and its hydrazine metabolites was obtained by analysing pooled plasma and urine samples. The coefficients of variation (ten samples of each analysed) are listed in Table II.

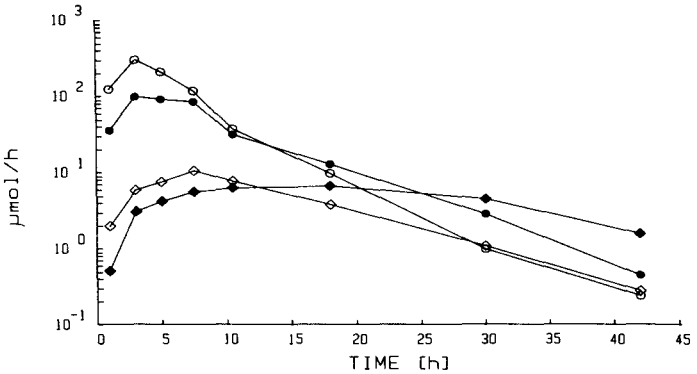

Fig. 4. Urinary excretion rates of isoniazid (○), acetylhydrazine (◇), acetylisoniazid (●) and diacetylhydrazine (◆) following intravenous administration of 10 mg/kg isoniazid to a slow acetylator patient.

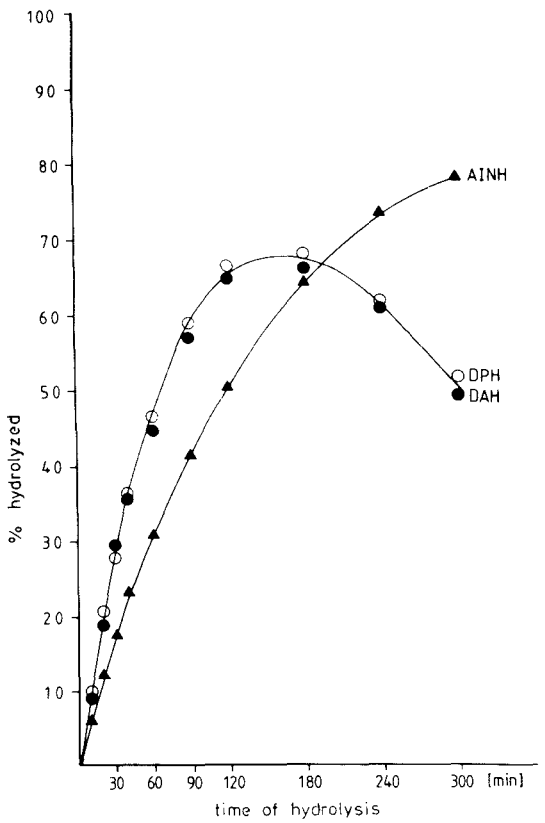

Fig. 5. Time course of the hydrolysis of diacetylhydrazine (●, DAH), dipropionylhydrazine (○, DPH) and acetylisoniazid (▲, AINH) to acetylhydrazine, propionylhydrazine and isoniazid.

TABLE I

LOWER LIMITS OF DETECTION FOR THE DETERMINATION OF ISONIAZID (INH), ACETYLHYDRAZINE (AH), ACETYLISONIAZID (AINH) AND DIACETYLHYDRAZINE (DAH) IN PLASMA AND URINE

Values are expressed in nmol/ml.

	INH	AH	AINH	DAH
Plasma	1.0	0.5	2.0	1.0
Urine	15.0	10.0	40.0	20.0

TABLE II

PRECISION OF THE DETERMINATION

Plasma and urine samples obtained from patients who were treated with isoniazid were pooled. Procedure 1: Ten samples of each pool were analysed on the same day. Procedure 2: Twelve samples of each pool were analysed over a period of six (plasma) or twelve (urine) months. Plasma concentrations (nmol/ml): INH, 22.0; AH, 10.1; AINH, 29.4; DAH, 4.8. Urine concentrations (nmol/ml): INH, 168.0; AH, 88.0; AINH, 1197.0; DAH, 147.0. The table shows the coefficients of variation (%).

	Procedure	INH	AH	AINH	DAH
Plasma	1	8.13	5.53	6.98	6.36
	2	8.59	6.39	8.79	8.51
Urine	1	1.61	1.36	4.02	4.05
	2	6.86	4.31	11.64	6.02

Day-to-day precision was obtained by analysing pooled urine and plasma samples on different days. For the plasma samples it was done within a period of six months, for urine samples within twelve months. The coefficients of variation are listed in Table II. In plasma and urine samples which were stored at −20°C no decrease in INH and metabolite concentrations could be observed over a period of six and twelve months, respectively.

Accuracy

The accuracy of the method was determined by adding known amounts of INH and INH metabolites to drug-free plasma and urine and analysing the samples as described. Tables III and IV show the results of these experiments. The coefficient of correlation found for plasma and urine was 0.99 for each compound.

Selectivity

Selectivity of this method was assessed by collecting the peaks of the INH, AH, AINH and DAH derivatives eluted from the column from plasma and urine samples of patients treated with isoniazid. The mass spectra of those peaks were identical with those obtained for reference compounds (direct inlet system). No other compounds could be detected during the complete evaporation of the samples.

TABLE III

ACCURACY OF THE DETERMINATION OF ACETYLHYDRAZINE (AH), ISONIAZID (INH), DIACETYLHYDRAZINE (DAH) AND ACETYLISONIAZID (AINH) IN PLASMA

Values are expressed in nmol/ml.

Sample	AH		INH		DAH		AINH	
	Added	Found	Added	Found	Added	Found	Added	Found
1	20	19.8	50	46.7	20	19.4	50	51.0
2	40	39.3	100	104.9	40	39.7	100	95.9
3	4	4.4	10	10.3	4	4.3	10	9.7
4	6	6.0	15	14.7	6	6.3	15	16.0
5	10	9.8	25	24.4	10	9.4	25	22.5
6	20	18.6	50	50.7	20	18.1	50	44.4
7	40	38.0	100	100.8	40	36.7	100	92.4
8	4	4.2	10	9.5	4	4.9	10	11.0
9	6	5.8	15	14.4	6	6.2	15	15.8
10	10	9.3	25	24.4	10	10.5	25	26.2

TABLE IV

ACCURACY OF THE DETERMINATION OF ACETYLHYDRAZINE (AH), ISONIAZID (INH), DIACETYLHYDRAZINE (DAH) AND ACETYLISONIAZID (AINH) IN URINE

Results are expressed as nmol/ml.

Sample	AH		INH		DAH		AINH	
	Added	Found	Added	Found	Added	Found	Added	Found
1	20	20.3	100	103.6	100	96.6	250	255.8
2	400	382.8	500	498.5	50	50.3	500	466.2
3	50	51.9	1000	985.5	100	94.8	150	141.5
4	200	183.9	500	500.0	100	110.9	1000	972.3
5	20	20.9	125	124.8	500	471.5	100	105.7
6	50	50.0	50	47.3	150	140.0	100	90.1
7	30	28.2	250	233.6	50	53.5	500	472.0
8	10	10.3	100	110.1	50	55.4	1000	962.7
9	200	182.9	50	48.4	100	100.4	1000	1083.1
10	100	97.3	500	494.0	200	187.8	500	546.0

DISCUSSION

The results indicate that the method described is sensitive, selective, precise and accurate. With the exception of the recently described method of Lauterburg et al. [8] which requires GC—MS, this is the only method which uses internal standards. The use of an internal standard is especially critical for the analysis of AINH and DAH. The measurement of these metabolites requires acidic hydrolysis to generate INH and AH which are then derivatized. In order to correct for differences in the kinetics of this hydrolysis reaction the use of an internal standard that is also hydrolysed is of the utmost importance.

In comparison to the use of aldehydes to form the corresponding imines — where only 90% is derivatized — derivatization with benzoyl chlorides yields a

nearly complete reaction (98%). A complete derivatization of INH and AH should be achieved, since non-derivatized INH and AH will interfere with the analysis of AINH and DAH. In adition, the benzoyl derivatives formed are stable in an aqueous solution at acidic pH in contrast to Schiff bases, which is of importance during the hydrolysis step since extraction of the derivatives is not complete. The use of two derivatizing reagents yields derivatives of different chromatographic behaviour and thus avoids interference from non-extracted derivatives formed before hydrolysis. Our initial attempt to separate and quantitate the chloro- and fluorobenzoyl derivatives in single-step chromatography was not possible, since one endogenous compound present in plasma and urine interfered with the determination of AINH.

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CHROMBIO. 2403

QUANTITATIVE DETERMINATION OF TOLAZOLINE IN SERUM AND URINE

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SUMMARY

A high-performance liquid chromatographic procedure is described for the analysis of tolazoline in serum and urine. This assay procedure is suitable for the analysis of micro-samples (50 or 100 μl serum and 100 μl urine). Samples are extracted in a single step and injected into a reversed-phase high-performance liquid chromatography system for detection at 210 nm. The clinical applicability of this assay is demonstrated by the determination of tolazoline serum and urine concentrations in neonates. In addition, the presence of urine conjugates and the extent of serum protein binding were investigated. This assay procedure has the required sensitivity (0.1 μg/ml), accuracy and precision for both routine monitoring and pharmacokinetic characterization of tolazoline in neonates and adults.

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INTRODUCTION

Tolazoline [4,5-dihydro-2-(phenylmethyl)-1H-imidazole] has been used as a vasodilator in the treatment of several peripheral vascular disorders in adults since the 1940s [1]. The pharmacology of this agent is complex, with studies demonstrating histaminergic, α-adrenergic blocking, sympathomimetic, parasympathomimetic and direct vasodilator activities [2, 3]. Several recent investigations have reported the use of tolazoline for the treatment of neonatal pulmonary hypertension [4, 5]. While tolazoline has been reported to be successful in the treatment of this disorder, a high incidence of adverse effects has been associated with its use in neonates [5, 6]. This narrow range between therapeutic and toxic doses and the paucity of information available on the pharmacokinetics of tolazoline in neonates [7] indicate the need to be able to monitor serum concentrations in these patients. Further, since the drug appears to be primarily excreted by the kidney [8], the determination of both serum and urine concentrations of tolazoline is required to accurately characterize its pharmacokinetics in neonates.

Several methods for the determination of tolazoline have previously been reported [8—11]. These methods were not suitable for the analysis of tolazoline in neonates because of the lack of specificity and sensitivity, and large sample volume requirements (> 200 μl). One previously published method has the desired sensitivity and sample volume size, but requires the use of a mass spectrometer and an extensive sample workup procedure involving extraction, freezing at 0°C and derivatization [12]. In addition, none of the previously published assay procedures has been utilized for the analysis of both serum and urine.

We now report a high-performance liquid chromatographic (HPLC) assay for the quantitation of tolazoline in serum and urine. This assay is a rapid, one-step extraction procedure and is suitable for the analysis of microsamples (50—100 μl). The clinical applicability of this method is shown by the analysis of tolazoline serum and urine samples from neonatal patients.

MATERIALS AND METHODS

Standards and reagents

Tolazoline hydrochloride was furnished by Ciba Pharmaceutical (Summit, NJ, U.S.A.). Naphazoline hydrochloride (internal standard), monobasic potassium phosphate (KH_2PO_4) and β-glucuronidase (EC 3.2.1.31) were obtained from Sigma (St. Louis, MO, U.S.A.). ACS reagent grade potassium bicarbonate ($KHCO_3$), potassium carbonate (K_2CO_3), sodium acetate, acetic acid and HPLC grade solvents (acetonitrile and methylene chloride) were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). HPLC quality water was obtained through a Millipore Milli-Q™ water purification system (Bedford, MA, U.S.A.) which was fed with distilled water.

The mobile phase was composed of phosphate buffer, 0.02 *M* KH_2PO_4, pH 3.75—acetonitrile (3:2, v/v). The phosphate buffer was filtered through a 0.45-μm Millipore HA filter and then passed through a Waters μBondapak® C_{18} column to remove organic compounds which had produced an unstable

chromatographic baseline. The buffer pH was adjusted to 3.75 with 85% phosphoric acid. The phosphate buffer and acetonitrile were then mixed and filtered through a Nuclepore polyester 0.4-μm membrane filter (Pleasanton, CA, U.S.A.).

Extraction procedure

The extraction of tolazoline and internal standard was performed in a single procedure. A serum or urine sample (50 or 100 μl) was placed into a 15-ml conical tube along with 30 μl of the internal standard aqueous solution (1.0 μg/ml for serum and 100 μg/ml for urine), 250 μl of extraction buffer (0.1 *M* $KHCO_3$, 0.1 *M* K_2CO_3, pH 10.0) and 5.0 ml of methylene chloride. The contents were sealed with a PTFE-lined cap, shaken mechanically for 20 min and centrifuged for 5 min at 1500 *g*. The aqueous layer at the top was removed by aspiration. The organic layer was transferred to a clean 75 × 100 mm tube and evaporated to dryness under a gentle stream of nitrogen in a water bath at 35°C. The dry residue was reconstituted with 100 μl (500 μl for urine samples) of 0.02 *M* KH_2PO_4, pH 3.75, and vortexed for 15 sec. The reconstituted serum extract was transferred to a WISP™ limited volume insert sample vial and 90 μl were injected into the chromatograph. For urine samples, the reconstituted extract was transferred into a limited volume glass insert (Sun Brokers, Wilmington, NC, U.S.A.) and 10 μl were injected into the chromatograph.

Liquid chromatographic conditions

A Waters Assoc. Model 6000A chromatography pump (Milford, MA, U.S.A.) was used to control the flow of the mobile phase at 1.2 ml/min. Samples were injected into the system using a WISP Model 710B sample processor (Waters Assoc.). A Whatman® guard column (5 cm × 2 mm I.D.) filled with Co:Pell ODS (Clifton, NJ, U.S.A.) was placed in series with the analytical column, a Waters Resolve® column, 5-μm Spherical C_{18}. The detection of the eluted peaks was accomplished by a Kratos Spectro-Flow 773 variable-wavelength detector (Westwood, NJ, U.S.A.) operated at a wavelength of 210 nm. The retention times for tolazoline and naphazoline were 7 and 10 min, respectively. The detector signal was recorded and peak area quantitated with a Hewlett-Packard 3390A integrator (Avondale, PA, U.S.A.).

Hydrolysis of urine conjugates

Since our preliminary pharmacokinetic studies in neonates found only about 80% of the dose excreted unchanged in the urine [13], three methods known to hydrolyze urine conjugates were performed to determine if conjugation represented another route for tolazoline elimination. For each hydrolysis method, three sets of samples were analyzed. The first set was drug-free urine to determine whether the hydrolysis procedure resulted in any compounds which would interfere with the assay. The second set consisted of drug-free urine spiked with known amounts of tolazoline to confirm the stability of tolazoline during the hydrolysis procedure. The third set was urine samples from patients receiving tolazoline. All samples were analyzed for tolazoline, using the above HPLC procedure, both before and after the hydrolysis procedure.

Acid hydrolysis was performed by adding 0.5 ml of concentrated hydrochloric acid to a 0.5-ml aliquot of urine. The samples were placed in PTFE-sealed glass tubes in boiling water for 1 or 6 h. A third set of samples was allowed to stand at room temperature for 1 h. After removal from the water bath, the samples were allowed to cool and then neutralized by the addition of 0.5 ml of 12 *M* sodium hydroxide. An aliquot (100 μl) was removed and extracted according to the procedure outlined above.

Basic hydrolysis was performed by adding 0.5 ml of 12 *M* sodium hydroxide to a 0.5-ml aliquot of urine. The samples were placed in PTFE-sealed glass tubes in boiling water for 1 or 6 h. A third set of samples was allowed to stand at room temperature for 1 h. After cooling, an aliquot (100 μl) was extracted according to the procedure outlined above.

Enzyme hydrolysis was performed by adding 0.5 ml of acetate buffer and 100 μl of β-glucuronidase (approx. 1000 U/ml) to a 0.5-ml aliquot of urine. The samples were incubated at 37°C for 24 h. An aliquot (100 μl) of the hydrolyzed sample was then extracted using the procedure outlined above.

Equilibrium dialysis procedure

The extent of tolazoline binding to serum proteins was examined by equilibrium dialysis. A Spectrum equilibrium dialysis system was used with 1.0-ml PTFE cells and Spectra/Por®-2 membranes (Spectrum Medical Industries, Los Angeles, CA, U.S.A.). Serum, 0.7 ml, was introduced into one compartment of the cell and dialyzed against 0.7 ml of 0.01 *M* phosphate buffer (pH 7.4) made isotonic with sodium chloride. During dialysis the cells were placed in a water bath at 37°C and rotated at 10 rpm. At the end of the dialysis procedure, the concentration of tolazoline in both compartments was assayed by the HPLC procedure described above. The time to reach equilibrium was determined by dialyzing blank adult serum spiked with a known amount of tolazoline for 0, 30, 45, 60, 90, 120 and 240 min. When the time to reach equilibrium had been established, replicate samples of adult (n = 3) and neonatal (n = 2) serum spiked with tolazoline were dialyzed on three different occasions to determine the extent of serum protein binding.

RESULTS AND DISCUSSION

The serum standard curve was linear over the range of tolazoline concentrations from 0.1 to 15.0 μg/ml. The standard curve was found to be stable over a seven-week period with a coefficient of variation for the slope of 4.2% for nine curves run over this time period. Pooled adult serum was used for the standard curve and control samples because of the lack of availability of large amounts of blank neonatal serum. No difference was observed in chromatograms obtained from adult and neonatal serum samples (Fig. 1).

In order to improve the sensitivity and allow for a smaller sample size as compared to the previously published HPLC procedure [11], a new extraction procedure was developed using a new internal standard. The new procedure resulted in an extraction efficiency of 95.6 ± 2.3% S.D. at a concentration of 1.0 μg/ml. The minimum detectable concentration was 0.1 μg/ml with a coefficient of variation for replicate samples (n = 5) of 10.3%. No interference

from endogenous compounds in the serum was observed (Fig. 1). The assay procedure was also examined for possible interference from hemolyzed serum or other drugs (Table I). Serum samples spiked with these drugs and a blank hemolyzed neonatal serum sample were extracted by the same procedure as the tolazoline serum samples. No peaks which would interfere with tolazoline or internal standard were observed.

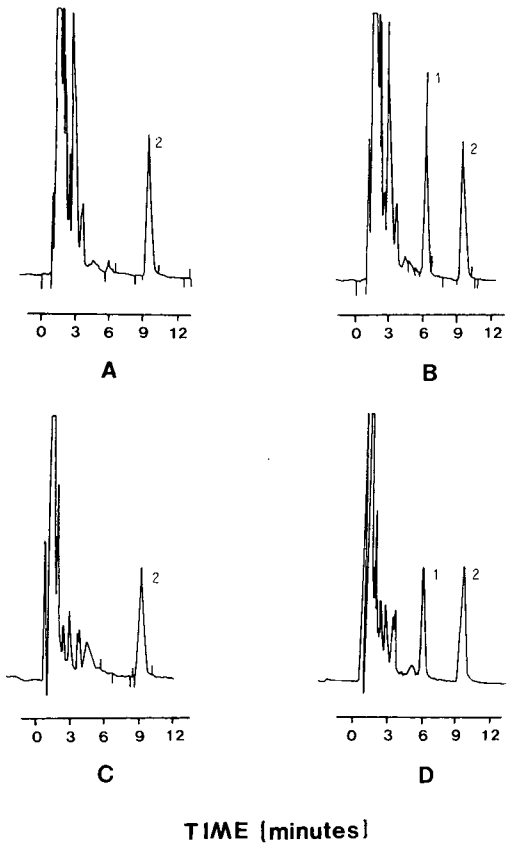

Fig. 1. Chromatograms comparing adult and neonate serum extracts. (A) Neonate blank with internal standard; (B) neonate patient with a measured tolazoline serum concentration of 0.76 μg/ml; (C) blank adult serum with internal standard; (D) adult serum spiked with 0.39 μg/ml tolazoline. Peaks: 1 = tolazoline; 2 = naphazoline, internal standard.

TABLE I

DRUGS TESTED FOR INTERFERENCE IN THE ASSAY PROCEDURE

Carbamazepine	Digoxin	Acetaminophen
Ethosuximide	Disopyramide	Chloramphenicol
Phenobarbital	Lidocaine	Desipramine
Phenytoin	Methotrexate	Dopamine
Primidone	Procainamide	Gentamicin
Valproic acid	N-Acetyl procainamide	Imipramine
	Propranolol	Salicylates
	Quinidine	
	Theophylline	

The accuracy and precision data for the serum assay based on five determinations at five different concentrations are summarized in Table II. Neonatal patients receiving the standard dosage regimens were found to have tolazoline serum concentrations ranging from 2.0 to 13.8 μg/ml. The intra-day and inter-day coefficients of variation in this concentration range were less than 5% and 8%, respectively.

The standard curve for the urine assay was linear over the concentration range 0.03—2.0 mg/ml and was stable over a ten-week period with a coefficient of variation for the slope of 3.9% for nine curves run over this time period. The accuracy and precision data for the urine assay based on four determinations at four different concentrations are summarized in Table III. Adult, drug-free urine was used to prepare standard curve and control samples. No difference was observed in chromatograms obtained from adult and neonatal urine samples (Fig. 2). Urine samples obtained from neonates receiving tolazoline had urine concentrations ranging from 0.03 to 0.8 mg/ml.

The results of the three urine hydrolysis procedures are summarized in Table IV. No evidence for the presence of urine conjugates of tolazoline could be found from any of the three procedures. The three urine samples spiked with a known amount of tolazoline had a slight decrease in tolazoline concentration after 1 and 6 h of acid hydrolysis owing to a slow breakdown of tolazoline in the acid medium. A similar decrease in tolazoline concentration was observed in the two neonatal patient samples. Following basic hydrolysis for 1 and 6 h, both spiked and patient samples had extensive breakdown of

TABLE II

SERUM TOLAZOLINE ASSAY PRECISION AND ACCURACY

Theoretical control concentration (μg/ml)	Mean measured concentration (μg/ml)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)
0.25	0.27	9.6	10.0
0.74	0.73	6.6	6.1
1.99	2.00	4.2	7.7
3.14	2.74	3.6	5.5
12.04	11.93	2.0	1.3

TABLE III

URINE TOLAZOLINE ASSAY PRECISION AND ACCURACY

Theoretical control concentration (mg/ml)	Mean measured concentration (mg/ml)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)
0.055	0.055	9.3	8.2
0.45	0.44	2.1	7.0
0.90	0.87	7.3	10.0
1.80	1.84	4.9	7.0

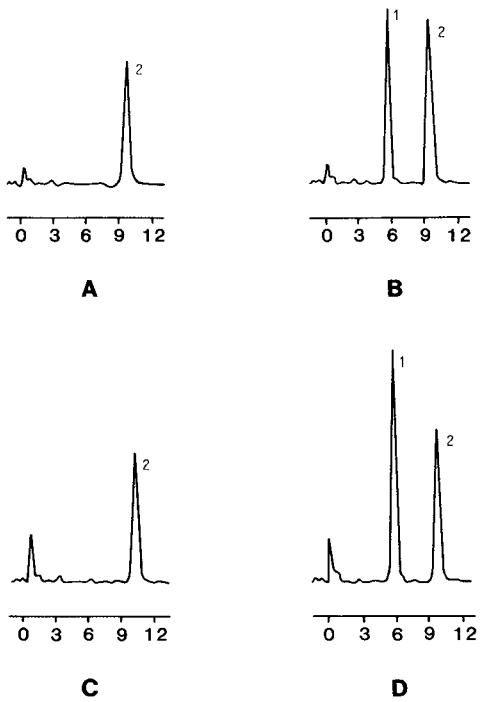

Fig. 2. Chromatograms comparing adult and neonate urine extracts. (A) Neonate blank with internal standard; (B) neonate patient with a measured tolazoline urine concentration of 0.09 mg/ml; (C) blank adult urine with internal standard; (D) adult urine spiked with 0.12 mg/ml tolazoline. Peaks: 1 = tolazoline, 2 = naphazoline, internal standard.

TABLE IV

URINE HYDROLYSIS

Results are expressed as percent of prehydrolysis tolazoline concentration.

Sample	Acid hydrolysis*			Basic hydrolysis*			Enzyme hydrolysis
	0 h**	1.0 h	6.0 h	0 h**	1.0 h	6.0 h	24 h
Spike No. 1	100.0	84.6	84.6	100.0	1.1	0	105.5
Spike No. 2	101.1	85.7	85.7	88.9	1.1	0	105.5
Spike No. 3	107.7	92.3	92.3	100.0	2.5	0	110.0
Patient No. 1	94.1	88.2	88.2	70.6	5.9	0	100.0
Patient No. 2	111.1	91.7	75.0	72.2	2.8	0	111.1

*Heated in a boiling water bath for time indicated.
**Allowed to stand at room temperature for 1 h.

tolazoline. The samples which were incubated in base at room temperature for 1 h showed only a minimal change in tolazoline concentration. Enzyme hydrolysis with β-glucuronidase produced no change in tolazoline concentration for either the spiked or neonatal patient samples. These results would

indicate that conjugation of tolazoline in neonates does not represent a major route of elimination.

The equilibrium dialysis procedure indicated minimal (< 10%) serum protein binding of tolazoline in either adults or neonates.. Equilibrium was achieved in 60 min. The mean recovery of tolazoline following the 60-min dialysis procedure was 100.4 ± 2.5% S.D. (n = 5). The intra-day and inter-day coefficient of variation for replicate samples (n = 3) with the dialysis procedure was 6.0 and 8.2%, respectively. The mean percent of tolazoline bound to serum proteins was 6.9% and 6.3% in adult and neonatal serum, respectively.

This assay procedure, therefore, has the sensitivity, accuracy and precision needed for both routine monitoring and pharmacokinetic characterization of tolazoline in neonates and adults. This method may be used to quantitate tolazoline in both serum and urine. While the procedure has been described here using 100-μl samples, the sensitivity of the assay procedure allows for a sample volume as small as 50 μl to be used when sample size is limited. This small sample size requirement and lack of interference from other drugs and hemolyzed samples make the procedure especially suitable for neonates where serum samples are difficult to obtain and sample volumes are limited. The simple and rapid extraction procedure allows the analysis of a large number of samples at one time. This method is currrently being used to study the pharmacodynamics and pharmacokinetics of tolazoline in neonates with pulmonary hypertension.

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CONCURRENT DETERMINATION OF VALPROIC ACID WITH OTHER ANTIEPILEPTIC DRUGS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We describe a method for the simultaneous determination of valproic acid with four other antiepileptic drugs (phenobarbital, phenytoin, carbamazepine, and primidone) in plasma by high-performance liquid chromatography. These drugs are extracted from plasma by adding a small volume of acetonitrile following saturation with ammonium sulphate. An aliquot of the extract is then injected on a reversed-phase column with a methanol—water mobile phase. The total time required for the whole analytical process including the plasma pretreatment and chromatography is approximately 20 min. The assay method is simple, rapid, reproducible and specific, and considered, therefore, suitable for both emergency and routine uses in monitoring these antiepileptic drugs simultaneously.

INTRODUCTION

Therapeutic monitoring of antiepileptic drug(s) (AED) is an aid in the clinical management of patients with seizure disorders [1]. Monitoring AEDs is desirable mainly for adjusting dosage, avoiding side-effects and assessing patient compliance [2]. Among AEDs the use of valproic acid (VPA) to treat epilepsy is increasing, because it has a wider spectrum of activity [3—5] and less apparent central nervous system toxicity than other AEDs [5]. VPA can be used not only alone [6, 7] but also in combination with other AEDs [3]. Other AEDs are known to affect the pharmacokinetics of VPA [8—10].

The simultaneous assay of VPA with phenobarbital (PB) [11] or ethosuximide [12] by the use of high-performance liquid chromatography (HPLC) has been reported. To our knowledge, there has been no report of a method for the simultaneous HPLC determination of VPA and two or more

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AEDs. The physicochemical characteristics of VPA include poor ultraviolet absorption and easy volatility. These are in contrast to other AEDs and seem to preclude the simultaneous determination by usual HPLC methods. Therefore, in order to determine VPA and other AEDs simultaneously with an HPLC method, several modifications of the usual analytical procedures are required.

The purpose of this report is to describe a method for the simultaneous HPLC quantification of VPA, PB, phenytoin (PHT), carbamazepine (CBZ) and primidone (PRM).

MATERIALS AND METHODS

Reagents

Acetonitrile, methanol and tetrahydrofuran (all of chromatography grade), hydrochloric acid, phosphoric acid, sodium phosphate dibasic and ammonium sulphate (all of reagent grade) were purchased from Wako (Osaka, Japan).

A 1 *M* solution of hydrochloric acid, saturated with ammonium sulphate was prepared by adding sufficient granular ammonium sulphate to 1 *M* hydrochloric acid until no more could be dissolved, and by agitating for a few minutes. A phosphate buffer, 0.05 *M*, pH 5.9, was prepared by dissolving 17.9 g of $Na_2HPO_4 \cdot 12H_2O$ in 1 l of distilled water. The pH was adjusted to 5.9 with phosphoric acid.

Standards

VPA in the form of sodium valproate was donated by Kyowa Hakko Kogyo (Tokyo, Japan), PB, PHT, and PRM by Dainippon Pharmaceutical (Osaka, Japan), and CBZ by Ciba-Geigy (Osaka, Japan). 4-Methylprimidone was purchased from Aldrich (Milwaukee, WI, U.S.A.).

A stock solution containing 1000 μg/ml sodium valproate (which is converted to approximately 867.6 μg/ml as VPA), 200 μg/ml PB, and 100 μg/ml each of PHT, CBZ and PRM was prepared in methanol. This solution was further diluted with methanol to the required concentration for each drug. Another stock solution of 10 μg/ml of the internal standard, 4-methylprimidone, was prepared in acetonitrile. All solutions were stored at 4°C.

Apparatus

Our HPLC system was composed of an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.), a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), and a UVILOG-5 III variable-wavelength ultraviolet detector (Oyo Bunko Kiki, Tokyo, Japan). Analyses were performed on a Hibar LiChrosorb RP-18 column (250 × 4 mm, I.D., 5 μm particle size). Detector output was recorded at 10 mV with a Hitachi Model 056 recorder (Hitachi, Tokyo, Japan). The chromatographic data were processed by a Model 5000E integrator (System Instruments, Tokyo, Japan). Other equipments included 1.5-ml stoppered conical polypropylene test tubes (Sarstedt), a bench-top vortex-type mixer, and centrifuge.

Assay procedure

Each 100-μl plasma sample was added to a 1.5-ml Sarstedt centrifuge tube

containing 200 μl of 1 *M* hydrochloric acid saturated with ammonium sulphate and the tube was vortexed for 20 sec. Then 60 μl of acetonitrile containing the internal standard were added to the tube, which was stoppered, vortexed for 20 sec and then centrifuged at 2700 *g* for 5 min to separate the acetonitrile layer from the aqueous phase. A 5—10 μl aliquot of this acetonitrile layer was injected into the chromatograph.

The chromatographic conditions were set as follows: column temperature, 50° C; mobile phase, methanol—tetrahydrofuran—phosphate buffer, 0.05 *M*, pH 5.9 (44:1:55, v/v); flow-rate, 1.1 ml/min; wavelength, 210 nm; detector range, 0.005—0.16 absorbance unit full scale (a.u.f.s.).

Quantitation

Plasma standards were prepared by adding known amounts of AEDs to pooled plasma free of any drug to give concentrations required for each of their calibration curves. The calibration curve was constructed for each drug, using analyte/internal standard peak height ratios. The ratio for an unknown sample was converted to the concentration by use of this calibration curve.

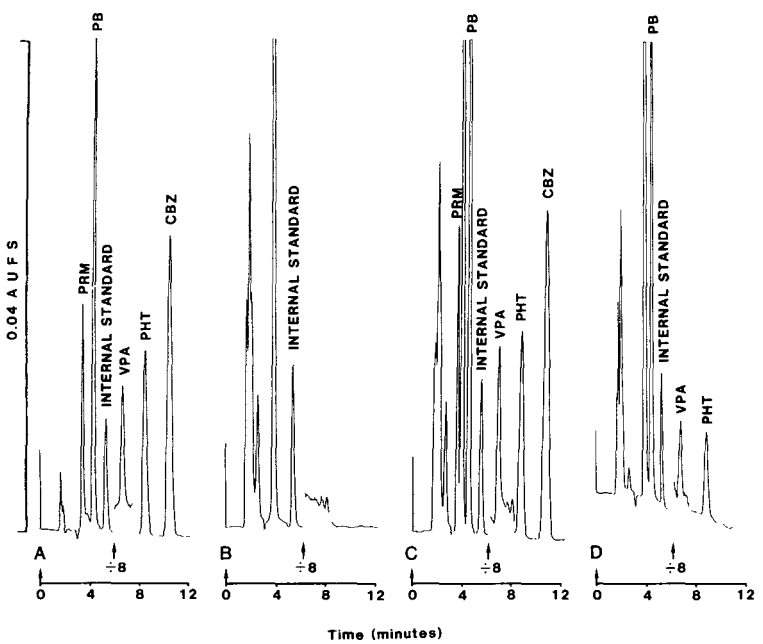

Fig. 1. Chromatograms obtained from: (A) a standard mixture of sodium valproate (1 μg), PB (0.2 μg), PHT (0.1 μg), CBZ (0.1 μg), PRM (0.1 μg) and internal standard (0.06 μg) without extraction; (B) blank plasma; (C) 100 μl of plasma spiked with sodium valproate (10 μg), PB (2 μg), PHT (1 μg), CBZ (1 μg) and PRM (1 μg); and (D) plasma of an epileptic patient, who was taking sodium valproate, PB and PHT concomitantly, with extraction according to the procedure. Plasma concentrations of AEDs of the patient are 45.5 μg/ml for VPA, 17.7 μg/ml for PB, and 4.9 μg/ml for PHT. Detector range was set at 0.04 a.u.f.s. except for VPA, for which it was set at 0.005 a.u.f.s. Multiplying the concentration of sodium valproate by the conversion factor of 0.8676 gives the concentration of VPA. PB = phenobarbital; PHT = phenytoin; CBZ = carbamazepine; PRM = primidone; VPA = valproic acid.

Solutions of all five analytes in drug-free plasma were assayed in triplicate. The concentration ranges employed were 10—500 μg/ml for sodium valproate (approximately 8.7—433.8 μg/ml as VPA), 2—100 μg/ml for PB and 1—50 μg/ml for each of PHT, CBZ and PRM. In all cases it was confirmed in the separate experiments that the calibration curves were linear for at least these concentration ranges. Linear regression analyses were performed for each analyte relative to the internal standard.

RESULTS

Chromatography

With our chromatographic conditions, VPA, PB, PHT, CBZ, PRM and the internal standard all exhibited symmetrical peaks. Fig. 1A shows a typical chromatogram for the drug standard. No interfering peak was observed when the blank plasma extract was analysed (Fig. 1B). Fig. 1C is a chromatogram from a spiked plasma sample containing known quantities of five AEDs [sodium valproate (100 μg/ml), PB (20 μg/ml), PHT, CBZ and PRM (10 μg/ml each)]. Fig. 1D is a chromatogram from plasma of an epileptic patient who was taking sodium valproate, PB and PHT concomitantly.

As can be seen in Fig. 1, it is necessary to switch the detector range from 0.04 to 0.005 a.u.f.s. to obtain an appropriate chromatographic peak for VPA. Except for VPA detected at 0.005 a.u.f.s., the peaks of the other four AEDs

TABLE I

PRECISION OF ASSAYS FOR ANTIEPILEPTIC DRUGS IN PLASMA

Drug	Concentration* (μg/ml)	C.V. (%)	
		Within-day (n = 5)	Day-to-day (n = 12)
Sodium valproate	500	4.2	5.6
	100	2.7	6.1
	25	3.0	7.2
Phenobarbital	100	1.6	2.1
	20	2.1	4.4
	5	1.0	2.7
Phenytoin	50	3.0	6.1
	10	4.5	6.0
	2.5	4.9	5.7
Carbamazepine	50	2.8	4.2
	10	3.5	5.0
	2.5	2.9	4.7
Primidone	50	2.9	5.3
	10	2.0	4.6
	2.5	4.2	4.4

*To convert concentrations of sodium valproate to those of valproic acid, multiply by 0.8676.

were adequate at 0.04 a.u.f.s. The capacity ratios (k') for PRM, PB, VPA, PHT, and CBZ were 1.00, 1.45, 2.75, 3.80, and 4.70, respectively, and 2.00 for the internal standard.

Analytical variables

Precision. We assessed the precision of the method by repeated analyses of plasma specimens containing known concentrations of the drugs being investigated. As given in Table I, the coefficient of variation (C.V.) for the within-day precision ranged from 1.0 to 4.9% (n = 5), and that of the day-to-day precision from 2.1 to 7.2% (n = 12).

Recovery. We measured the absolute analytical recovery from plasma of the

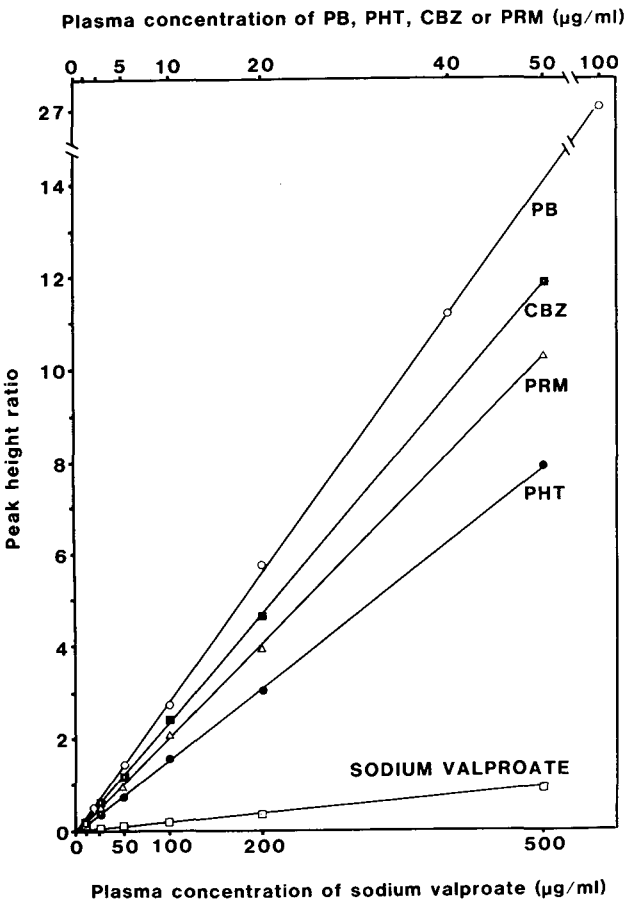

Fig. 2. Calibration curves for sodium valproate, PB, PHT, CBZ and PRM in plasma. A three-point standard curve was prepared by plotting on the ordinate the ratio of each compound's peak height to that of the internal standard for each concentration. Linear regression analysis of calibration curve data indicated no significant deviation from linearity (r = 0.9986—0.9997). In addition, intercept values did not significantly differ from zero. (□) Sodium valproate; (○) phenobarbital (PB); (●) phenytoin (PHT); (■) carbamazepine (CBZ); (△) primidone (PRM). Multiplying the concentration of sodium valproate by the conversion factor of 0.8676 gives the concentration of valproic acid.

five drugs in the following way. The drugs were added to drug-free plasma to achieve the midpoint concentrations as given in Table I. This plasma was then analysed by our method. Carefully measured aliquots of the acetonitrile layer were then injected and their peak heights measured. Absolute recovery was calculated by comparing these peak heights with the peak heights obtained by the direct injection of the pure drug standards. Absolute recoveries of AEDs were 89.6%, 104.0%, 79.2%, 83.9%, and 91.8% for VPA, PB, PHT, CBZ, and PRM, respectively.

Linearity and sensitivity. Plasma standards were prepared containing various known amounts of each drug. A constant amount of the internal standard was added to each sample. Concentration and peak height ratio correlated linearly with each other for all AEDs examined. All the calibration curves passed through the origin (Fig. 2).

With the 100-μl volume of sample used in the present assay method, the sensitivity was such that the drugs can be detected at plasma concentrations as low as 3 μg/ml for sodium valproate (approximately 2.6 μg/ml for VPA), and at least as low as 0.05 μg/ml for the other four AEDs.

Interference. To determine the potential clinical usefulness of our assay method, we examined the possible chromatographic interference from several other compounds including pharmacologically active metabolites derived from their parent drugs (CBZ and PRM) and clinically commonly used drugs, which may be administered concurrently with VPA. Table II summarizes the data for capacity ratios of the tested compounds. None of the compounds showed any potential interference with the present assay method.

TABLE II

CAPACITY RATIOS FOR SELECTED COMPOUNDS

Compound	Capacity ratio k'	Compound	Capacity ratio k'
Acetaminophen	0.21	4-Methylprimidone	2.00
Salicylic acid	0.25	Valproic acid	2.75
Ethylphenylmalonamide	0.38	Methylphenobarbital	3.10
Theophylline	0.39	Phenytoin	3.80
Caffeine	0.45	Glutethimide	4.37
Ethosuximide	0.53	Pentobarbital	4.42
Primidone	1.00	Carbamazepine	4.70
Chloramphenicol	1.35	Lidocaine	7.80
Phenobarbital	1.45	Diazepam	15.25
Carbamazepine-10,11-epoxide	1.57		

DISCUSSION

Various HPLC techniques for simultaneously determining the plasma concentrations of AEDs have been reported [11—22]. However, to our knowledge, there have been only two reports where VPA and PB [11] or VPA and ethosuximide [12] were measured simultaneously by HPLC.

There seem to be several reasons that simultaneous assays for VPA and other AEDs have rarely been developed. First, VPA has a poor absorbance in the

ultraviolet range. Derivatization for detection enhancement [23—26] or other technical alterations (i.e. a colorimetric procedure based on variation in colour of a solution of bromocresol purple [11] or controlled evaporation using the Technicon Evaporation-to-Dryness Module and a low wavelength of 210 nm for adequate detection sensitivity [12]) seem to be necessary. In contrast, AEDs other than VPA show good ultraviolet absorbance at low wavelengths, and no derivatization is required. Secondly, since VPA is a volatile compound [12, 26, 27], extreme care must be taken to avoid losses due to volatilization when concentrating sample extracts. In general, when compounds have a low ultraviolet absorbance, one would attempt to derivatize them for detection enhancement and/or to evaporate the extract for their enrichment. Obviously, the volatility of VPA precludes any evaporation procedure. Thirdly, the chemical structure of VPA differs from that of other AEDs, VPA being a branched-chain carboxylic acid and lacking nitrogen and a ring moiety [3]. The optimum conditions for separating VPA from other AEDs on a chromatogram, where VPA and other AEDs are simultaneously detected within a given time period and the method used for clinical application, seem somewhat difficult to be set because of their different chemical structures and associated physicochemical properties.

In order to overcome the several difficulties associated with the simultaneous determination of VPA and the four other AEDs as discussed above, we attempted several modifications. First, we tried to derivatize VPA according to the various methods reported [23—26]. However, those methods were found not to be specific for VPA; namely, the structural portion ($\succ C{=}O$) of VPA and the other AEDs reacted on derivatization with phenacyl bromide [23, 26], 1-chlormethylisatin [24] and 4-bromophenacyl bromide [25]. We cannot find any other method by which VPA is specifically derivatized. Even if such method were found, obtaining adequate capacity ratios of VPA and other AEDs on one chromatogram would be very difficult. Secondly, we tried to extract VPA and other AEDs simultaneously by using organic solvents such as diethyl ether and ethyl acetate and then extracting back into an alkaline aqueous phase without evaporation to avoid loss of VPA due to its easy volatility. However, this procedure resulted in a low recovery of the five analytes (approximately 41—75%). Thirdly, we pretreated samples simply by adding a water-soluble organic solvent to the plasma, and injected the supernatant into the chromatograph. However, this pretreatment method was considered to be inadequate since the volume required becomes larger and the concentration of VPA is decreased.

Therefore, we tried to separate the mixture of water and water-soluble organic solvent into two layers. AEDs except for CBZ are acidic drugs and they should, therefore, be extracted into a water-soluble organic layer when plasma is acidified (with 1 *M* hydrochloric acid). CBZ was also considered likely to dissolve in a water-soluble organic layer. In order to achieve the separation of water and water-soluble organic solvent mixture into the two phases, we subjected the plasma samples to salting-out procedure. The salts tested were sodium chloride, potassium chloride, sodium dihydrogen phosphate, zinc sulphate, magnesium sulphate and ammonium sulphate. Water-soluble organic solvents tested were methanol, ethanol and acetonitrile. The combination of

ammonium sulphate and acetonitrile yielded the best extraction efficiency for all the analytes. In order to increase the enrichment of VPA with this separation step, we set the detector wavelength rather low (210 nm, Fig. 1). Furthermore, we adjusted the pH of the mobile phase to 5.9 since AEDs except for CBZ are acidic drugs with different pK_a values. Finally, we added tetrahydrofuran to the mobile phase to adjust the AEDs' capacity ratios. These procedures were found to be necessary for obtaining satisfactory chromatograms of all the analytes examined.

The extraction technique described for analytes in plasma is simple, rapid, reproducible and specific enough for application to therapeutic monitoring of AEDs. The time required for the pretreatment procedure is 8 min and that for the chromatographic run is 12 min, resulting in a total of 20 min. The coefficients of variation (C.V., %) for both within-day and day-to-day analyses (Table I) were within the performance limits required for medical management [28]. Furthermore, pharmacologically active metabolites that are derived from CBZ and PRM, and several drugs that may be co-administered with VPA and/or AEDs, did not interfere with our assay. The absolute recovery of all the analytes from plasma was > 79%. An excellent linear calibration curve (r = 0.9986—0.9997), passing through the origin, was obtained for each analyte (Fig. 2).

The common use of VPA with other AEDs [3] and the consequent need not only to monitor plasma concentrations of VPA with [3] or without other AED(s) [6, 7] but also to detect any interactions between VPA and other AED(s) [3, 8—10] makes multiple drug analyses in one HPLC system an attractive option. Finally, the HPLC method reported here is the first for simultaneously determining VPA and two or more AEDs. Our method appears to be specific for VPA, PB, PHT, CBZ and PRM, and is simple, rapid, and of sufficient precision for clinical application.

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ANALYSIS OF MEPERIDINE AND NORMEPERIDINE IN SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the simultaneous analysis of meperidine and normeperidine in serum and urine. A 1-ml sample aliquot is extracted into hexane, then back-extracted into a small volume of dilute acid which is injected onto a cyanopropyl analytical column. Absorbance of the column effluent is monitored at 205 nm. Two internal standards are employed, diphenhydramine for meperidine and nordiphenhydramine for normeperidine. Chromatography of the four compounds takes 4 min. Serum concentration—time curves of meperidine and normeperidine are presented for eight healthy subjects following single 70-mg bolus injections of meperidine.

INTRODUCTION

Meperidine (Demerol®, Winthrop) is a narcotic analgesic commonly used for pre-operative sedation and the control of post-operative pain. It is widely used in obstetrics and bone fracture clinics. As with other opioids, the drug has a history of abuse by drug addicts due to its euphoric effect.

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The analysis of meperidine (M) and its primary N-demethylated metabolite, normeperidine (NM) in serum, blood and urine has been accomplished mainly using gas chromatography (GC). Flame-ionization [1, 2], nitrogen—phosphorus [3], electron-capture [4] and mass spectrometric [5] detection have been used. Meperidine itself has presented few analytical difficulties. However, normeperidine has always been derivatized to ensure reproducible recoveries from serum and blood extracts. The above methods have used trifluoroacetic anhydride [1], heptafluorobutyric anhydride [2, 3], trichloroethyl chloroformate [4] and N-methyl-bis-trifluoroacetamide [5] as derivatizing agents. All procedures are labour-intensive, requiring at least three extractions prior to derivatization followed by an evaporation or wash step prior to the chromatography. Radioimmunoassays are described [6] in which the two antibodies raised in rabbits were specific for either M or NM. High-performance liquid chromatography (HPLC) has been described for the analysis of M in pharmaceutical preparations using a reversed-phase C_{18} column [7]. An HPLC procedure has not previously been described for the analysis of M and/or NM in biological materials.

The following is a rapid but precise method for the simultaneous analysis of both compounds from 1 ml of serum or 100 μl of urine. No derivatization is required; chromatography is complete within 4 min. M and NM are quantified in the same chromatogram by using diphenhydramine (D) and nordiphenhydramine (ND) as their respective internal standards.

EXPERIMENTAL

Equipment

HPLC was performed isocratically on a Series 2/2 pumping system coupled to an LC-85 variable-wavelength dual-beam detector which was fitted with a 1.4 μl flow cell (Perkin-Elmer, Norwalk, CT, U.S.A.). Samples were introduced via a Rheodyne 7125 injection valve fitted with a 50-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). Peak area ratios were automatically converted to concentration units with a Sigma 15 integrator (Perkin-Elmer).

A Supelcosil® LC-PCN, 150 mm × 4.6 mm I.D. analytical column protected by a Supelguard® LC-CN guard column, 20 mm × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.) were used. The analytical and guard columns both contained 5-μm spherical silica particles coated with cyanopropyl stationary phase. Narrow bore, 0.07 mm I.D., stainless-steel connection tubing was used from the injector to detector in order to reduce peak broadening.

Mobile phase

All solvents were HPLC grade and were used as received (Fisher, Pittsburgh, PA, U.S.A.). A 15 mmol/l phosphate buffer was prepared by dissolving 2.6 g of dipotassium hydrogen phosphate in 1 l of double-distilled water. This solution was brought to pH 7.0 with 0.9 mol/l orthophosphoric acid and then was passed through a 0.22-μm pore filter under suction (Millipore, Bedford, MA, U.S.A.). The mobile phase was acetonitrile—phosphate buffer—methanol (55:25:20, v/v/v). The solution was degassed by mixing for 20 min. The mobile phase was pumped at 2.5 ml/min and the column effluent was monitored at 205 nm at a sensitivity of 0.04 a.u.f.s.

Serum and urine standards

Meperidine · HCl and normeperidine · HCl were gifts from Winthrop Labs. (Aurora, Ontario, Canada). The internal standard diphenhydramine · HCl was purchased from Sigma (St. Louis, MO, U.S.A.). The second internal standard, nordiphenhydramine · HCl was a gift from Warner-Lambert (Ann Arbor, MI, U.S.A.).

Stock solutions of M, NM, D and ND were prepared in 1 mmol/l orthophosphoric acid at concentrations of 100 μg/ml, expressed as the free bases. The working internal standard solution was prepared from the stock solutions to contain both D and ND concentrations of 1000 ng/ml in 1 mmol/l orthophosphoric acid. Appropriate volumes of stock M and NM solutions were combined in drug-free serum and urine pools to give 50 ml of each standard at 1000, 500, 200, 100, 50 and 10 ng/ml. Aliquots of 1.5 ml were stored in 3-ml polypropylene vials at −20°C. Sera containing M and NM have been reported to be stable for at least eighteen months when frozen at −20°C [2]. The serum- and urine-based standards were used for precision, linearity and recovery studies.

All glassware including extraction tubes, conical tubes and storage containers for stock solutions were silylated with dimethyldichlorosilane (Pierce, Rockford, IL, U.S.A.) in toluene (1:5) for 2 h. Glassware was consecutively rinsed in methanol, chloroform and hexane, then dried in an oven at 100°C.

Sample analysis

To 1 ml of serum in a 20-ml (150 mm × 16 mm) extraction tube were added 100 μl of the combined D + ND internal standard solution, 100 μl of 1 mol/l sodium hydroxide and 5.0 ml hexane. The tube was closed with a PTFE-lined cap and mixed by rotation (60 rpm) for 15 min. After a brief centrifugation, 4.0 ml of the hexane layer were transferred to a 13-ml conical centrifuge tube. The drugs were back-extracted into 80 μl of 1 mmol/l orthophosphoric acid by vortexing vigorously for 30 sec. The two phases were allowed to separate and 50 μl of the aqueous phase were injected. The analysis of urine specimens proceeded in the same way except that 1.0 ml saturated sodium borate buffer, pH 10.2 was used as an alkalinizing agent. The higher concentration of M and NM in urine of clinical specimens necessitated an initial 1:10 dilution into drug-free urine before analysis.

Results were calculated from peak area ratios by the Sigma 15 integrator. It was calibrated daily from extracted 100 ng/ml serum or urine standards. D was used as the internal standard for M while ND was used as the internal standard for NM.

Within-day precision was calculated from ten replicates of each serum and urine standard. Since all volumes were handled quantitatively, the peak area data from the within-day precision study were also used in the calculation of drug recoveries. Drug solutions were prepared in 1 mmol/l orthophosphoric acid at ten times the corresponding serum standard concentrations. A 50-μl injection of these solutions gave peak areas representing 100% recovery for each drug. Recovery was calculated from the ratio of corresponding peak areas in the extracted and unextracted standards. Linearity was also assessed from the within-day precision data by comparing the observed to the expected concentrations for the standards. The average values from the ten injections of

each concentration were used in regression analyses. Day-to-day precision data were obtained from single analyses of all standards on 22 days. The data were gathered over a two-month period during which time over 800 measurements on clinical specimens were also made.

Single-dose response

Eight healthy male volunteers who were receiving no medication each received a 70-mg intravenous dose of M · HCl over 2 min. Blood was drawn via an indwelling heparin lock in a forearm vein contralateral to the injection site. Blood samples (5 ml) were collected into plain Vacutainer® (Becton Dickenson, Rutherford, NJ, U.S.A.) tubes at 5, 10, 15, 20, 30, 45, 60, 75, 90 min and at 2, 3, 4, 5, 6, 8 and 11 h post dose. Venipunctures were used to collect 5-ml blood samples at 24, 48, 72, and 96 h post dose. The tubes were centrifuged, the serum transferred to polypropylene vials and stored frozen at −20°C until analysis. Aliquots of 24-h urine collections were also frozen. All analyses were performed in duplicate.

RESULTS AND DISCUSSION

Typical chromatograms from serum and urine extracts are displayed in Fig. 1. M, D, NM and ND elute at 1.5, 1.9, 2.6 and 2.9 min, respectively. A valley in the baseline is evident at about 4 min. Its exact elution time varied with each batch of mobile phase but did not interfere with drug peak area quantitations. The valley appears following the injection of aqueous or organic solvents other than the mobile phase. Although each batch of phosphate buffer was brought

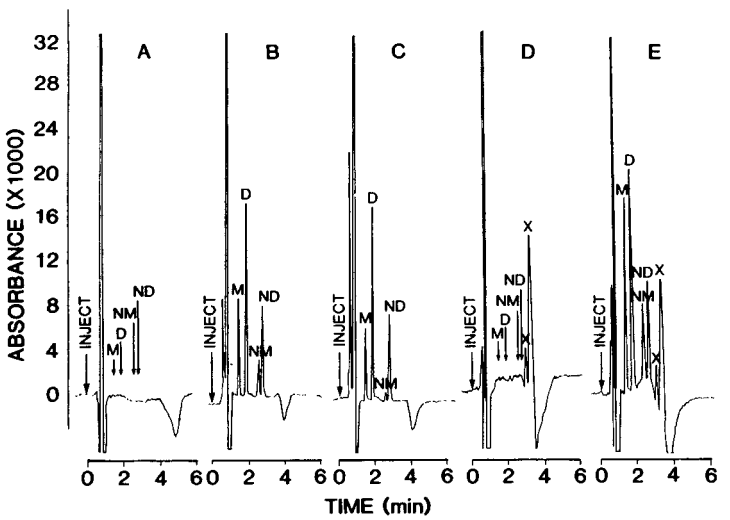

Fig. 1. Chromatograms from serum and urine extracts. (A) Serum blank; (B) serum standard containing meperidine and normeperidine each at 100 ng/ml; (C) serum from volunteer 8 h post 70-mg meperidine · HCl bolus injection, meperidine = 84 ng/ml, normeperidine = 27 ng/ml; (D) urine blank; (E) 24-h urine collection from volunteer post 70-mg meperidine · HCl bolus injection, meperidine = 150 × 10 = 1500 ng/ml, normeperidine = 170 × 10 = 1700 ng/ml. Peaks: M = meperidine; D = diphenhydramine; NM = normeperidine; ND = nordiphenhydramine; X = unknown.

to pH 7.0, it was necessary to optimize the pH of the mobile phase with a few drops of 1 mol/l sodium hydroxide or 0.9 mol/l orthophosphoric acid. The addition of alkali moved all four peaks to earlier elution times and improved the separation between the NM and ND peaks. It was sometimes necessary to add acid to lengthen all retention times so that baseline could be established before the elution of M. The addition of acid or base has little influence upon the measured pH of the phosphate buffer (pH = 6.95—7.05) or the apparent pH of the moble phase (pH = 8.9—9.0). Once the pH of the mobile phase was optimized, it remained stable and required no further adjustment. Each batch of mobile phase could be recycled three times after which the baseline became too noisy.

All four compounds displayed absorption maxima below 200 nm but the baseline was too unsteady to be of practical value. Above 210 nm there was insufficient response from NM to accurately quantify low serum concentrations. Optimal signal-to-noise was observed at 205 nm. Chromatograms from drug-free serum and urine extracts are shown in Fig. 1a and d. The baseline is clean at the elution times for M, D, NM and ND. Two additional unknown peaks appeared in urine extracts just prior to the baseline valley. Their presence did not compromise the analysis of urine specimens. M and NM are largely excreted in urine in their free and conjugated forms. The other two major metabolites are meperidinic acid and normeperidinic acid which account for 9.0—27.8% and 4.4—13.0% of the administered dose as measured by 24-h cumulative urinary excretion, respectively [8]. Based on the GC method for analysis of meperidinic acid and normeperidinic acid in urine [8], neither acid metabolite would be expected to extract into hexane at alkaline pH, and hence would not interfere with the analysis of M and NM. Attempts to procure the two acid metabolites were unsuccessful.

Results from precision studies are shown in Table I. For M, within-day coefficients of variation (C.V.) on serum standards ranged from 2.3% to 10.1%. The corresponding C.V. values for NM were slightly higher, 2.8—12.4%. During the developmental phase of the method, D was used as an internal standard for both M and NM. The precision for NM was somewhat poorer at all concentrations and most noticeable below 100 ng/ml. For comparison, serum within-day precision data have been included for NM using both D and ND as the internal standard. The secondary amine ND compensated more fully for adsorption losses of NM onto glassware than did the tertiary amine, D. The corresponding concentrations of M and NM in serum and urine standards show similar within-day C.V. values. Day-to-day and within-day C.V. values from urine standards are alike for both M and NM at low concentrations. However, the day-to-day C.V. values at 1000 ng/ml are three times as large as the corresponding values for the within-day precision. This can be attributed to the daily one-point recalibration at 100 ng/ml. All data were taken from one calibration when the within-day precision was evaluated.

Good linearity was observed for both M and NM from serum and urine standards. Calculated correlation coefficients were all 1.000; the slopes ranged from 1.02 to 1.09. The limits of detection, taken as three times maximum baseline noise, were 2 ng/ml for M and 5 ng/ml for NM.

Results from recovery experiments are summarized in Table II. Mean

TABLE I

PRECISION STUDIES

Drug concentration (ng/ml)	Within-day (n = 10)						Day-to-day (n = 22)			
	Meperidine		Normeperidine*		Normeperidine**		Meperidine		Normeperidine*	
	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)
Serum										
1000	1014	2.3	1090	2.8	1124	4.0	972	7.9	1018	10.6
500	522	2.2	520	5.4	526	6.1	486	5.3	505	7.8
200	211	5.1	192	8.6	183	11.4	201	7.6	201	8.8
100	104	6.6	93	8.1	85	11.1	102	6.4	100	6.8
50	52	5.5	50	7.9	46	14.8	53	8.1	51	9.6
10	10	10.1	11	12.4	13	30.8	11	11.0	11	13.4
Urine										
1000	1043	3.7	1034	2.6			962	7.7	980	9.8
500	510	6.9	508	6.2			508	6.8	485	6.8
200	213	3.2	205	5.1			198	7.7	194	7.0
100	101	3.9	95	9.2			101	5.1	101	7.4
50	53	5.2	50	5.9			52	8.8	54	10.2

*Using nordiphenhydramine as internal standard.
**Using diphenhydramine as internal standard.

TABLE II

RECOVERY STUDIES (PERCENT ± S.D.)

In all cases n = 10.

Drug concentration (ng/ml)	Meperidine	Normeperidine	Diphenhydramine	Nordiphenhydramine
Serum				
1000	86 ± 2	75 ± 2		
500	87 ± 3	68 ± 5		
200	80 ± 5	54 ± 8		
100	86 ± 5	55 ± 8	78 ± 5	75 ± 9
50	81 ± 10	55 ± 12		
10	91 ± 13	73 ± 16		
Urine				
1000	90 ± 5	63 ± 5		
500	93 ± 5	55 ± 5		
200	89 ± 5	58 ± 5		
100	82 ± 6	53 ± 5	78 ± 4	73 ± 6
50	77 ± 7	44 ± 7		

recoveries from either serum or urine ranged between 77% and 93% for M and between 44% and 75% for NM. Mean D and ND recoveries were 78% and 75% respectively in serum. There was no difference in recovery from urine. M has been shown to extract from urine equally well at pH 7—11. However, NM was extracted optimally at pH 10 [1]. These observations were confirmed by ourselves. M, D and ND all extracted equally well at pH 8—12.5. NM was recovered maximally between pH 9.5—12.5. In the proportions used in the

method, sodium hydroxide as an alkalinizing agent raised serum to pH 10.4—10.8. Borate buffer raised urine to pH 9.8—10.0.

During the developmental phase of this work, it was observed that less than 10% of all four compounds were recovered from a pooled urine-based standard when 100 μl of 1 mol/l sodium hydroxide was used in the extraction procedure. However, saturated borate buffer gave recoveries similar to those observed in serum using sodium hydroxide. This anomaly was pursued by examining recoveries from standards prepared in drug-free urines obtained from eight normal volunteers. The urine from one of the eight consistently resulted in standards giving low drug recovery when sodium hydroxide was used but expected recovery when borate buffer was used. All other urine-based standards gave the same recovery with sodium hydroxide or borate buffer. The person in question has no health problems. An explanation for the disparity is not apparent.

The Supelcosil LC-PCN column provided reliable chromatography over a three-month period. Approximately 1000 injections were made before the column was replaced. Stability and longevity of cyanopropyl columns have been a concern to others [9, 10]. In our experience other brands of cyanopropyl columns have not provided the same extended performance. Subtle differences in peak resolution and retention times are also inherent to the source of bulk packing material. Back-extraction into a small volume of 1 mmol/l orthophosphoric acid adequately recovered all four drugs from the initial hexane extract. This step served to simultaneously clean up

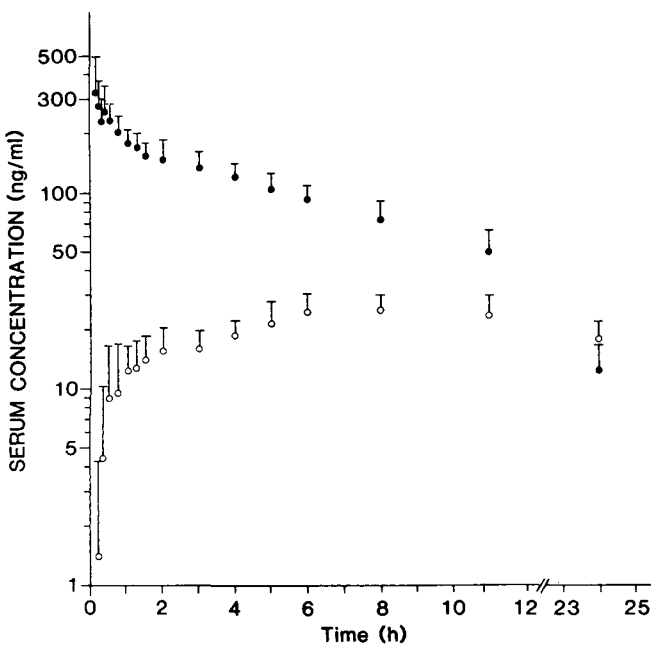

Fig. 2. Mean (+S.D.) serum concentration versus time curves for meperidine (•) and normeperidine (○) in eight healthy male volunteers following a 70-mg intravenous bolus dose of meperidine ◦ HCl.

and concentrate the extract suitable for analysis by HPLC. No deleterious effect resulted from the injection of dilute acid onto the column. As opposed to GC procedures, it was unnecessary to perform a derivatization step in order to recover secondary amines adsorbed onto glassware.

The mean (+S.D.) serum M and NM concentration versus time curves for eight normal volunteers are displayed in Fig. 2. Serum concentrations for M and NM beyond the 24-h sample were all less than 5 ng/ml. Similar plasma concentrations have been reported for M and NM in healthy persons following a single dose [3, 5, 6, 11, 12]. The observed half-life, volume of distribution at steady state, total body clearance and 24-h urinary excretion ranges for M were 4.8—8.1 h, 3.6—5.8 l/kg, 0.40—0.73 l/kg/h, and 1.5—9.9 mg, respectively. The 24-h urinary excretion of NM ranged from 2.6 to 6.1 mg. A discussion of the effect of cimetidine on the pharmacokinetics of M and NM is forthcoming [13].

After single doses of M, the peak serum concentrations of NM ranged from 10 to 40 ng/ml. However, multiple doses of M as used clinically result in accumulation of NM owing to the long elimination half-life of the metabolite. Patients with cancer and patients with significant renal impairment are especially predisposed to this accumulation. NM concentrations of 50—1800 ng/ml have been documented in these patients [14]. We have noted M and NM concentrations of 208 and 511 ng/ml, respectively, in a renal failure patient developing seizures thought to be secondary to NM toxicity. This NM concentration is close to those reported by Szeto et al. [14] in patients developing NM-induced seizures (670 and 1800 ng/ml).

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DICLOFENAC AND ITS MONOHYDROXYLATED METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

Sensitive and selective high-performance liquid chromatographic assays for diclofenac and its monohydroxylated metabolites in biological fluids are described. Using ultraviolet detection at 282 nm, diclofenac is assayed in plasma at concentrations down to 10 ng/ml; total (free + conjugated) diclofenac and its monohydroxylated metabolites (the sum of 3'- + 4'-hydroxydiclofenac and 5-hydroxydiclofenac) are assayed in urine after chemical hydrolysis at concentrations down to 200 ng/ml. The applicability of the described assays is shown.

INTRODUCTION

Diclofenac sodium (Voltaren®) is a non-steroidal anti-inflammatory drug used in the treatment of rheumatic diseases. Several methods for its assay in plasma have been reported including thin-layer chromatography (TLC) [1], high-performance liquid chromatography (HPLC) [2—4] and gas chromatography (GC) [5—8].

The GC methods are based on the formation of an indolinone [5], a methyl ester [7,8], or an acetylated [6] derivative. Those involving electron-capture detection [5,7,8] are extremely sensitive; however, they require an extensive sample clean-up (three-step extraction) before derivatization.

The TLC method [1] as well as two of the HPLC methods reported [2,3] lack the sensitivity required for pharmacokinetic investigations.

Chan et al. [4] recently described a simple and sensitive HPLC method, with single-step extraction and direct UV detection without derivatization. The overall recovery in the range 5—1000 ng/ml was 94.8 ± 7.8%, indicating good precision and accuracy. However, the data reported suggest that the repro-

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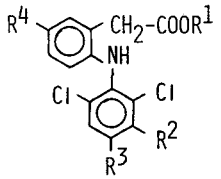
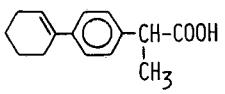
Diclofenac and its known metabolites



Compound		R^1	R^2	R^3	R^4	24-h Renal elimination in percentage of dose (free + conjugated)
Diclofenac sodium	D	Na	H	H	H	5–10
3'-Hydroxydiclofenac	3'-OH-D	H	OH	H	H	<5
4'-Hydroxydiclofenac	4'-OH-D	H	H	OH	H	20–30
5-Hydroxydiclofenac	5-OH-D	H	H	H	OH	5–10
4',5-Dihydroxydiclofenac	4',5-OH-D	H	H	OH	OH	5–10

Internal standards

Assay I: 2-(*p*-cyclohexen-1'-yl-phenyl)propionic acid (I)



Assay II: 4'-hydroxy-5-chlorodiclofenac (II)
R^1=R^2=H; R^3=OH; R^4=Cl

Fig. 1. Chemical structures of diclofenac, its known metabolites and internal standards.

ducibility at low levels was not fully satisfactory. The coefficient of variation of the peak height ratio was 20% for 5 ng/ml (three replicates) and 21.7% for 10 ng/ml (four replicates). The recovery for samples spiked with 9 ng/ml ranged from 67% to 144%.

A similar HPLC method, with better precision and accuracy, was developed for the assay of unchanged diclofenac in plasma (Assay I).

Diclofenac sodium is extensively metabolized in man: 35—65% of an oral dose is excreted in urine as conjugates of the parent drug, mono- and dihydroxylated metabolites [9]. The unconjugated compounds account for less than 1% [10]. 3'-, 4'-, and 5-hydroxydiclofenac (3'-, 4'- and 5-OH-D) and 4',5-dihydroxydiclofenac (4',5-OH-D) (see Fig. 1 for chemical structures) have been identified in human urine as conjugates [11]. They account, respectively, for less than 5%, 20—30%, 5—10% and 5—10% of the administered dose [9].

A method based on extractive alkylation and GC has been described for the determination of the total (free + conjugated) monohydroxylated metabolites in urine [12]. However, the derivatives of the three compounds are not resolved on the column and are measured as a single peak.

A procedure based on extractive alkylation, GC with capillary column and electron-capture detection is the only method available for the simultaneous and specific assay in urine of the parent drug and its four mono- and dihydroxylated metabolites [13].

This paper describes an HPLC method for the assay of diclofenac and its monohydroxylated metabolites in urine after chemical hydrolysis (Assay II). It is selective for diclofenac and 5-hydroxydiclofenac. The sum of 3'- and

4′-OH-D is measured as a single compound since they have identical retention times. The dihydroxylated metabolite cannot be assayed because of interference.

MATERIALS AND METHODS

Chemicals

Diclofenac sodium, its metabolites and the internal standards [2-(*p*-cyclohexen-1′-yl-phenyl)propionic acid (I) for Assay I and 4′-hydroxy-5-chlorodiclofenac (II) for Assay II] were supplied by Ciba-Geigy (Basle, Switzerland) (see Fig. 1 for chemical structures).

Acetonitrile for spectroscopy (Uvasol from E. Merck, Darmstadt, F.R.G.) was used. All the other solvents and reagents were of analytical grade quality. Diisopropyl ether was purified just before use by passing it through a column filled with basic alumina (Woelm, Eschwege, F.R.G.).

Assay I — unchanged diclofenac in plasma

Chromatography. The chromatography was performed on a Hewlett-Packard instrument, Model 1084 B, equipped with a variable-wavelength detector set at 282 nm and a variable-volume injection system. The separation was achieved with a prepacked Merck column (25 cm × 4 mm I.D., LiChrosorb RP-8 10-μm packing). A precolumn (5 cm × 4.7 mm I.D.) filled with Co-Pell ODS 30—38 μm (Whatman, Clifton, NJ, U.S.A.) was used to protect the analytical column. The degassed mobile phase, methanol—pH 7 phosphate buffer (Titrisol from Merck) (60:40, v/v), was used at a flow-rate of 1.3 ml/min. The mobile phase and the columns were at room temperature.

The peak heights were measured manually, the peak areas were given by the integrator—recorder of the Hewlett-Packard 1084 B instrument (79850 A LC terminal).

Sample preparation. To 1 ml of plasma in a 10-ml glass tube were added 100 μl of the solution of the internal standard I (added amount 1.25 μg). The sample was acidified with 2 ml of 0.83 *M* phosphoric acid and 4 ml of hexane—isopropyl alcohol (90:10) were added. The tubes were capped, shaken for 10 min on a rotating shaker (Infors) at 250 rpm and then centrifuged at 1500 *g* for 10 min. The aqueous phase was frozen, the organic layer transferred into a 10-ml conical glass tube and evaporated to dryness under a nitrogen stream at room temperature. Just prior the analysis, the residue was redissolved in 300 μl of mobile phase. After shaking for 15 sec on a vortex mixer, 70 μl were injected onto the column.

Calibration curves. Calibration samples were prepared by adding 100 μl of reference solutions of diclofenac sodium and 100 μl of the solution of the internal standard I (all prepared in water from methanolic stock solutions) to 1 ml of blank plasma. The amounts corresponded to plasma concentrations of diclofenac sodium ranging from 10 ng/ml to 3 μg/ml.

The calibration curves were obtained by plotting versus the concentrations, either the peak height ratio diclofenac sodium/internal standard for the concentration range 10—250 ng/ml or the peak area ratio for the range 0.25—3 μg/ml. Their equations were calculated by the least-squares method using linear

regression. For routine analysis, a calibration curve must be established every ten days.

Assay II — unchanged drug and monohydroxylated metabolites in urine

Chromatography. The equipment was that used in Assay I.

The separation was achieved with a column (25 cm × 4.7 mm I.D.) filled with Nucleosil C_{18}, 10 μm particle size (Macherey Nagel, Düren, F.R.G.) using the balanced-density slurry packing technique. The slurry, made of 3.5 g of Nucleosil C_{18} (10 μm particle size) dispersed in a mixture of 17 ml methanol and 4 ml of 10^{-2} *M* sodium acetate solution, was forced into the column with methanol under a pressure of 500 bars. The degassed mobile phase methanol—acetonitrile—pH 7 buffer (30:17:53, v/v/v) was used at a flow-rate of 1.3 ml/min. The mobile phase and the column were at room temperature.

Sample preparation. A 100-μl volume of the methanolic solution of internal standard II was introduced into a 10-ml disposable glass ampoule (added amount 250 ng). Methanol was evaporated under a nitrogen stream. Then 250 μl of urine, about 100 mg of ascorbic acid and 250 μl of 5 *M* sodium hydroxide were introduced into the ampoule. After shaking for a few seconds on a vortex mixer, the ampoule was capped and heated at 75°C for 1 h. After cooling, 1.2 ml of 1 *M* hydrochloric acid were added, followed by 1 ml of pH 7 buffer and 4 ml of peroxide-free diisopropyl ether. The ampoule was closed with a polyethylene stopper, shaken for 15 min on a rotating shaker at 250 rpm and centrifuged at 1500 *g* for 5 min.

The aqueous phase was frozen. The organic layer was transferred into a 10-ml glass ampoule and 500 μl of a potassium carbonate solution (20 g/l) were added. The ampoule was closed, shaken for 5 min at 300 rpm and centrifuged for 3 min. The aqueous phase was frozen and the organic layer discarded. About 100 mg of ascorbic acid, 1 ml of pH 6 buffer (citric acid—sodium hydroxide; Titrisol from Merck, four-fold concentrated), 100 μl of 1 *M* hydrochloric acid and 4 ml of peroxide-free diisopropyl ether were added. After shaking for 5 min at 300 rpm and centrifugation for 3 min, the aqueous phase was frozen. The organic layer was transferred into a 5-ml disposable glass ampoule and taken to dryness under a nitrogen stream at 37°C. The dry residue was taken up into 300 μl of methanol—pH 4 buffer (sodium citrate—hydrochloric acid, according to Sörensen) mixture (50:50). After shaking for 15 sec on a vortex mixer, 100 μl were injected onto the column. The samples must be injected as soon as possible after preparation since degradation of 5-OH-D was observed.

Calibration curves. Calibration solutions of diclofenac sodium, 4'- and 5-OH-D as well as the solution of the internal standard II were prepared in methanol. Ascorbic acid was added to the 5-OH-D solution to prevent oxidation.

Calibration samples were prepared by introducing aliquots of the methanolic solutions of each compound into an ampoule. The solvent was evaporated and 250 μl of blank urine were added.

The range of the calibration curves for each compound was 0.20—20 μg/ml. A log—log plot of the peak area or peak height ratio (compound/internal standard) versus concentration was used. Near the limit of quantitation, a curve with the peak height ratio was used.

A calibration curve remains valid over one week for 4′- and 5-OH-D, and over about one month for diclofenac.

RESULTS AND DISCUSSION

Assay I

Plasma interference. Diclofenac and the internal standard I were well separated from the plasma components with UV detection at 282 nm (Fig. 2). More interferences were observed at 215—220 nm, as proposed by Chan et al. [4].

Linearity. A linear relationship was obtained in the range 10—250 ng/ml with the peak height ratios, in the range 0.25—3 μg/ml with the peak area ratio (correlation coefficients higher than 0.9990).

Accuracy, precision and reproducibility. Table I shows that concentrations of diclofenac down to 10 ng/ml can be accurately and precisely measured using 1-ml plasma samples. This limit corresponds to a peak height of about 0.5 cm.

Selectivity. The known metabolites (Fig. 1) do not interfere in the assay of diclofenac. Their retention times were 3.12, 3.24, 3.29 and 3.80 min, respectively, for 3′-OH-D, 4′-OH-D, 4′,5-OH-D and 5-OH-D, compared to 5.8 min for diclofenac and 7.2 min for the internal standard I.

The retention times of other anti-inflammatory drugs, relative to that of diclofenac, were 1.04 min for indomethacin, 0.54 min for ketoprofen, and 0.40 min for piroxicam.

Stability. Diclofenac sodium and internal standard I solutions were stable over one month at 5°C. The dry residue remains stable for one night when stored as such in the refrigerator. Once dissolved in the mobile phase, the solution must be injected on the day of preparation.

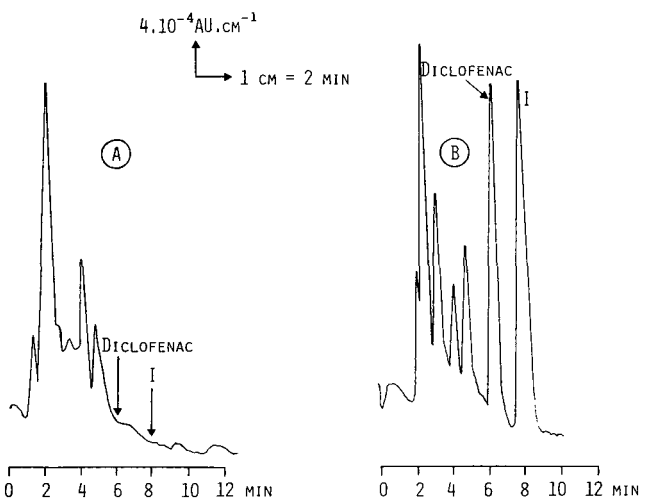


Fig. 2. Chromatograms corresponding to extracts of (A) a 1-ml sample of blank human plasma and (B) a 1-ml sample of human plasma spiked with 200 ng/ml diclofenac sodium and 1.25 μg of internal standard (I).

TABLE I

ACCURACY, PRECISION AND REPRODUCIBILITY OF ASSAY I

Plasma samples were spiked with diclofenac sodium and assayed within a day or on several days

Added (ng/ml)	Found, mean (ng/ml)	*n*	C.V. (%)	Mean recovery* (%)
Within-day reproducibility				
10	10.5**	12	8.5	105
15	15.9**	8	5.9	106
20	20.4	6	5.8	102
1498	1510	6	3.7	101
Day-to-day reproducibility				
20	18.8***	9§	6.7	95
200	202***	10§	3.3	101
1498	1472***	9§	7.2	98

*Mean of the individual recovery values.
**Spiked samples prepared with plasma from six volunteers.
***Spiked samples prepared with plasma from three volunteers.
§One or two samples per day over six days.

Application. Fig. 3 displays the mean plasma concentration—time curve of diclofenac in six subjects after administration of a single 75-mg dose as suppository.

Assay II

Urine interferences. Diclofenac and monohydroxylated metabolites were well separated from the endogenous components extracted from urine after chemical hydrolysis (Fig. 4).

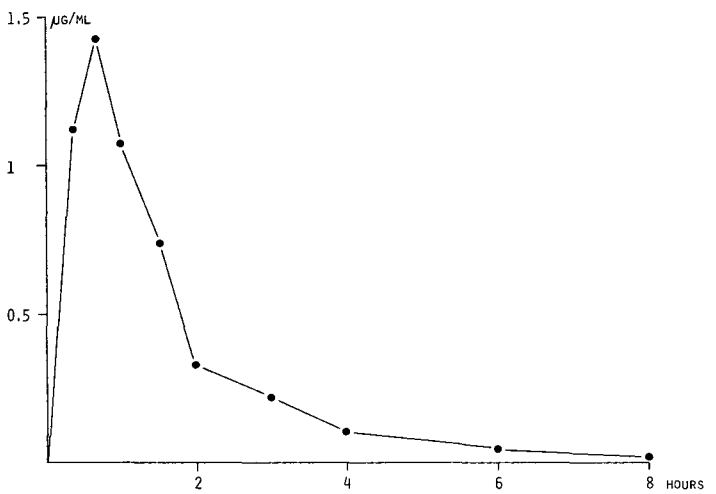


Fig. 3. Plasma concentrations of diclofenac after rectal administration of a 75-mg suppository (each point is the mean of six subjects).

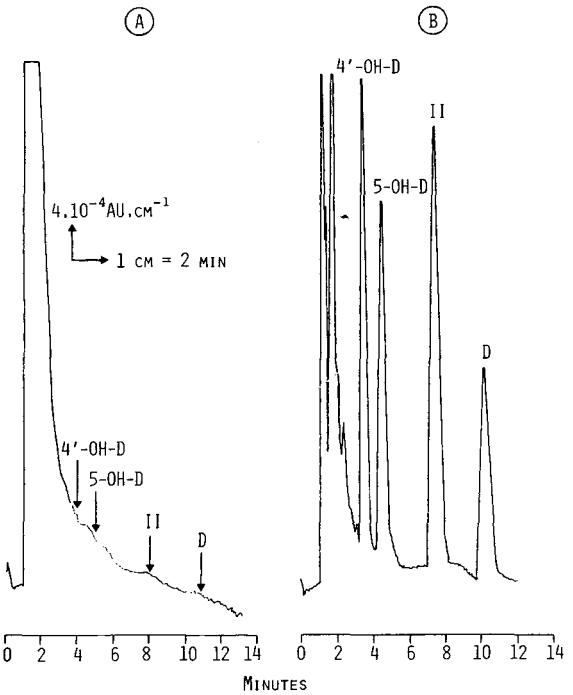


Fig. 4. Assay II. Typical chromatograms of diclofenac and its metabolites extracted from urine after chemical hydrolysis: (A) Urine blank; (B) urine spiked with 1 μg/ml diclofenac (D), 4′-hydroxydiclofenac (4′-OH-D) and 5-hydroxydiclofenac (5-OH-D) and 250 ng of internal standard (II).

Linearity. A good linear relationship was obtained in the range 200 ng/ml to 20 μg/ml for each compound (correlation coefficients higher than 0.9990).

Selectivity. The relative retention times were respectively 1, 1.74, 1.78, 2.25, 3.90 and 5.29 for 4′,5-OH-D, 4′-, 3′- and 5-OH-D, 4′-hydroxy-5-chlorodiclofenac (II) and diclofenac. 3′- and 4′-OH-D give a single peak.

According to Riess et al. [9], the latter accounts for 20—30% of the total ^{14}C-radioactivity recovered in urine after oral administration whereas the former represents less than 5%. Therefore, the interference of 3′-OH-D in the assay of 4′-OH-D is within 15—20%.

The dihydroxylated metabolite, although well separated, could not be assayed because of interferences by endogenous components.

Accuracy, precision and reproducibility. 3′- and 4′-OH-D were not resolved and gave a single peak. For this reason, the accuracy and precision were assessed with samples spiked only with one of these two compounds, namely 4′-OH-D. Spiked samples prepared with urines from five volunteers by adding the unconjugated compounds were analysed. The results (Table II) show that diclofenac and its monohydroxylated metabolites can be precisely and accurately assayed in urine after chemical hydrolysis at concentrations down to 200 ng/ml.

The reproducibility was also assessed on samples collected after oral administration of diclofenac sodium, and analysed twice at a five-month interval. The

TABLE II

ACCURACY, PRECISION AND REPRODUCIBILITY OF ASSAY II

Urine samples were spiked with unconjugated compounds and assayed within a day.

Compound	Added (ng/ml)	Found, mean (ng/ml)	n	C.V. (%)	Mean recovery* (%)
Diclofenac	200	219	8	14.2	109
	500	486	4	5.6	97
	4000	4023	9	4.8	100
4'-Hydroxydiclofenac	200	184	8	6.7	93
	500	474	4	4.4	95
	4000	3884	8	3.5	97
5-Hydroxydiclofenac	200	200	8	9.3	100
	500	496	4	4.4	100
	4000	4025	7	8.8	101

*Mean of the individual recovery values.

slope of the regression line for second (HPLC 2) versus first (HPLC 1) analysis for the three compounds combined was 1.01 (r = 0.9988 for 35 values).

Stability. The methanolic solutions of diclofenac, 4'-OH-D and the internal standard II are stable for one month, that of 5-OH-D for only ten days (with ascorbic acid). Degradation of 5-OH-D was observed when the sample preparation was done in glass tubes used repeatedly instead of disposable glass ampoules.

Urine samples appeared stable for five months at −20°C. Three freezing—thawing cycles during this period had no influence on the stability.

Application. Urinary excretions in the study mentioned above (Fig. 5) demonstrate the applicability of Assay II. Recoveries are in good agreement with literature values [9,13].

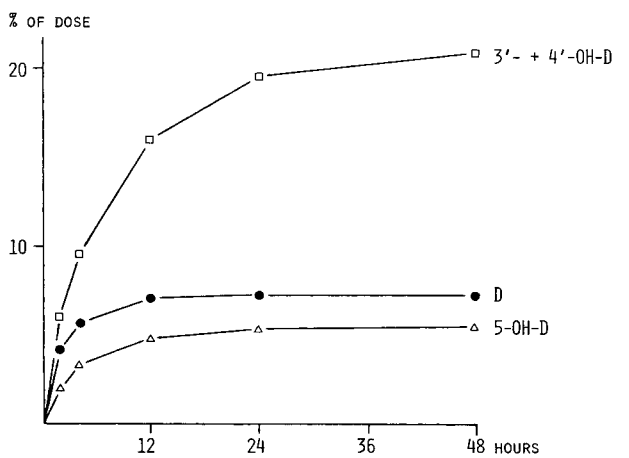


Fig. 5. Cumulative excretion of total (free + conjugated) diclofenac and its monohydroxylated metabolites in urine after a 75-mg dose given as a suppository (each point is the mean of six subjects). For abbreviations, see legend to Fig. 4.

CONCLUSION

Although 3′- and 4′-OH-D cannot be separated and 4′,5-OH-D cannot be measured as in the capillary GC method [13], the HPLC method described for diclofenac and its monohydroxylated metabolites in urine appears valuable and very useful for bioavailability evaluations. The HPLC technique developed for the assay of unchanged diclofenac in plasma is suited to the analysis of large numbers of samples.

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DETERMINATION OF CAPTOPRIL AND ITS MIXED DISULPHIDES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method has been developed which enables sensitive determination of captopril and its mixed disulphides in plasma and urine after oral administration of a new antihypertensive agent, 1-(D-3-acetylthio-2-methylpropanoyl)-L-prolyl-L-phenylalanine (DU-1219, I). Captopril is derivatized with a new reagent, N-(4-benzoylphenyl)maleimide and the derivative is extracted with chloroform and assayed using a liquid chromatograph equipped with an ultraviolet detector at 254 nm. Mixed disulphides of captopril with thiol compounds such as cysteine, glutathione and plasma proteins are reduced with tributylphosphine to form captopril, followed by derivatization with N-(4-benzoylphenyl)maleimide.

Accurate determinations are possible over a concentration range of 10—500 ng/ml captopril in plasma, and 100—2500 ng/ml captopril in urine. The coefficients of variation of captopril in plasma (200 ng/ml) and urine (500 ng/ml) are 3.7% and 2.6%, respectively, and those of mixed disulphides of captopril are similar to those of captopril. Plasma levels and urinary excretion of captopril and its mixed disulphides in healthy volunteers following single oral administration of I (50 mg) have also been determined.

INTRODUCTION

The compound 1-(D-3-acetylthio-2-methylpropanoyl)-L-prolyl-L-phenylalanine (DU-1219, I, Fig. 1) is an orally active antihypertensive agent, designed to inhibit angiotensin-converting enzyme in vivo [1, 2]. Disposition and metabolic studies of ^{14}C-labelled I in rats have revealed that I was considerably converted to an active metabolite, captopril, and captopril and its mixed disulphides were detected in plasma and urine after dosing of I [3]. In order to

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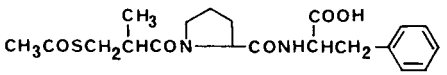
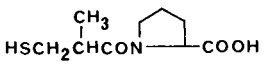
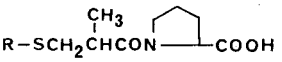


I

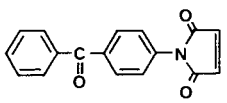


Captopril



Mixed disulphides

R = cysteine,
glutathione,
proteins,
captopril,
etc.



BPM

Fig. 1. Chemical structures of I, captopril and mixed disulphides of captopril and BPM.

study the pharmacokinetics of I in man, a sensitive and specific assay method for captopril and its mixed disulphides is necessary.

Captopril in blood or urine has been determined by gas chromatography (GC) [4], gas chromatography—mass spectrometry (GC—MS) [5], a radiochemical method [6] and high-performance liquid chromatography (HPLC) [7—9]. However, the GC method is limited by sensitivity, and the GC—MS method of Funke et al. [5] and the HPLC methods of Jarrott et al. [8] and Shimada et al. [9] require specific detectors.

In this paper we describe a HPLC method with ultraviolet detection at 254 nm. The method, based on extraction and derivatization using a new thiol derivatizing reagent, N-(4-benzoylphenyl)maleimide (BPM), is selective and sensitive for the determination of captopril and its mixed disulphides in plasma and urine.

EXPERIMENTAL

Materials

Captopril, I, 1,1′-[dithiobis(2-D-methyl-3-propanoyl)]-bis-L-proline (captopril dimer) and (4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid (II) were synthesized in our laboratories. Thiosalicylic acid and disodium EDTA were obtained from Wako (Osaka, Japan). The derivatizing reagent of thiol compounds, N-(4-benzoylphenyl)maleimide (BPM), was also synthesized in our laboratories [10] (Fig. 1). Human serum albumin conjugated captopril (HSA—captopril) was prepared by the methods of Harrap et al. [11] and Meredith [12], and stored in 0.01 *M* phosphate buffer (pH 7.4) (6 μg of captopril per 10 mg of protein per ml). Silicic acid (Kieselgel 60, 70—230 mesh; E. Merck, Darmstadt, F.R.G.) and silica gel 60 plates F_{254} (Merck) were used for column chromatography and thin-layer chromatography (TLC), respectively. All other chemicals were of analytical-reagent grade.

Instruments

HPLC was carried out using a Waters Model ALC/GPC 204 liquid chromato-

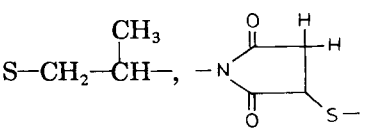
graph, equipped with a dual-delivery pump (Model 6000), an automatic sampler (Model 710B), a UV detector (Model 440) with a 254-nm filter and a recorder with integration (Data Module, Model 730) (Waters Assoc., MA, U.S.A.), and a Shimadzu Model LC-4A liquid chromatograph, with a Rheodyne 7125 sample loop injector and a recorder with Shimadzu Chromatopac C-R2A (Kyoto, Japan).

A stainless-steel column (30 × 0.4 cm I.D.) packed with μBondapak C_{18} (10 μm; Waters Assoc.) was used. The following mobile phases were used at a flow-rate of 1.0 ml/min: (1) acetonitrile—methanol—1% acetic acid (45:11:75) for captopril in plasma; (2) 42.5:11:74 for the mixed disulphides in plasma; (3) 42.5:8.2:47.3 for captopril in urine; and (4) acetonitrile—1% acetic acid (47:53) for the mixed disulphides in urine.

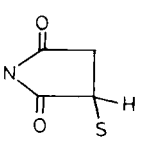
Nuclear magnetic resonance (NMR) spectra were recorded on a Varian FT-80A NMR spectrometer. Field desorption (FD) mass spectra were recorded on a JEOL Model JMS-D300 mass spectrometer.

Synthesis of captopril—BPM adduct

To 0.11 g of captopril in 2 ml of water were added 0.15 g of BPM in 2 ml of acetone and one drop of triethylamine. The mixture was allowed to stand at room temperature for 15 min and evaporated to dryness in vacuo. Oily residue was purified by silica gel chromatography (column, 15 × 0.9 cm I.D.). The eluate with chloroform—methanol (19:1, v/v) was evaporated to give 0.1 g of captopril—BPM adduct as a white amorphous solid. A TLC spot of captopril—BPM was obtained with R_F = 0.29 (chloroform—methanol—water, 100:10:0.1, v/v/v) detected by UV lamp at 254 nm and iodine vapour. ^{1}H-NMR (in deuterated dimethyl sulphoxide, DMSO-d_6): δ = 1.1 [d, J = 6 Hz, 3H, CH_3]; δ = 1.7—2.3 [m, 4H, proline 3-CH_2, 4-CH_2]; δ = 2.6—3.8 [m, 7H, proline 5-CH_2,



]; δ = 4.2 [m, 2H, proline 2-CH,



];

δ = 7.4—8.0 [m, 9H, aromatic]; δ = 12.3 [bs, 1H, COOH]. FD—MS: *m/z* 494 (M^+).

Thiosalicylic acid—BPM adduct (TLC: R_F = 0.52) and II—BPM adduct (TLC: R_F = 0.18), used as the internal standards, were prepared by the same method as described above.

Assay procedure for captopril and its mixed disulphides in plasma and urine

(a) Captopril in plasma. To 1 ml of plasma in a glass-stoppered 15-ml centrifuge tube were added 2 ml of 0.1 *M* phosphate buffer (pH 6.0) and 0.5 ml of 0.5% BPM acetone solution. The tube was vortex-mixed for 15 sec and allowed to stand at room temperature for 10 min. To the mixture 2 ml of 0.5 *M* phosphate buffer (pH 7.0) and 0.1 ml of internal standard solution A (4 μg of II—BPM adduct in 0.1 ml of acetone) were added. The mixture was washed twice

with 4 ml of diethyl ether, acidified with 0.5 ml of 6 *M* hydrochloric acid and extracted with 7 ml of chloroform. The organic layer was evaporated to dryness below 40°C under a gentle stream of air.

The clean-up procedure was carried out as follows. The residue was dissolved in 2 ml of 0.5 *M* phosphate buffer (pH 7.0) and washed twice with 4 ml of diethyl ether. After acidification with 6 *M* hydrochloric acid the aqueous layer was again extracted with 7 ml of chloroform. The organic layer was evaporated to dryness. The residue was dissolved in 100 μl of methanol and a 20-μl aliquot of solution was injected into the liquid chromatograph.

(b) Total captopril (captopril and its mixed disulphides) in plasma. To 1 ml of plasma sample in a glass-stoppered 15-ml centrifuge tube were added 2 ml of 0.1 *M* phosphate buffer (pH 6.75), 0.2 ml of 0.1 *M* EDTA solution and 0.2 ml of 0.8% tributylphosphine methanol solution. The mixture was incubated at 50°C for 60 min. After the reaction was stopped in the ice box for a few minutes, 1 ml of 0.5 *M* phosphate buffer (pH 7.0) and 0.1 ml of internal standard solution A were added, and the mixture was then washed with 4 ml of diethyl ether. To the aqueous layer was added 0.5 ml of 0.5% BPM acetone solution; the mixture was vortex-mixed for 30 sec, stood at room temperature for 30 min, and was then washed with 4 ml of diethyl ether. The aqueous layer was acidified and extracted with 7 ml of chloroform. The organic layer was evaporated to dryness and the residue was then treated by the clean-up procedure described above. The residue was dissolved in 100 μl of methanol and a 10-μl aliquot of the solution was injected into the liquid chromatograph.

(c) Protein-conjugated captopril in plasma. To 1 ml of plasma sample in a glass-stoppered 15-ml centrifuge tube was added 0.2 ml of 3 *M* perchloric acid; the tube was vigorously vortex-mixed for 30 sec and centrifuged at 700 *g* for 10 min. The supernatant was removed, and the precipitated pellet was then resuspended in 2 ml of water and neutralized with 1 *M* tripotassium phosphate. To the mixture were added 1 ml of 0.5 *M* phosphate buffer (pH 7.0), 0.2 ml of 0.1 *M* EDTA solution and 0.2 ml of 1% tributylphosphine solution. The mixture was incubated at 50°C for 60 min. Captopril in the mixture was determined as described in the above procedure (a).

(d) Captopril in urine. To 0.2 ml of urine sample in a glass-stoppered 15-ml centrifuge tube were added 0.2 ml of 0.5% BPM solution and 0.2 ml of 0.1 *M* phosphate buffer (pH 6.5), and the tube was allowed to stand at room temperature for 15 min. To the mixture were added 2.5 ml of 0.5 *M* phosphate buffer (pH 7.0) and the mixture was washed with 4 ml of diethyl ether. To the aqueous layer was added 0.1 ml of internal standard solution B (1 μg of thiosalicylic acid—BPM adduct in acetone). The mixture was acidified with 0.25 ml of 6 *M* hydrochloric acid, and extracted with 6 ml of chloroform. The organic layer was evaporated to dryness. The residue was dissolved in 200 μl of acetonitrile and a 20-μl aliquot of the solution was injected into the liquid chromatograph.

(e) Total captopril in urine. To 0.2 ml of urine sample in a glass-stoppered 15-ml centrifuge tube were added 2 ml of 0.1 *M* phosphate buffer (pH 7.0), 0.1 ml of 0.1 *M* EDTA solution and 0.1 ml of 2% tributylphosphine solution. The mixture was incubated at 50°C for 60 min and kept in the ice box for a few minutes. To the mixture was added 0.5 ml of 0.5% BPM solution. After

30 min the mixture was washed twice with 4 ml of diethyl ether. To the aqueous layer was added 0.2 ml (2 μg) of internal standard solution B. Captopril in the mixture was determined as described in the above procedure (d).

Calibration curves for captopril and its mixed disulphides in plasma and urine

Calibration curves were constructed by assaying plasma and urine spiked with known amounts of captopril, captopril dimer or HSA—captopril by the methods described above. The peak height ratios of captopril to the internal standard were plotted against the plasma or urine concentrations seeded.

Stability of captopril—BPM adduct

The stability of captopril—BPM in plasma and urine was determined. The derivatized captopril in plasma (0.1—0.5 μg/ml) or in urine (0.2—10 μg/ml) was stored in a freezer (about −20°C) for 30 days.

Human study

The study was carried out under the supervision of physicians. Four healthy volunteers fasted overnight, then orally received 50 mg of I. Blood was taken into a heparinized 10-ml tube (JMS plavetest, Japan Medical Supply, Tokyo, Japan) containing a solution of 0.1 *M* EDTA and 0.1 *M* ascorbic acid [8]. The samples were immediately centrifuged for 2 min. For the determination of captopril, plasma and urine samples were treated with BPM as described in the assay procedure and kept frozen until analysis.

RESULTS AND DISCUSSION

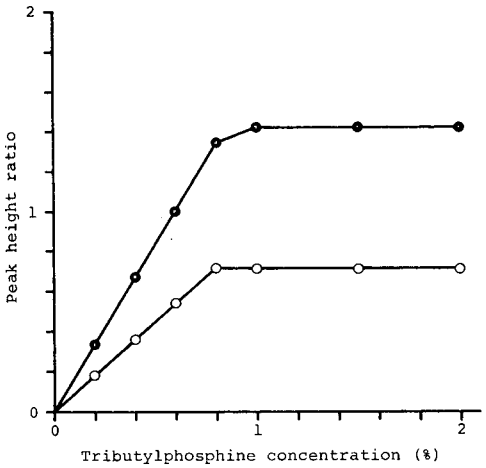
Derivatization of captopril

It is well known that thiol compounds are unstable in biological fluids and undergo rapid oxidation to form the disulphides and other unidentified products [6,13], indicating the necessity for the rapid derivatization of captopril to a suitable adduct immediately after sample collection. In addition, captopril has no prominent absorption properties in the UV spectrum. The use of a derivatization reagent having functions reactive toward the thiol group and high absorption in the UV spectrum would thus seem suitable. BPM was chosen from 30 maleimide compounds synthesized in our laboratories for this purpose [10].

When captopril was treated with BPM, captopril—BPM adduct was rapidly formed in phosphate buffer solution and showed a high ϵ value ($1.8 \cdot 10^4$ at 254 nm). The structure of the adduct was characterized by NMR and MS. Effects of time and pH on the reaction of captopril with BPM were examined. The derivatization of captopril was complete in 5 min at both 0°C and 24°C. The pH of the reaction was optimum at pH 4—7.5. Therefore, captopril was reacted with BPM at pH 6—7 at room temperature for 10—30 min.

Reduction of mixed disulphides of captopril

The mixed disulphides represent a fraction of captopril covalently binding to thiol compounds such as captopril itself, cysteine, glutathione and plasma



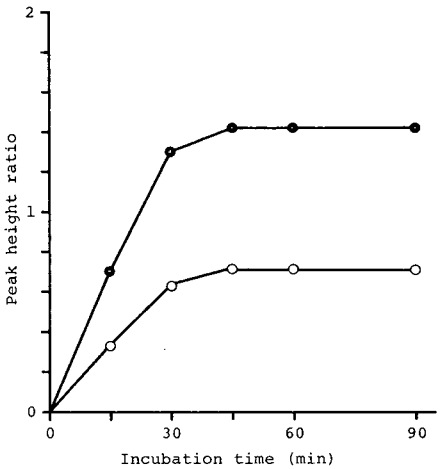


Fig. 2. Effect of tributylphosphine concentration on the reduction of captopril dimer at 50°C. The reaction mixture consisted of 1 ml of plasma, 0.2 ml of EDTA solution, 0.2 ml of tributylphosphine (0.2—2.0% in methanol) and 1 ml of 0.1 *M* phosphate buffer (pH 6.75) containing 2 μg (●) or 1 μg (○) of captopril dimer.

Fig. 3. Effect of incubation time on the reduction of captopril dimer at 50°C. Captopril dimer: (●), 2 μg; (○), 1 μg.

proteins [14—17]. These compounds were reduced to captopril with tributylphosphine [7,18]. The reduction of captopril dimer and HSA—captopril was complete in 45 min at 50°C when the concentration of tributylphosphine was 0.8—2%, as shown in Figs. 2 and 3. Dithiothreitol and sodium borohydride [12,19] were also used for the reduction of the disulphides in plasma. In this case we did not succeed in separating the peaks of captopril from those owing to plasma.

Determination of captopril and its mixed disulphides in plasma

A typical chromatogram of plasma containing 200 ng/ml captopril is shown in Fig. 4 with the assay procedure a. The peaks of captopril and internal standard were separated from those of control plasma and solvent. The calibration curve obtained with 10—250 ng of captopril in 1 ml of plasma was rectilinear and passed through the origin. Quantitative reduction of captopril

TABLE I

ACCURACY AND PRECISION OF THE METHOD FOR DETERMINATION OF CAPTOPRIL AND ITS MIXED DISULPHIDES IN HUMAN PLASMA

Assay procedure a			Asay procedure b			Assay procedure c		
Captopril (ng/ml)		C.V. (%)	Captopril dimer (ng/ml)		C.V. (%)	HSA—captopril (ng/ml)		C.V. (%)
Added	Found ± S.D.*		Added	Found ± S.D.*		Added	Found ± S.D.*	
500	500.7 ± 26.7	5.3	500	498.7 ± 6.3	1.3	500	501.6 ± 14.1	2.8
200	200.3 ± 7.3	3.7	200	197.5 ± 8.7	4.4	200	196.7 ± 8.0	4.0
100	100.3 ± 2.8	2.8	100	98.1 ± 0.6	0.6	100	98.0 ± 1.0	1.0
50	48.3 ± 1.6	3.2	50	49.2 ± 5.1	10.2	50	50.1 ± 2.9	5.8
25	25.5 ± 1.9	7.6	25	23.4 ± 4.0	16.0	25	27.5 ± 5.4	21.6
10	12.4 ± 0.6	6.0						

*n = 6.

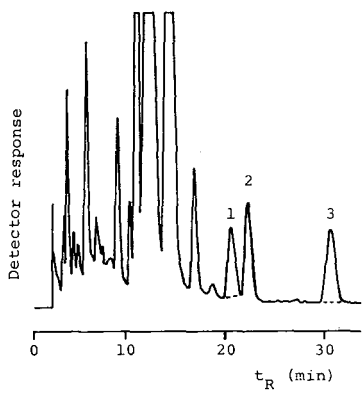


Fig. 4. Typical chromatogram of captopril in plasma (200 ng/ml). Broken lines represent the background from control plasma. HPLC conditions as described in the text. Peaks: 1 = captopril—BPM; 2 = BPM; 3 = internal standard.

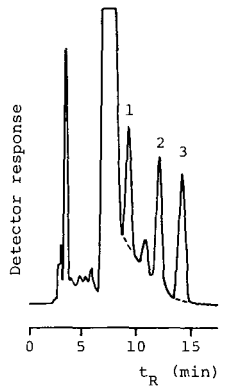


Fig. 5. Typical chromatogram of captopril in urine (200 ng in 0.2 ml of urine). Peaks: 1 = captopril—BPM; 2 = BPM; 3 = internal standard.

dimer and HSA—captopril was observed in the range of 25—500 ng/ml as captopril.

Table I shows data on accuracy and precision of the assay. The coefficients of variation at the 200 ng/ml level of captopril and captopril dimer were 3.7% and 4.4%, respectively. The assay limit was 10 ng/ml captopril.

Determination of captopril and total captopril in urine

A typical chromatogram of urine containing captopril is shown in Fig. 5. Good linearity was obtained in the range 100—2500 ng/ml captopril and 250—5000 ng/ml captopril dimer. Accuracy and precision of this method are shown in Table II. The coefficients of variation at the 500 ng/ml level of captopril and captopril dimer were 2.6% and 8.2%, respectively. The minimum detectable concentration was 50 ng/ml captopril.

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR DETERMINATION OF CAPTOPRIL AND TOTAL CAPTOPRIL IN HUMAN URINE

Assay procedure d			Assay procedure e		
Captopril (ng/ml)		C.V. (%)	Captopril dimer (ng/ml)		C.V. (%)
Added	Found ± S.D.*		Added	Found ± S.D.*	
100	120 ± 4.0	4.0	250	234 ± 6.5	2.6
250	258 ± 11.9	4.8	500	553 ± 41.0	8.2
500	504 ± 12.7	2.6	1000	957 ± 85.0	8.5
1000	982 ± 31.2	3.1	2500	2503 ± 51.3	2.1
2500	2504 ± 107.6	4.3	5000	4984 ± 322.3	6.4

*n = 4.

Stability of captopril—BPM adduct in plasma and urine

Captopril—BPM in plasma and urine were stable for at least 30 days in a freezer.

Plasma levels and urinary excretion of captopril and its mixed disulphides in man receiving 50 mg of I

Plasma levels of captopril, protein-conjugated captopril and total captopril following oral administration of I are shown in Fig. 6. Plasma levels of captopril reached a maximum 1 h after dosing with a level of 246 ng/ml. Plasma levels of protein-conjugated captopril and total captopril were maximal 1 h after dosing with levels of 552 ng/ml and 932 ng/ml, respectively. Plasma levels of mixed disulphides of captopril were three to four times higher than those of free captopril.

Urinary excretion of captopril and total captopril for 24 h after dosing were 29.0% and 62.5% of the dose, respectively.

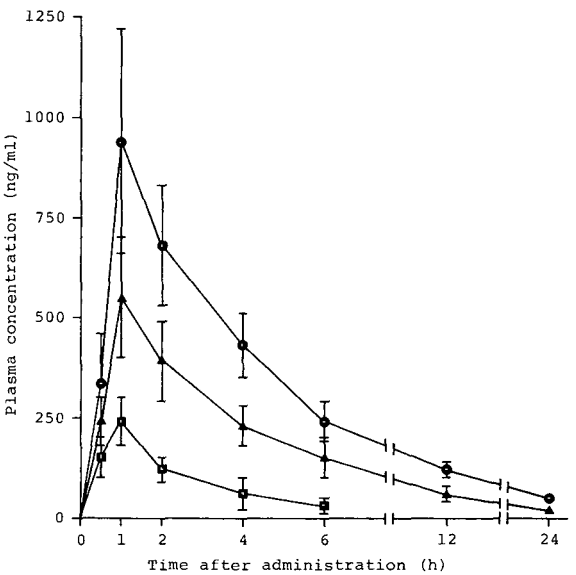


Fig. 6. Plasma levels of captopril (□), protein-conjugated captopril (▲) and total captopril (●) in healthy volunteers following oral administration of 50 mg of I. Data points are mean values ± S.E. from four subjects.

CONCLUSION

We have developed a selective and sensitive HPLC method for the determination of captopril and its mixed disulphides in plasma and urine. This method should permit pharmacokinetic studies in man.

ACKNOWLEDGEMENTS

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the analytical section of the laboratories for the measurement of mass spectra and NMR spectra.

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CHROMBIO. 2425

DETERMINATION OF CITALOPRAM, AMITRIPTYLINE AND CLOMIPRAMINE IN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The determination of citalopram, amitriptyline, clomipramine and their desmethyl metabolites after alkaline diethyl ether extraction from plasma is achieved by high-performance liquid chromatography using two internal standards and μBondapak C_{18} as stationary phase. Elution is carried out isocratically at 0.5 or 1 ml/min with a mixture of acetonitrile—potassium dihydrogen phosphate—distilled water (45:50:5). Detection is monitored by absorption at 254 nm. The detection limit is less than 5 ng/ml for each compound. The coefficients of variation are between 1.3% and 9.4% for 8—360 ng/ml. Interference from 22 possible co-medications is discussed. The technique can be used for therapeutic monitoring of these antidepressants as well as in analytical toxicology.

INTRODUCTION

The tricyclic antidepressants (Fig. 1) amitriptyline and clomipramine are widely prescribed for the treatment of depression [1, 2]. Citalopram (Lu 10-171) (Fig. 1), a new bicyclic antidepressant, is a specific potent serotonin re-uptake inhibitor [3—7]. The early onset of action and the rare side-effects of citalopram make the drug easy to apply and probably enhance compliance. The correlation between plasma levels of some antidepressants, even their major metabolites, and therapeutic effects suggests that measurement of plasma levels may provide valuable information for improving the clinical management of patients [8—16]. The use of high-performance liquid chromatography (HPLC) for clinical analyses has increased considerably during the past few years and the technique has been used routinely for many drug analyses [17].

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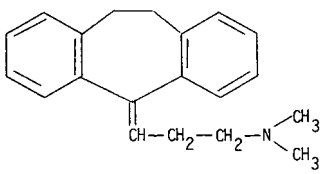
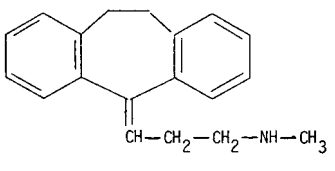
Tricyclics



(AMITRIPTYLINE)



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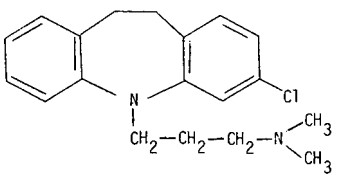
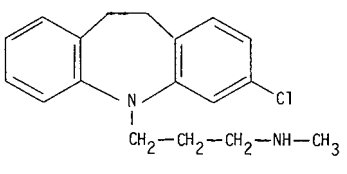
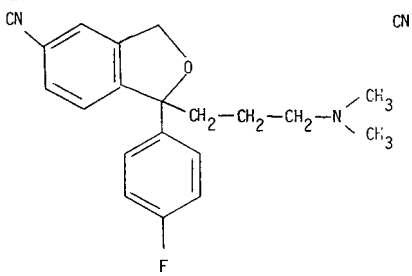


(CLOMIPRAMINE)

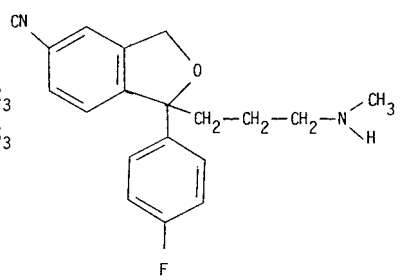


(DESMETHYLCLOMIPRAMINE)

Bicyclics



(CITALOPRAM)
(Lu 10-171)



(MONODESMETHYLCITALOPRAM)
(Lu 11-109)

Fig. 1. Chemical structures of compounds of interest.

HPLC procedures generally involve extraction of the tricyclic antidepressants from plasma or serum, followed by various modes of chromatographic separation and detection [18—24]. Recently, an analytical procedure for the determination of citalopram and its metabolites in plasma by HPLC was published [25].

The present study was undertaken to develop a common procedure for the routine clinical determination of citalopram, amitriptyline and clomipramine, as well as their desmethyl metabolites in plasma.

EXPERIMENTAL

Reagents

All reagents were of analytical grade. Methanol (RS per HPLC), acetonitrile (RS per HPLC), diethyl ether (RPE), 2 *M* sodium hydroxide (RPE) and acetone were from Carlo Erba. Potassium dihydrogen phosphate, 0.025 *M* was from Prolabo, and 0.5 *M* sulphuric acid from E. Merck.

Standards

Citalopram · HBr (Lu 10-171) and monodesmethylcitalopram · HCl (Lu 11-109) were supplied by Lundbeck. Amitriptyline · HCl was supplied by Roche. Nortriptyline · HCl was supplied by Squibb. Clomipramine · HCl, desmethylclomipramine · HCl and desipramine · HCl were supplied by Ciba-Geigy.

Stock solutions of each drug are prepared in methanol at a concentration of 1 μg/μl and stored at 4° C. They were diluted to 10 and 1 ng/μl for preparation of calibration standards.

Apparatus and chromatographic conditions

A Waters Model M45 pump fitted with a Waters U6K injector was used. A μBondapak C_{18} column, 10 μm particle size (30 cm × 3.9 mm I.D.) was connected to a Waters Model 441 detector monitored at 254 nm. The mobile phase was a mixture of acetonitrile—0.025 *M* potassium dihydrogen phosphate—distilled water (45:50:5) at a flow-rate of 0.5 ml/min for the bicyclic drug and 1 ml/min for the tricyclic compounds. Retention times are indicated in Table I. The increased flow-rate for tricyclic drugs allows retention times of less than 15 min.

TABLE I

RETENTION TIMES OF DRUGS

Drug	Retention time (min)	
	Flow-rate 0.5 ml/min	Flow-rate 1 ml/min
Citalopram	11.34	
Monodesmethylcitalopram	10	
Amitriptyline	18.66	10
Nortriptyline	15.66	8
Clomipramine	24	13
Monodesmethylclomipramine	19.84	10.20
Desipramine	13.85	7.20

Glassware

All glassware was washed with a mixture of sulphuric acid—potassium bichromate solution before use. All glass centrifuge tubes were rinsed with acetone and ether.

Extraction procedure

Into a centrifuge tube measure 100 μl of internal standard solution. Desipramine (1 ng/μl) is used as internal standard for citalopram and clomipramine analysis; clomipramine (1 ng/μl) is used for amitriptyline and nortriptyline analysis. Add 1—2 ml of plasma, 1 ml of 2 *M* sodium hydroxide and 10 ml of diethyl ether. Shake for 10 min and centrifuge for 5 min at 2800 *g*. Transfer the organic phase to another centrifuge tube and shake for 10 min with 2 ml of 0.5 *M* sulphuric acid. Centrifuge for 5 min at 2800 *g* and discard the top layer. To the aqueous layer add 3 ml of 2 *M* sodium hydroxide and re-

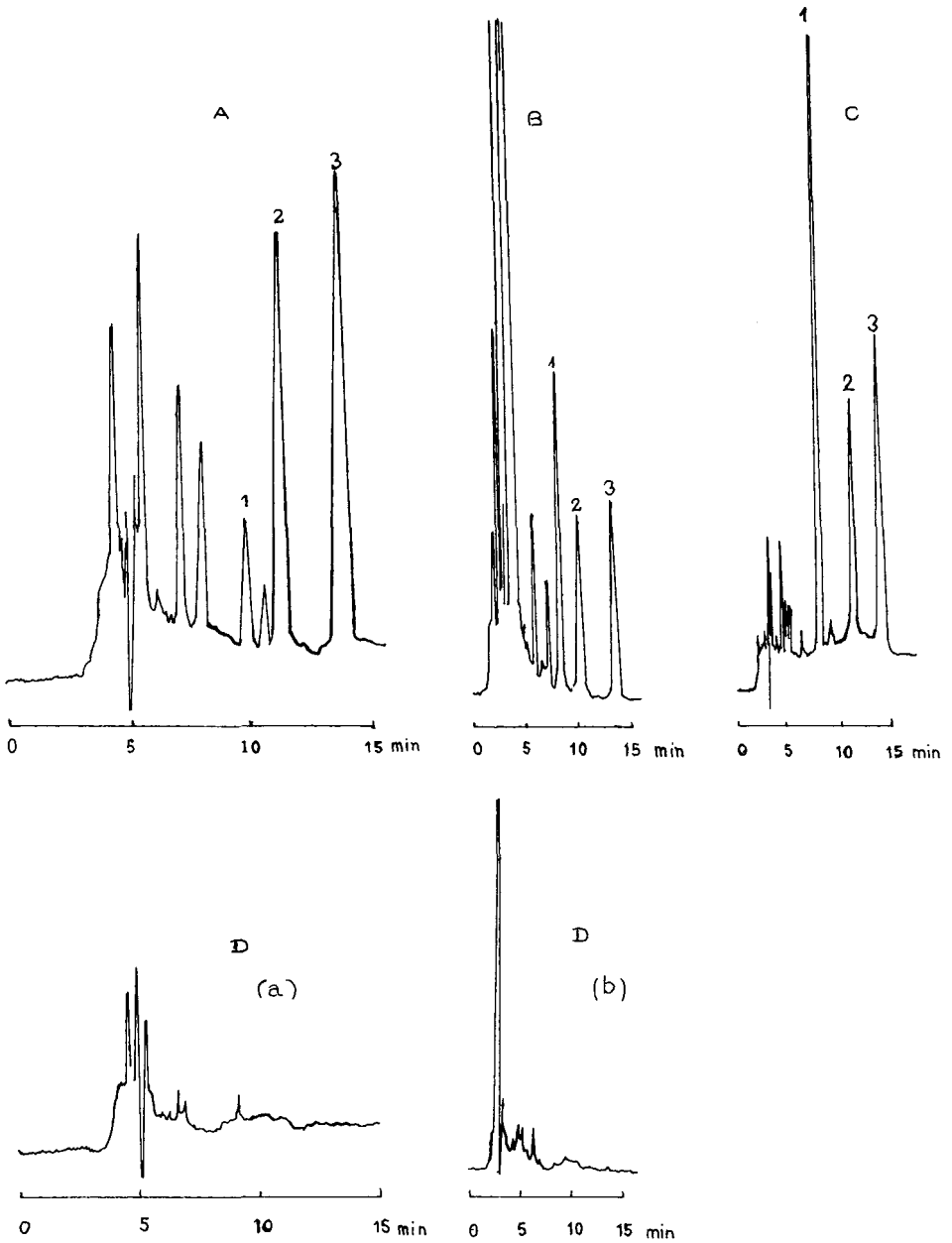


Fig. 2. (A) Chromatogram of a 1-ml plasma extract from a patient receiving daily 60 mg of citalopram • HBr for one month. Peaks: 1 = monodesmethylcitalopram, 2 = citalopram, 3 = desipramine (internal standard). (B) Chromatogram of a 1-ml plasma extract from a patient receiving daily 50 mg of amitriptyline • HCl intramuscularly for two weeks. Peaks: 1 = nortriptyline, 2 = amitriptyline, 3 = clomipramine (internal standard). (C) Chromatogram of a 1-ml plasma extract from a patient receiving daily 75 mg clomipramine • HCl for two months. Peaks: 1 = desipramine (internal standard), 2 = desmethylclomipramine, 3 = clomipramine. (D) Chromatogram of a 1-ml blank plasma extract; flow-rate 0.5 ml/min (a) or 1 ml/min (b).

extract with 10 ml of diethyl ether. After centrifugation, remove the organic phase and evaporate to dryness under nitrogen. Dissolve the residue in 100 µl of the mobile phase on a whirlmixer. Inject 20—50 µl of this solution into the chromatograph for analysis.

The ratio between the peak area of the analysed drug and that of the internal standard is calculated and plotted against the concentration of the tested drug after analysis of plasma samples spiked, respectively, with increasing amounts of each drug (10—400 ng/ml) and a constant amount of the appropriate internal standard (100 ng). The linear regression data for calibration curves were determined: the relations are linear between 10 and 300 ng/ml for bicyclics and between 10 and 400 ng/ml for tricyclics.

Chromatograms of plasma extracts from psychiatric patients receiving daily 60 mg of citalopram for one month, 50 mg of amitriptyline for two weeks, or 75 mg of clomipramine for two months are presented in Fig. 2.

RESULTS

Recovery experiments

The percentage extraction of each drug (10—400 ng/ml) was measured using the analytical conditions described. For the assay, the tested drugs are added before the extraction procedure and appropriate internal standard in the last organic phase. For the blank, drugs and internal standard are added together to the last organic phase. The recoveries were 84% and 80% for citalopram and its metabolite, 92% and 93% for amitriptyline and nortriptyline, 88% and 84% for clomipramine and monodesmethylclomipramine, respectively.

Detection limit

The detection limits for quantitative determination were 2—4 ng/ml for amitriptyline and nortriptyline, 2.5—5 ng/ml for clomipramine and desmethylclomipramine, 4—5 ng/ml for citalopram and monodesmethylcitalopram (the use of a UV—LC Philips detector at 239 nm for these bicyclic compounds allows a better limit, 1 ng/ml).

Reproducibility

The reproducibility of the analysis, within day (7—9 determinations) and day to day (three determinations), is indicated in Tables II and III. The within-day coefficients of variation are between 3.7% and 9.4% for the lower concentrations (8 or 9 ng/ml) and less than 6.5% for the upper concentrations (20—360 ng/ml). The day-to-day coefficient of variation is between 1.5% and 5.4% for four determinations over a period of a month (the samples were frozen for seven to thirty days).

Selectivity

Twenty-two drugs were tested for possible interference (Table IV). For analysis of bicyclics, carbamazepine, norclobazam and desmethylflunitrazepam, which might be partially extracted, were not resolved from either citalopram or desipramine (internal standard). For analysis of tricyclics, triazolam, nordiazepam, clobazam, trimipramine, diazepam, norclobazam and metabolites

TABLE II

WITHIN-DAY REPRODUCIBILITY

Drug	Concentration (ng/ml)	n	Mean $r_{S/IS}$* ± S.D.	C.V. (%)
Citalopram	8	9	0.028 ± 0.0016	5.7
	20		0.065 ± 0.0035	5.4
	40		0.146 ± 0.007	4.8
	80		0.295 ± 0.012	4
Monodesmethylcitalopram	9	9	0.040 ± 0.0015	3.7
	23		0.102 ± 0.0036	3.5
	45		0.209 ± 0.0038	2
	90		0.441 ± 0.010	2.3
Amitriptyline	9	7	0.110 ± 0.00818	8.4
	22		0.248 ± 0.01603	6.4
	44		0.490 ± 0.0196	4
	88		1.187 ± 0.057	4.8
	176		2.09 ± 0.0412	2
	352		4.284 ± 0.058	1.3
Nortriptyline	9	7	0.118 ± 0.0087	7.4
	22		0.33 ± 0.0085	2.6
	44		0.648 ± 0.0234	3.6
	88		1.308 ± 0.0850	6.5
	176		2.380 ± 0.122	5.1
	352		4.936 ± 0.1562	3.1
Clomipramine	9	7	0.059 ± 0.005	8.4
	45		0.009 ± 0.129	4.1
	90		0.567 ± 0.0256	4.5
	180		1.102 ± 0.0297	2.7
	340		2.007 ± 0.055	2.7
Desmethylclomipramine	9	7	0.068 ± 0.00642	9.4
	45		0.350 ± 0.014	4
	90		0.584 ± 0.018	3
	180		1.352 ± 0.034	2.5
	360		2.335 ± 0.066	2.8

*$r_{S/IS}$ = ratio between peak area of the analysed drug (S) and that of the internal standard (IS).

TABLE III

DAY-TO-DAY REPRODUCIBILITY

Drug	Added (ng/ml)	Found (ng/ml)				Mean ± S.D.	C.V. (%)
		Day 1 (n = 3)	Day 7 (n = 3)	Day 15 (n = 3)	Day 30 (n = 3)		
Citalopram	20	20.19	20.70	19.30	19.92	20.20 ± 0.63	3.15
Monodesmethylcitalopram	20	19.33	19.66	17.04	19.13	18.77 ± 1.016	5.40
Amitriptyline	176	182.40	176.80	174.20	174.40	177 ± 3.3	1.86
Nortriptyline	176	180.50	178.50	169	171.70	174 ± 2.36	1.5
Clomipramine	180	188	171.5	168	172	175 ± 7.6	4.4
Desmethylclomipramine	180	178	168.2	170.70	168.24	171.27 ± 3.8	2.22

TABLE IV

ELUTION OF CITALOPRAM, AMITRIPTYLINE, CLOMIPRAMINE AND THEIR METABOLITES, AND SOME SUBSTANCES TESTED FOR POSSIBLE INTERFERENCE

Flow-rate 0.5 ml/min.

Substance	Retention time (min)	Substance	Retention time (min)
Solvent front	4	*Desipramine*	13.85
Endogenous substances	4.10—7	7-Acetamidonitrazepam	15
Meprobamate	4.10	7-Aminonitrazepam	15.16
Caffeine	6.66	Triazolam	15.16
Viloxazine	6.70	*Nortriptyline*	15.66
Dibenzepine	7.84	Nordiazepam	16.66
Indalpine	8.10	Imipramine	16.90
Monodesmethylcitalopram	10.00	Flunitrazepam	17
Amineptine	11.20	Levomepromazine	17.66
Citalopram	11.34	Clobazam	18.34
Carbamazepine	11.66	*Amitriptyline*	18.66
Desmethylflunitrazepam	12.34	Trimipramine	18.90
Nitrazepam	12.50	*Desmethylclomipranine*	19.84
Doxepine	12.66	*Clomipramine*	24
Estazolam	13.34	Diazepam	24.50
Norclobazam	13.50		

of nitrazepam might interfere with either of these antidepressants or with desipramine (internal standard for clomipramine and its metabolite). Twelve drug-free plasmas from healthy subjects were extracted and analysed for possible interference by endogenous constituents, but no background interference was observed. Therefore citalopram cannot be quantitated in samples which also contain carbamazepine, clobazam and flunitrazepam. For analysis of tricyclics it is not possible to use diazepam (except for analysis of amitriptyline), triazolam, clobazam, or trimipramine as co-medications. These tests also indicate that the method can be extended to the quantitation of other antidepressants in plasma. We are now studying the possible applications for determining imipramine and desipramine, metapramine and its main metabolites.

CONCLUSIONS

The proposed method provides excellent sensitivity and reproducibility for the HPLC analysis of citalopram, amitriptyline, clomipramine and their desmethyl metabolites. Its selectivity could cause some inconvenience because of the possible interference from some drugs commonly administered as co-medications. The method is suitable for therapeutic monitoring and for analytical purposes in cases of possible intoxication.

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CHROMBIO. 2426

15-HYDROXYCYPROTERONE ACETATE AND CYPROTERONE ACETATE LEVELS IN PLASMA AND URINE

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(First received July 19th, 1984; revised manuscript received September 6th, 1984)

SUMMARY

15β-Hydroxycyproterone acetate (15HOCPA) and cyproterone acetate (CPA) have been quantitated in human plasma and urine by a selective high-performance liquid chromatographic assay. The levels of 15HOCPA in plasma are generally twice those of its precursor CPA, although both compounds appear to have similar clearance rates. Approximately 6% of the dose is excreted into the urine, predominantly (> 90%) as the free form of 15HOCPA and CPA.

INTRODUCTION

Cyproterone acetate (CPA) is a synthetic steroid, whose efficacy as an antiandrogen in the treatment of acne and hirsutism is well documented [1]. Some controversy exists, however, whether CPA or one of its metabolites is the active compound [2—4]. The main evidence for the latter inference is based either on the lack of CPA activity when applied topically [2, 3] or the increase in patient responders when CPA is administered intramuscularly [4].

The major metabolite of CPA, in both plasma and urine, has been identified as the 15β-hydroxy analogue (15HOCPA) [5]. While assays for plasma CPA based on either radioimmunoassay (RIA) or high-performance liquid chromatography (HPLC) have been reported [6, 7], no assays for 15HOCPA have been published.

The significance of 15HOCPA is that while its antiandrogen properties are comparable to CPA, its progestational effect is considerably less [3, 8]. These results infer that 15HOCPA is potentially better suited than CPA for the treatment of androgen-induced problems in both women and men.

In this paper we describe a HPLC assay which allows quantitation of both

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CPA and HOCPA in either plasma or urine. Application of this assay allows the accurate simultaneous assessment of CPA and 15HOCPA levels.

EXPERIMENTAL

Chemicals

Cyproterone acetate and cyproterone (CP) were donated by Professor F. Neuman and 15β-hydroxycyproterone acetate by Professor I.D. Cooke.

All solvents were of the highest analytical grade and were redistilled prior to use. Organic extracts were dried using anhydrous sodium sulphate.

Thin-layer chromatography (TLC) was performed on Eastman precoated silica-gel sheets, in chloroform as solvent.

Synthesis of cyproterone propionate

A heterogenous mixture of cyproterone (1 g, 2.6 mmol) and Amberlite IR 120 resin (2 g) was stirred in redistilled propionic anhydride (5 ml) at 50°C for three days. The mixture was then poured into water, stirred for a further 1 h and then extracted with diethyl ether (20 ml three times). The ethereal extract was then washed with saturated sodium bicarbonate solution, water, brine and then dried. The solvent was removed using a rotary evaporator to leave a solid which was then chromatographed on a silica column.

Elution with diethyl ether—hexane (3:5, v/v) and recrystallization of the solid from diethyl ether gave cypropterone propionate, CPP (513 mg, 47%) as a pale yellow solid, m.p. 193—194°C. The product was homogenous by HPLC, TLC and had expected spectroscopic properties i.e. UV, IR and mass spectrometric (MS).

High-performance liquid chromatography

A Waters instrument was used (Waters Assoc., Milford, MA, U.S.A.) fitted with a univeral liquid injector and a variable-wavelength UV detector, set at 282 nm.

The column (300 × 3.9 mm) was an octadecylsilane reversed-phase (μBondapak, 10 μm), operated at ambient temperature, using acetonitrile—water (65:35, v/v) as the eluting solvent mixture.

Extraction procedure

Patient plasma (0.25—0.5 ml) or urine (0.5 ml) was added to the internal standard, CPP, (100 ng for plasma, 300 ng for urine) and the mixture sonicated for 30 min. Diethyl ether was then added (5 ml) and the mixture was shaken for 30 min, the ether extract was then separated and washed with aqueous sodium hydroxide (1 ml, 0.25 *M*), water (1 ml) and then dried. The ether extract was then transferred to another tube and the sodium sulphate washed with a further aliquot of diethyl ether (2.5 ml).

The combined ether extracts were evaporated to dryness at ambient temperature and then reconstituted in diethyl ether (1 ml) prior to Florisil column chromatography (300 mg). The column was developed with *n*-heptane (5 ml), diethyl ether (5 ml) and the required fraction then eluted with methanol—diethyl ether (1:10, v/v, 5 ml). The latter fraction was evaporated

to dryness, reconstituted in the HPLC solvent (0.3 ml) and a portion (0.015 ml) injected onto the column.

Quantitation was based on peak height measurements with respect to the standard curves. The relative retention times of the compounds are for 15HOCPA 0.41, CP 0.56, CPA 0.72 and CPP 1.00.

Standard curves

For each assay blank plasma or urine (i.e. no CPA therapy) was used to establish the response between CPA, 15HOCPA and CPP. Amounts of CPA and 15HOCPA added comprised of two ranges: (a) 10, 20, 30, 40, 50, 60, 70 and 80 ng; and (b) 30, 50, 100, 150 and 200 ng. Work-up was identical to that specified above for patient samples.

Radioimmunoassay of CPA

RIA was performed at the Institute of Medical and Veterinary Science (Adelaide, Australia) using an antibody to CPA supplied by Schering. Full details of the methodology have been previously published by Nieuweboer and Lubke [6].

Urinary conjugates of CPA and 15HOCPA

The urine sample was first extracted to quantitate the free fraction. The urine, which then only contained conjugates, was split into two equal fractions. One fraction was subjected to sulphate hydrolysis, while the other was treated with a glucuronidase enzyme [9]. Each sample was then processed in the usual manner.

Verification of 15HOCPA in patient samples

15HOCPA was converted to the respective 15-β-acetate (15ACCPA) by the standard acetic anhydride—pyridine procedure. The synthesised 15ACCPA had R_F 0.38 compared to 0.27 for 15HOCPA on TLC, and a retention time 0.75 min longer on HPLC.

Several plasma samples were processed in the normal manner and the final extracts subjected to preparative HPLC with respect to the peak assigned as 15HOCPA. Acetylation of the isolated compound gave a single major component with identical properties to 15ACCPA on both TLC and HPLC.

Other plasma samples were extracted and then the final solution prior to HPLC partitioned. One aliquot was chromatographed as the control. The other aliquot was acetylated and then subjected to HPLC. The retention time of the peak attributed to 15HOCPA in the control was absent after acetylation, with a new peak appearing at the retention time associated with 15ACCPA.

Patient samples

In this institution plasma and urine samples were collected from women undergoing CPA therapy on the reverse sequential regimen of CPA (100 mg, days 5—15) and ethinyl estradiol (0.05 mg, days 5—25). Samples were taken on different days of the cycle, however, the length of time each individual patient had been on treatment was not constant.

The samples used for the RIA versus HPLC comparison were supplied by

IMVS. These represented a number of varied regimens, including both continuous and intermittent treatment, as well as the standard reverse sequential course.

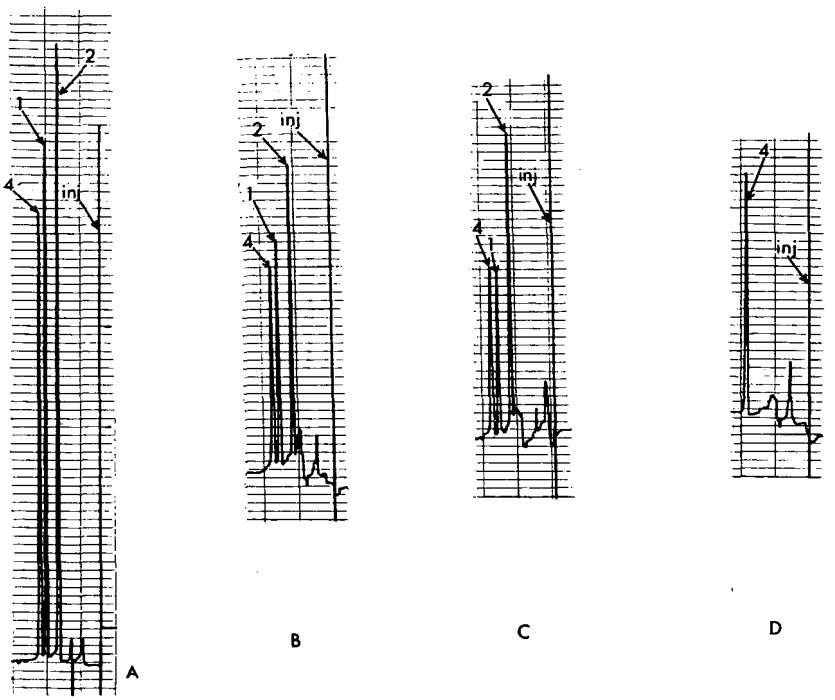

Fig. 1. HPLC profiles of (A) standards; (B) actual plasma sample; (C) previous sample spiked with authentic 15HOCPA; (D) plasma sample from subject not on CPA therapy. Peaks: 1 = CPA; 2 = 15HOCPA; and 4 = CPP.

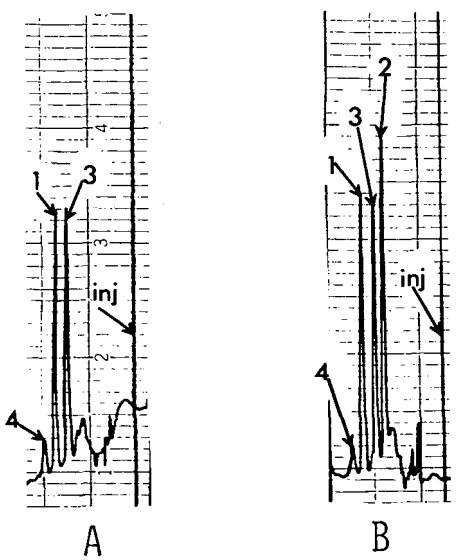

Fig. 2. HPLC profiles of (A) CPA plasma sample after acetylation; and (B) same sample spiked with 15HOCPA. Peaks: 1 = CPA; 2 = 15HOCPA; 3 = 15ACCPA; and 4 = CPP.

RESULTS

Initial experiments showed that simple extraction of CPA and 15HOCPA from plasma resulted in many interfering peaks in the HPLC trace. Consequently, a step involving Florisil chromatography was added. Fig. 1 shows typical

TABLE I

INTER-ASSAY PRECISION OF HPLC METHOD

Units are nmol/l and nmol/ml for plasma and urine, respectively.

Sample	n	CPA			15HOCPA		
		Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
Plasma	6	111	11.9	11	241	45.5	19
Plasma	6	290	16.9	6	771	169.0	21
Plasma	3	472	38.0	8	349	40.0	11
Plasma	8	900	158.0	17	2695	515.0	19
Urine	3	1.71	0.28	16	5.99	0.84	14

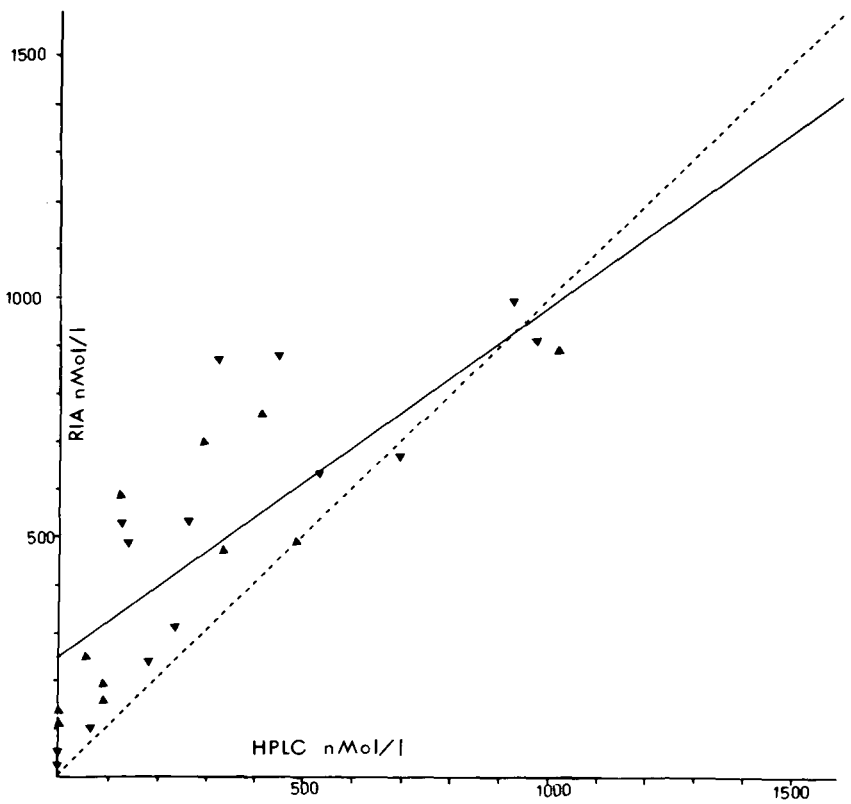

Fig. 3. Comparison of CPA values measured on the same plasma sample by RIA and HPLC. The dashed line represents the optimum 1:1 response slope. Intercept = 252.8; slope = 0.775; r = 0.81; and S_{yx} = 177.2.

HPLC traces for the procedure described in Experimental. Identity of the two analytes CPA and 15HOCPA was not only confirmed by spiking with authentic samples, but also by collection and chemical derivatisation for 15HOCPA (see Experimental and Fig. 2).

To obviate any problems associated with recovery from plasma or urine a homologous analogue of CPA was synthesised as the internal standard. Other experiments established that the absolute recoveries for CPA and 15HOCPA through the whole procedure were 78% and 84%, respectively. Standard curves of CPA versus CPP and 15HOCPA versus CPP were linear with correlation coefficients > 0.99 in all cases. The minimum detectable concentration was approximately 20—40 nmol/l for both CPA and 15HOCPA.

The precision of the HPLC method was assessed by repeated measurements on different patient samples (Table I). Comparison of CPA values by RIA and HPLC are shown in Fig. 3. The results indicate the existence of both proportional (slope) and constant (intercept) errors between the assays.

The levels of 15HOCPA and CPA in the same plasma samples are shown in

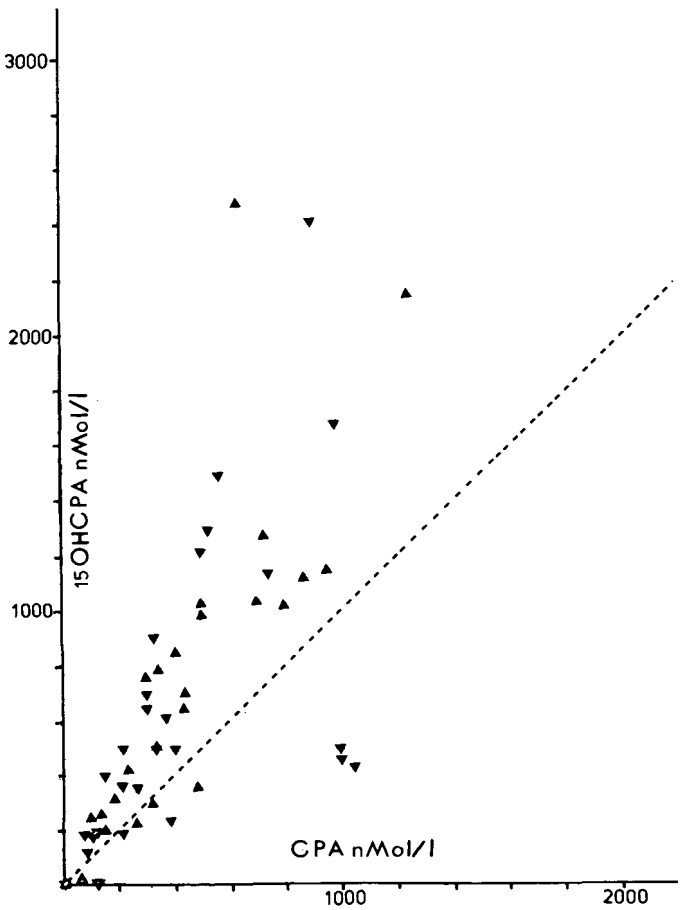

Fig. 4. Comparison of CPA and 15HOCPA values in the same plasma sample as measured by HPLC. The dashed line represents equal molar values.

Fig. 4. Ratios of 15HOCPA/CPA ranged from 0.38 to 3.2, with a median value of 1.85. No relationship was apparent between the 15HOCPA/CPA ratio and the day of the cycle (5—15) on which the medication was taken. Similarly, absolute values of CPA and 15HOCPA were not clearly related to the number of times the drug regimen had been completed. Analysis of plasma samples taken on day 5, prior to the commencement of a new course, showed negligible levels of CPA and 15HOCPA.

Analysis of the urinary results (Table II) shows that CPA and 15HOCPA are excreted almost exclusively in the free form. The relative amount of 15HOCPA to CPA (7.3, 4.3 and 3.9), however, appears more elevated than in plasma. This high ratio of 15HOCPA/CPA is diminished for the urinary conjugates. These results imply that the higher excretion of free 15HOCPA may be related to a greater water solubility than CPA.

TABLE II

URINARY EXCRETION OF CYPROTERONE ACETATE AND 15β-HYDROXYCYPROTERONE ACETATE

	Subject I	Subject II	Subject III
Free			
CPA (nmol/ml)	2.31	3.13	1.71
15HOCPA (nmol/ml)	17.0	13.4	6.63
Glucuronides			
CPA (nmol/ml)	0.48	0.62	n.m.*
15HOCPA (nmol/ml)	1.51	1.12	n.m.
Total (nmol/ml)	21.30	18.27	
Percentage free	90.7	90.5	
Volume (ml)	660	770	S**
24 h Total (μmol)	14.06	14.06	
Percentage 100 mg dose	5.92	5.92	

*n.m. = value not measured.
**S = sample not a 24-h sample.

DISCUSSION

The HPLC assay described herein is shown to allow reliable quantitation of CPA and 15HOCPA in both plasma and urine. No assay has previously been published for 15HOCPA (see ref. 4). The advantages of HPLC assay, in comparison to RIA techniques, centre on the increased specificity and the ability to simultaneously measure more than one analyte.

The results of the present investigation, with respect to CPA plasma levels, are similar to those previously published [4]. Plasma levels of 15HOCPA were found to be approximately twice those of CPA (Fig. 4). The plasma clearance rate of both CPA and 15HOCPA, however, appears to be similar based on the following observations. First, negligible amounts of either CPA or 15HOCPA were present, prior to initiation of a new drug course. Secondly, the 15HOCPA/CPA ratio did not show any marked change between days 5 and 15 of the drug regimen.

Approximately 6% of the typical daily CPA dose (100 mg), was found to be excreted in the urine (Table II). Only a minor fraction (10%) of the urinary products, however, were conjugated. The conjugates were glucuronides, with no evidence for sulphates. Based on previous radioactive studies, 5% and 30%, respectively, of the CPA dose had been reported as excreted in the urine [10, 11]. Our finding of substantial amounts of free urinary 15HOCPA and CPA agrees with the earlier work cited for males [5].

The present study shows that a major part of the administered CPA dose is rapidly metabolised to 15HOCPA, and that 15HOCPA has a similar plasma clearance to CPA. This finding would add support to the suggestion that 15HOCPA replaces CPA in the reverse sequential therapy, because of its reduced progestational effect relative to its antiandrogen properties [4]. Practical questions of 15HOCPA bioavailability and cost of synthesis however, would also need to be addressed.

ACKNOWLEDGEMENTS

We thank Schering for the supply of cyproterone and cyproterone acetate. The collaboration of J.D. Gilliland (IMVS) in the RIA versus HPLC comparison is gratefully acknowledged. The assistance of Drs. C.D. Matthews, B. Higgins and in particular M. McEvoy in the supply of patient samples is also acknowledged.

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Note

Urinary excretion of N-acetylamino acids

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(Received August 23rd, 1984)

N-Acetylation is a pathway in the metabolism of amino acids, to which little attention has been paid. There are only a few reports on single N-acetylamino acids detected in urine. Small amounts of N-acetyltryptophan [1], N^{α}-acetyllysine [2] and N-acetylhistidine [3] were found to be excreted in urine. Increased urinary N^{α}-acetyllysine was observed in a case of hyperlysinaemia and lysinuria [2], and elevated urinary N-acetylhistidine in patients with histidinaemia [3]. The N-acetyl derivative of a more unusual amino acid, aminooctanoic acid, has also been described [4]. During our analysis of organic acids a series of N-acetylamino acids was found in normal urine.

EXPERIMENTAL

Materials and samples

Alanine, valine, leucine, aspartic acid and glutamic acid from E. Merck (Darmstadt, F.R.G.) were used to prepare N-acetyl derivatives. The reference substance N-acetyl-O-methyltyrosine methyl ester was purchased from Sigma Chemie (Taufkirchen, F.R.G.).

Several 24-h urine samples were collected from healthy individuals and analysed directly after the collection period.

Analysis of organic acids in urine

The sample preparation and the gas chromatographic (GC) and gas chromatographic—mass spectrometric (GC—MS) studies were carried out according to the procedure previously described [5]. The reference compounds were analysed by the same GC and GC—MS conditions as the urinary constituents.

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Synthesis of N-acetylamino acid methyl esters

One milligram of each of the reference amino acids was allowed to react in screw-capped vials (Macherey-Nagel, Düren, F.R.G.) with 1 ml of a solution of 10% acetyl chloride in methanol for 10 min at 110° C. The reaction mixture was evaporated to dryness and 1 ml of acetic anhydride was added. After a reaction of 30 min at 110° C, the solution was evaporated to dryness again and the resulting N-acetylamino acid methyl ester was redissolved in 1 ml of methanol.

RESULTS AND DISCUSSION

Detection of N-acetylamino acids within the profile of the organic acids

The N-acetyl metabolites of alanine, valine, leucine, aspartic acid and glutamic acid are constituents of fraction 3d, N-acetyltyrosine a component of fraction 4 of the urinary organic acids. Both are polar fractions. Fig. 1 demonstrates that the N-acetylamino acids of fraction 3d belong to the compounds of medium to low concentration. The same is observed for N-acetyltyrosine. Their recognition and identification in urine by GC—MS is facilitated by the pre-fractionation technique, through which they are enriched in the subfractions. Quantification has not yet been attempted.

Mass spectrometric fragmentation and identification of the N-acetylamino acid methyl esters

The MS fragmentation of the N-acetylamino acid methyl esters is characterized by a number of general features. For the substances identified as urinary constituents these characteristics are systematically described in Table I, even though the spectra of the methyl esters of N-acetylleucine [6] and N-acetylglutamic acid [7] have been published.

The molecular ion can be distinguished as a small peak only in the methyl esters of N-acetylalanine and N-acetyltyrosine. A major fragment in all of the mass spectra is the ion $(M—COOCH_3)^+$. By further loss of ketene from the acetyl group resulting in $(M—COOCH_3, —CH_2CO)^+$, another ion with high

TABLE I

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF THE N-ACETYLAMINO ACIDS IDENTIFIED IN URINE

The values represent the relative intensities (%) of the fragments listed.

Substance	MW*	M^+	$(M—COOCH_3)^+$	$(M—COOCH_3, —CH_2CO)^+$	*m/e* 43
N-Acetylalanine	145	4	69	100	45
N-Acetylvaline	173	—	60	100	53
N-Acetylleucine	187	—	51	100	47
N-Acetylaspartic acid	203	—	53	100	64
N-Acetylglutamic acid	217	—	19	27	54
N-Acetyltyrosine**	251	2	75	11	29

*Molecular weight.

**Methylated at the phenolic —OH group.

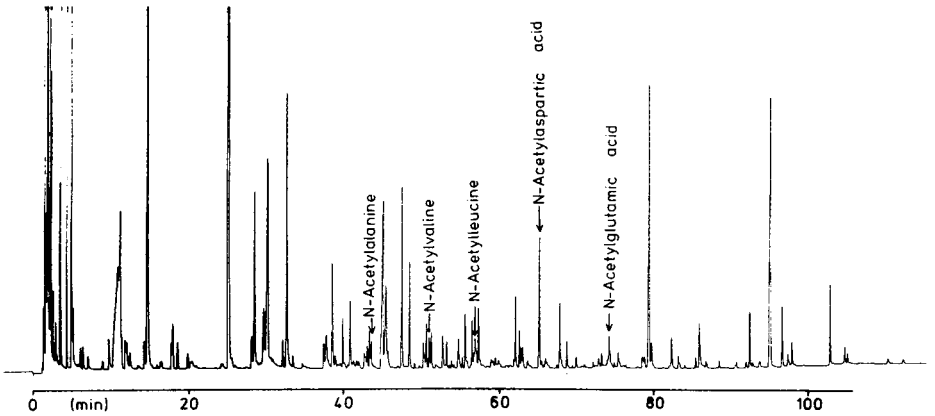

Fig. 1. Gas chromatogram of fraction 3d of the methyl esters of the organic acids in urine of a healthy individual. The N-acetylamino acids are labelled.

abundance, in some cases the base peak, is formed. An intense fragment in all of the spectra is also the peak at *m/e* 43, corresponding to the acetyl ion. The loss of the side-chain R of the amino acid derivative and of a ketene molecule leads to an ion at *m/e* 88, which has its highest abundance in the spectrum of N-acetylvaline methyl ester with R being an isopropyl group. A fragment of low intensity, however, always present, is $(M-CH_3CO)^+$.

McLafferty rearrangement at the carbomethoxy group leads to *m/e* 131 in the fragmentation of N-acetylvaline and N-acetylleucine. As in the spectra of regular dicarboxylic acid methyl esters, the fragmentation of the N-acetylaspartic acid (Figs. 2 and 3) and N-acetylglutamic acid methyl esters includes the ion $(M-COOCH_3, -CH_3OH)^+$. By additional loss of ketene the fragment $(M-COOCH_3, -CH_3OH, -CH_2CO)^+$ is formed which is the base peak of glutamic acid methyl ester. Further characteristic peaks of low abundance in the spectra of the aminodicarboxylic acid methyl esters are $(M-CH_3O)^+$ and $(M-CH_3OH)^+$. In the N-acetyltyrosine methyl ester in which the phenolic —OH group is methylated as well by the procedure applied to the urinary acids, the base peak corresponds to the aromatic ion *m/e* 121.

m/e 88	$(M-CH_3CO)^+$	*m/e* 131	$(M-COOCH_3, -CH_3OH)^+$	$(M-COOCH_3, -CH_3OH, -CH_2CO)^+$
11	3	—	—	—
42	1	2	—	—
24	2	11	—	—
10	2	—	3	26
9	2	—	35	100
6	2	—	—	—

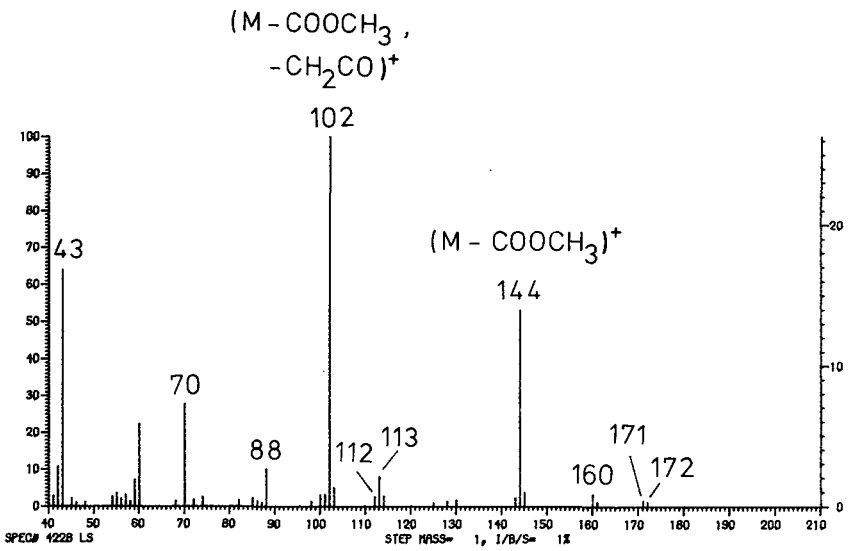

Fig. 2. Mass spectrum of the methyl ester of N-acetylaspartic acid identified in urine of a healthy individual.

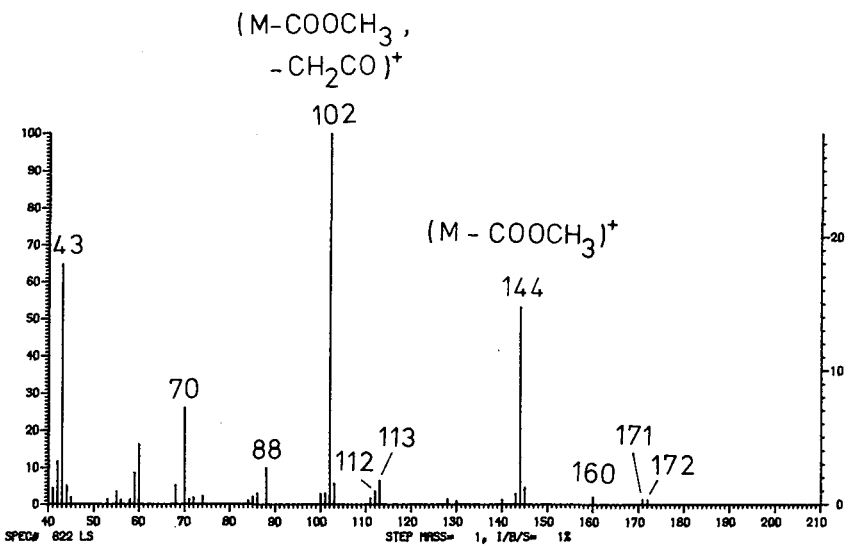

Fig. 3. Mass spectrum of the reference N-acetylaspartic acid methyl ester.

On the basis of the discussed general characteristics of the MS fragmentation of the N-acetylamino acid methyl esters, the recognition of possibly occurring further metabolites should be feasible.

N-Acetylamino acids in urine

The six to our knowledge not so far reported N-acetylamino acids are regularly found in normal urines. Together with N-acetyltryptophan, N^{α}-acetyllysine and N-acetylhistidine, nine acetyl derivatives of common amino acids are now identified. We conclude from these findings that N-acetylation of the

normal amino acids is a metabolic pathway of minor extent but occurring regularly. The biochemical mechanism, the role of enzymes in the N-acetylation and the location where this metabolic reaction takes place, remain to be investigated.

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Note

Determination of adult sheep plasma catecholamines using [^{3}H]norepinephrine as the internal standard

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(Received September 17th, 1984)

Recent publications have described the use of liquid chromatography with electrochemical detection for the determination of plasma catecholamines [1, 2]. All are based on a liquid—solid extraction of the catecholamines onto alumina, followed by elution with dilute perchloric acid which serves as the injectable extract. An internal standard, 3,4-dihydroxybenzylamine (DHBA), is added to each plasma sample to be extracted. We have found the method to work well with plasma from humans, monkeys, pigs, rats, and fetal sheep. However, when neonatal or adult sheep plasma is extracted, the recovery of the DHBA is very low. This has also been reported to be a problem when extracting dog plasma [3]. We describe below a technique using [^{3}H]norepinephrine ([^{3}H]NE) as the internal standard to calculate the recovery of catecholamines in neonatal and adult sheep.

MATERIALS AND METHODS

Sample extraction was run in 1.5-ml polypropylene tubes to which the following had been added: 50 μl of 5 m*M* sodium hydrogen sulfite, 50 μl of 0.1 *M* perchloric acid or 50 μl of [^{3}H]NE (New England Nuclear, diluted to 0.0033 μCi per 50 μl of 0.1 *M* perchloric acid) and 50 μl of DHBA at a concentration of 1 ng per 50 μl of 0.1 *M* perchloric acid.

Differing amounts of pooled adult sheep plasma were then added. When using less than 1 ml of plasma, the volume was brought up to 1 ml with 0.1 *M* phosphate buffer, pH 7.0. Next, 20 mg of aluminum oxide (Fisher, activated by the method of Anton and Sayre [4]) were added to each tube followed by 600 μl of 1.5 *M* Tris buffer. The sample was then vigorously shaken for 15 min,

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the supernatant aspirated and the alumina washed three times with distilled water. After the final washing the alumina was aspirated dry. Next, 200 μl of 0.2 *M* perchloric acid were added to the alumina, the tubes shaken for 15 min, then centrifuged. A 100-μl aliquot of the perchloric acid extract was then injected into the HPLC system. In the samples where [^{3}H]NE was used as the internal standard, 50 μl of the perchloric acid extract were also counted in 5 ml of Aquasol with a Packard Tri-Carb liquid scintillation counter.

The high-performance liquid chromatograph consisted of an M-45 dual-piston pump (Waters Assoc.), a WISP 710B automated sample injector (Waters Assoc.), a 25-cm ODS 5-μm column (IBM Instruments), a glassy carbon working electrode with an Ag/AgCl reference electrode (BioAnalytical Systems), an LC-4B amperometric detector and an LC-22A temperature controller (BioAnalytical Systems), and an M730 Data Module (Waters Assoc.).

The mobile phase contained 100 m*M* sodium phosphate monobasic, 2.75 m*M* octanesulfonic acid, sodium salt (Eastman Kodak), 0.1 m*M* EDTA, disodium salt, and 5.5% acetonitrile. The flow-rate was 1.2 ml/min at 30°C with a working electrode potential of 0.65 V and a sensitivity of 0.5 nA.

RESULTS AND DISCUSSION

Our laboratory recently began measuring catecholamines in sheep plasma using the same methodology as that for human plasma. This method had already been successfully used for measuring catecholamines in monkeys, pigs, and rats with DHBA recoveries of 70—80%. It was observed that as seen with human plasma, 1 ml of fetal sheep plasma had a DHBA recovery of 70—80%. However, adult sheep showed a much lower recovery. We found that as the

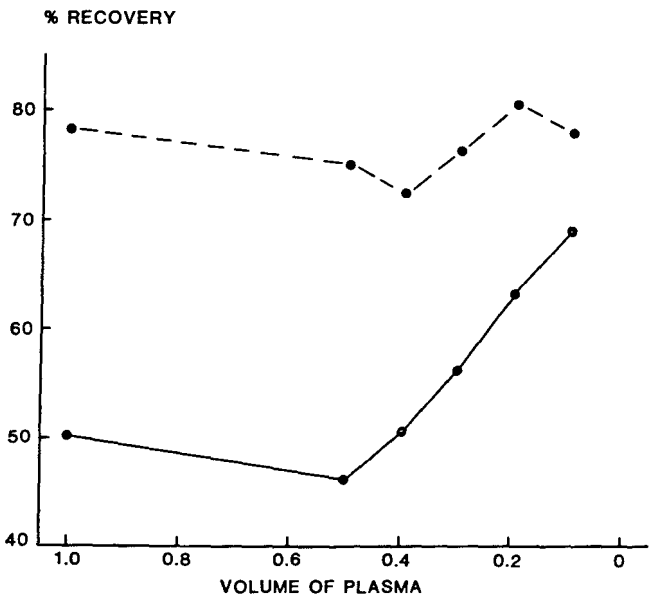

Fig. 1. Percent recovery of DHBA (—) and [^{3}H]NE (- - -) from differing volumes of adult sheep plasma.

TABLE I

CALCULATED VALUES FOR POOLED ADULT SHEEP PLASMA NE

Amount of plasma extracted (ml)	Recovery of DHBA (%)	NE using DHBA recovery (pg/ml)	Recovery of [^{3}H]NE (%)	NE using [^{3}H]NE recovery (pg/ml)
1.0	50.2	2364	78.2	1519
0.5	46.2	2312	75.2	1412
0.4	50.4	2275	72.4	1584
0.3	56.2	1667	76.3	1227
0.2	63.3	1815	80.7	1424
0.1	68.9	1580	78.0	1398

amount of adult sheep plasma used in the extraction procedure decreased, the recovery of DHBA increased (Fig. 1). The peak heights of NE, when corrected for the amount of plasma extracted, were comparable, showing NE recovery was not effected. The variation in DHBA recovery caused the calculated values of NE to be erroneously high (Table I).

Because we found the recovery of NE not to be effected, we chose [^{3}H]norepinephrine as our internal standard. The extraction procedure is the same with the exception of [^{3}H]NE which was diluted in 0.1 *M* perchloric acid being substituted for 0.1 *M* perchloric acid alone. NE values were calculated from the chromatogram, subtracting the amount of NE added, then using the recovery of [^{3}H]NE (Table I).

The reason for the low recovery of DHBA is not known but may be, as speculated by Robie and DuSapin [3], the result of protein binding which does not occur with NE. In sheep this protein appears in the plasma shortly after birth since the recovery of DHBA in fetal sheep is high.

In summary, when measuring catecholamines in neonatal or adult sheep, an internal standard other than DHBA must be used because the DHBA recovery by alumina extraction is low as compared to that of NE giving incorrect high values for NE. We have found [^{3}H]NE to be a satisfactory substitute.

ACKNOWLEDGEMENTS

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Note

Determination of bile acids in rat bile by high-performance liquid chromatography

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The determination of individual bile acids in biological fluids is important for studying bile acid metabolism in hepatobiliary and other diseases. The rat has been widely used in studies of bile acid metabolism, in which bile acids have been analysed by gas chromatography (GC) [1—3] and gas chromatography—mass spectrometry (GC—MS) [4]. In recent years, high-performance liquid chromatography (HPLC) has been applied to the analysis of bile acids in human bile [5—7] and serum [8—11]. However, the results for rat samples from this procedure with HPLC have not been satisfactory, because rats, like mice, possess several peculiar bile acids, such as α-, β-, and ω-muricholic acid (MCA), which complicate the bile acid separation.

This report describes a method for separating and quantifying the eight bile acids α-MCA, β-MCA, cholic acid (CA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA) in rat bile by HPLC, with an

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immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) column reactor for detection.

EXPERIMENTAL

Materials

Most of the standard bile acids and 5β-androstane-3α,11α,17β-triol were purchased from Sigma (St. Louis, MO, U.S.A.) and Steraloids (Wilton, NH, U.S.A.). α-MCA, β-MCA and ω-MCA were synthesized according to the methods of Hsia and co-workers [12, 13]. 3α,7β,12α-Trihydroxy-5β-cholanoic acid was synthesized according to the method of Samuelsson [14]. NAD was obtained from Oriental Yeast Co. (Tokyo, Japan) and 3α-HSD from Sigma. The other chemicals of reagent grade were purchased from Wako (Osaka, Japan).

Apparatus

An HPLC Model LC-3A (Shimadzu, Kyoto, Japan) equipped with a stepwise elution unit SGR-1A (Shimadzu) was used. A Zorbax C_8 column (particle size 5 μm, 250 mm × 4.6 mm I.D.; Dupont Instruments, Wilmington, DE, U.S.A.) was used at 40°C in a column oven CTO-2A (Shimadzu). 3α-HSD-bound amino glass beads (120—200 mesh) prepared by the glutaraldehyde method [15] were packed in a stainless-steel tube (50 mm × 2.1 mm I.D.) and used as a reactor at 20°C in a water bath. The reagent containing NAD was delivered by a peristaltic pump PRR-2A (Shimadzu). A spectrofluorophotometer RF-530 (Shimadzu) was used as a detector to monitor NADH fluorescence. A schematic flow diagram of the system is shown in Fig. 1.

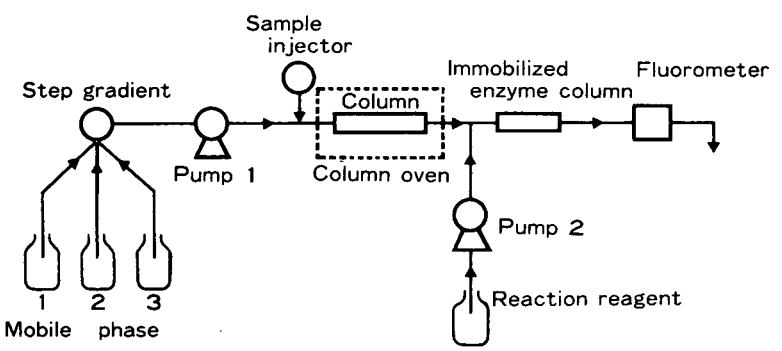


Fig. 1. Schematic flow diagram of the system.

Liquid chromatography

Individual bile acids were separated by reversed-phase chromatography with stepwise elution at a flow-rate of 0.5 ml/min. The initial mobile phase was 100 m*M* potassium phosphate buffer, pH 6.8—acetonitrile (10:4, v/v), the second was 20 m*M* potassium phosphate buffer, pH 6.8—acetonitrile (10:4, v/v), and the last was 1 m*M* potassium phosphate buffer, pH 6.8—acetonitrile (10:5, v/v). The time schedule for the stepwise elution was 8, 12 and 10 min for the first, the second and the third mobile phase, respectively. The eluate from the column was mixed with the NAD solution delivered at a flow-rate of 0.7

ml/min and migrated through the immobilized 3α-HSD column reactor. The NAD solution was prepared by dissolving NAD at the concentration of 0.1% in 100 m*M* Tris—HCl buffer (pH 8.0) containing 0.1% disodium EDTA. NADH formed in the reactor was monitored by the fluorescence detector at excitation 340 nm and emission 460 nm.

Standard bile acid solution

Eight standard bile acids (α-MCA, β-MCA, CA, UDCA, HDCA, CDCA, DCA, and LCA) and 5β-androstane-3α,11α,17β-triol as an internal standard were dissolved in the initial mobile phase.

Rat bile preparation

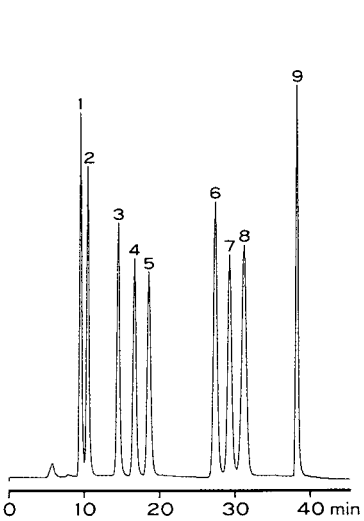
Wistar strain male rats weighing about 300 g were used; fresh bile was obtained by bile duct cannulation. Rat bile was prepared according to a slight modification of the method described previously [1]. To 10 μl of rat bile were added 100 μl of the internal standard solution (100 μg/ml in ethanol). Ethanol (2 ml) was added to the mixture and the solution was boiled for about 5 min and centrifuged at 650 *g* for 15 min. The supernatant solution was transferred into another tube. The residue was further extracted twice with 2 ml of ethanol twice. The extracts were combined to the supernatant solution and evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of 1.25 *M* sodium hydroxide solution and hydrolysed at 120°C for 6 h. The reaction mixture was acidified to pH 1.0—2.0 with 2 *M* hydrochloric acid solution and extracted three times with 6 ml of diethyl ether. The combined extracts were evaporated to dryness and the residue was dissolved in 1 ml of the initial mobile phase. A sample of 10—20 μl of the solution was used for HPLC.

RESULTS AND DISCUSSION

In addition to CA, CDCA, DCA, UDCA and LCA, rat bile contains α-MCA, β-MCA which are not found in human bile [1—4]. Since these bile acids could not be separated practically in a short time by HPLC with an isocratic elution system, we adopted a stepwise elution system. Fig. 2 shows a chromatogram of the eight standard bile acids and the internal standard. The components were resolved within 40 min into nine peaks with good separation. The relationship between the amount of each bile acid and the detector response was linear until 1.2 μg and the detection limit ranged from 0.8 to 1.5 ng for a signal-to-noise ratio of 2. Table I shows the reproducibility of the present method for 300 ng of the individual bile acids and the internal standard. The coefficient of variation (C.V.) for the retention time of each bile acid was less than 0.23% and that for the peak area was less than 1.04%.

In the preparation of rat bile, the bile acids were extracted effectively after hydrolysis. Recovery of each bile acid was in the range 93.9—98.3% (C.V. = 0.86—2.31%, $n = 5$). The internal standard was also recovered at 94.3% (C.V. = 0.94, $n = 5$).

Fig. 3 shows a chromatogram of the bile acids in normal rat bile. α-MCA, UDCA, HDCA, CDCA and DCA were detected with CA and β-MCA being the major components, but LCA could hardly be found. Several other unidentified



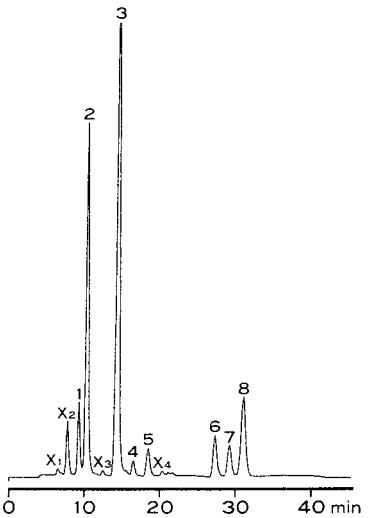


Fig. 2. Separation of standard bile acids and an internal standard. A mixture of 300 ng of each was injected. Peaks: 1 = α-muricholic acid; 2 = β-muricholic acid; 3 = cholic acid; 4 = ursodeoxycholic acid; 5 = hyodeoxycholic acid; 6 = chenodeoxycholic acid; 7 = deoxycholic acid; 8 = 5β-androstane-3α,11α,17β-triol (internal standard); 9 = lithocholic acid.

Fig. 3. Typical chromatogram of bile acids extracted from normal rat bile. Peaks: 1 = α-muricholic acid; 2 = β-muricholic acid; 3 = cholic acid; 4 = ursodeoxycholic acid; 5 = hyodeoxycholic acid; 6 = chenodeoxycholic acid; 7 = deoxycholic acid; 8 = 5β-androstane-3α,11α,17β-triol (internal standard); X_{1-4} = unknown.

TABLE I

REPRODUCIBILITY OF THE PRESENT METHOD FOR THE DETERMINATION OF INDIVIDUAL BILE ACIDS AND AN INTERNAL STANDARD

Column: Zorbax C_8, 250 × 4.6 mm I.D. Mobile phase: stepwise gradient programmed 8, 12 and 10 min for the first, second and third mobile phase, respectively. First mobile phase: 100 m*M* potassium phosphate, pH 6.8—acetonitrile (10:4, v/v); second mobile phase: 20 m*M* potassium phosphate, pH 6.8—acetonitrile (10:4, v/v); third mobile phase: 1 m*M* potassium phosphate, pH 6.8—acetonitrile (10:5, v/v). Flow-rate: 0.5 ml/min.

	Retention time		Relative peak area*	
	Mean (min)	C.V.** (%)	Mean (min)	C.V.** (%)
Internal standard***	31.08	0.12	—	—
α-Muricholic acid	9.35	0.10	0.74	1.03
β-Muricholic acid	10.32	0.15	0.69	0.71
Cholic acid	14.38	0.07	0.64	0.44
Ursodeoxycholic acid	16.53	0.13	0.62	0.54
Hyodeoxycholic acid	18.43	0.11	0.65	0.41
Chenodeoxycholic acid	27.22	0.07	0.93	0.49
Deoxycholic acid	29.11	0.10	0.82	0.32
Lithocholic acid	37.94	0.22	0.85	0.35

*Relative peak area was defined as the ratio of the peak area of individual bile acids to the area of the internal standard.

**$n = 5$.

***5β-Androstane-3α,11α,17β-triol.

peaks (X_1—X_4) were also present. These peaks were due to 3α-hydroxysteroids, since none of them appeared when the rat bile was analysed without NAD in the reagent.

Table II shows the retention times of other bile acids which should be present in rat bile. Their values differ from those of the eight standard bile acids and the internal standard we studied. The unidentified peak X_2 seemed to correspond to 3α-hydroxy-7,12-dioxo-5β-cholanoic acid. Peak X_3 appeared very close to that of hyocholic acid, but differed in its retention time. The small peak X_4 corresponded to 3α-hydroxy-7-oxo-5β-cholanoic acid or 3α-hydroxy-12-oxo-5β-cholanoic acid, which could not be separated by the present method. These unknown peaks, however, require further investigation to be identified. Peaks X_1 and X_3 and the small peaks behind X_4 had different retention times from any bile acid we tested.

TABLE II

RETENTION TIMES OF OTHER BILE ACIDS

Conditions as in Table I.

Bile acids	Retention time (min)	C.V.* (%)
3α,7β,12α-Trihydroxy-5β-cholanoic acid	7.16	0.21
3α-Hydroxy-7,12-dioxo-5β-cholanoic acid	7.78	0.47
ω-Muricholic acid	8.52	0.05
Hyocholic acid	12.29	0.06
3α-Hydroxy-12-oxo-5β-cholanoic acid	20.24	0.02
3α-Hydroxy-7-oxo-5β-cholanoic acid	20.25	0.04

*$n = 3$.

The present method using HPLC in combination with the immobilized 3α-HSD enzymatic detection offers selectivity for 3α-hydroxysteroids and does not require any derivatization of bile acids, which is essential in GC analysis. Deterioration of immobilized enzyme is generally of concern. The immobilized 3α-HSD column reactor in the present method reduced its sensitivity of detection to about 80% but it maintained almost the same reproducibility of intra-assay even after 200 times repetition of analysis. This indicates that our method is a reliable and simple one for analysing individual bile acids in rat bile.

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Note

Determination of homocysteine in urine*

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Homocysteine is a product of methionine metabolism rarely detectable in normal human tissue fluids [1]. This thiol-containing amino acid is present in excessive amounts in the blood and urine of persons suffering from homocystinuria (an enzyme deficiency blocking the metabolic pathway between homocysteine and cystathionine) [2, 3]. Homocystinurics exhibit a wide range of physical manifestations including skeletal defects and mental retardation; if allowed to continue unchecked, cardiovascular disease invariably kills the patient at any time after their early teen years [4, 5].

The prematurity and pronounced nature of this vascular damage, coupled with research showing that an increased level of homocysteine in the circulation is a major causative factor in accelerated cardiovascular injury [6, 7], has led us to develop a more sensitive, routine analytical technique for measuring homocysteine in biological fluids and to look at the possibility that hormone treatment may lead to increased levels of homocysteine which may predispose to cardiovascular damage.

Surveys suggest that women taking oral contraceptives have an increased risk of suffering thrombosis as compared to non-users [8, 9]. It has been proposed

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that oral contraceptives may interfere with sulfur amino acid metabolism [10, 11], leading to altered homocysteine production and/or breakdown in the methionine pathway, and elevated homocysteine levels in tissue fluids. This would increase cardiovascular damage, perhaps leading to thrombotic episodes.

This report describes a technique for determining the homocysteine concentration in urine. The procedure involves separation on an ion-exchange column followed by electrochemical detection (ED) at a hanging mercury drop. Preliminary studies involving urine from rats treated with estrogen and progestogen used in oral contraceptives are also reported.

METHOD

Chemicals

Buffers were made up with sodium dihydrogen phosphate (A.R. grade, BDH) in deionized, distilled water. The pH was adjusted with A.R.-grade orthophosphoric acid (BDH) and prior to chromatographic work, buffer filtration was carried out using a 0.45-μm filter (Millipore). Mercury was triply distilled (Englehard). Amino acid standards were: L-cysteine and L-methionine (Puriss grade, Fluka) and D,L-homocysteine (Sigma). Urine specimens were treated with acid (either orthophosphoric or hydrochloric acid, both A.R. grade, BDH) to prevent oxidation of the thiol groups prior to analysis.

Analytical system

An anodic reaction of cysteine at mercury electrodes leads to the formation of an adsorbed inorganic—metallic species:

$$2R{-}SH + Hg \rightarrow (R{-}S)_2Hg + 2H^+ + 2e$$

This has been the basis of several methods for the electrochemical detection of cysteine, as well as other biologically important thiols such as penicillamine and glutathione [12—17]. The reaction has been used to detect homocysteine in biological samples [12, 13, 17], but only recently has it been shown that the formation of an adsorbed homocysteine—mercury complex following oxidation is the basis for homocysteine detection [18]. Most electrodes for flowing systems utilize mercury-coated gold surfaces, operated amperometrically at a potential sufficiently positive to allow the anodic reaction to occur when the thiol group passes the electrode surface.

Adsorption products build up on the electrode with each subsequent detection, leading to a reduced electrochemical response over time, and rather than clean and renew the solid electrode surface each time, we have incorporated a static mercury drop electrochemical detector (Model 310, EG and G Princeton Applied Research), so a new mercury drop of specified size can be formed whenever significant surface contamination occurs. The thiol detection limit is about 0.5 ng, making it similar to freshly prepared mercury-coated gold electrodes. This is the first time this method has been used to detect biologically important thiols, although it has been used with success for other compounds [19].

Homocysteine is polarographically inseparable from cysteine [18], and strong cation-exchange high-performance liquid chromatography (HPLC)

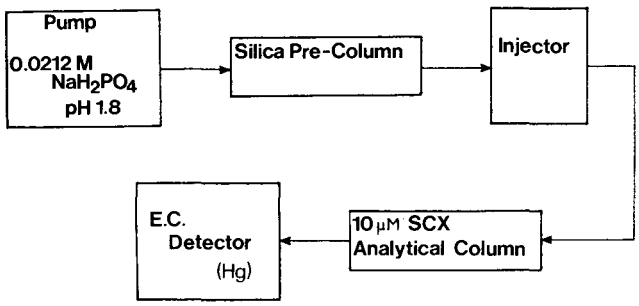

Fig. 1. Schematic representation of the HPLC—ED system for homocysteine determination.

(25 cm × 4.6 mm I.D. stainless-steel column containing Partisil-10 SCX 10 μm, Whatman) has been incorporated into the system to allow separation of the thiols prior to detection. The choice of ion exchange as the chromatographic medium is based upon previously developed methods to separate thiol-containing compounds [12, 13, 16, 17]. A unique feature of our method (Fig. 1) is the use of a silica pre-column (Silica Pre-Column Kit, Whatman) prior to the injector in the HPLC system, for a very acidic eluent is needed to allow satisfactory separation of homocysteine from other electrochemically active species. Without this silica pre-column, the silica backbone of the ion-exchange column can degenerate within 24 h, rendering the column useless. As the buffer passes through the silica pre-column first, silica dissolves and saturates the buffer, thus greatly reducing dissolution of silica from the backbone of the analytical column, resulting in extended column life [20—22]. Incorporation of the pre-column kit has enabled use of an analytical column on a daily basis for two months (about 800 injections) with no appreciable drop in column efficiency. Inclusion of the silica pre-column before the injection does not affect the separation of the substances of interest, since they only pass through the analytical column.

Urine collection

Rats used were female Sprague—Dawley. They were fed rat pellet food and water ad libitum during the experiments, except for the one fed a methionine (0.35%, w/w) loaded diet, where the amino acid was pre-mixed into the food. Urine was collected over 24-h periods from rats isolated in metabolic cages into vials containing known volumes of acid (either orthophosphoric or hydrochloric acid) to act as antioxidant, preventing thiols forming disulfides from atmospheric contact. Prior to chromatographic analysis, the samples were centrifuged (EBA 35, Hettich) at 500 *g* for 5 min to separate any solid material, and then the supernatant was diluted 1:1 with Nanopure water (Waters Assoc.) to give a more manageable injection volume.

Ethynyl estradiol (Sigma) was dissolved in absolute alcohol (0.83 mg/ml) as was levonorgestrel (Wyeth International) (0.83 mg/ml). Estradiol valerate (Sigma) was dissolved in 1:1 benzyl benzoate—castor oil (0.03 mg/ml).

RESULTS AND DISCUSSION

An acidic eluent (pH 2.2, 0.02 *M* sodium dihydrogen phosphate) was

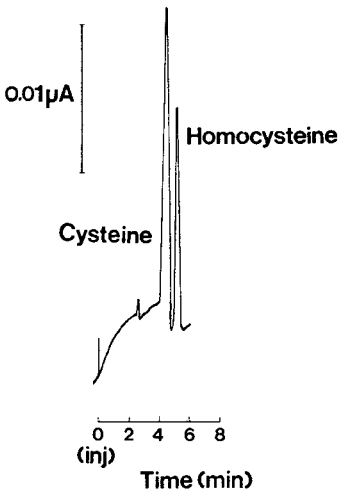

Fig. 2. HPLC profile obtained using a Partisil SCX-10 column followed by electrochemical detection at a hanging mercury drop electrode (at −0.15 V versus Ag/AgCl) of an injected sample containing $2.5 \cdot 10^{-5}$ *M* concentrations of homocysteine and cysteine in 5 μl of eluting buffer. Eluting buffer is 0.02 *M* sodium dihydrogen phosphate, pH 2.2 at a flow-rate of 1.0 ml/min.

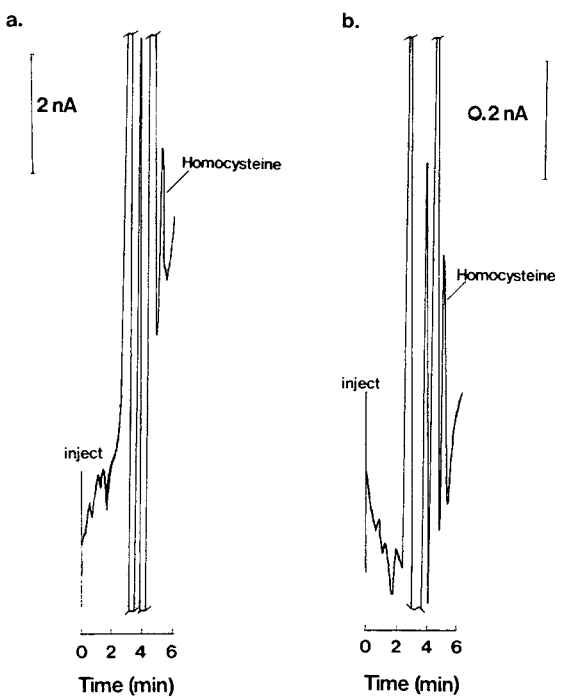

Fig. 3. HPLC profile obtained using a Partisil SCX-10 column followed by electrochemical detection at a hanging mercury drop electrode (at −0.15 V versus Ag/AgCl) of (a) 10 μl of a rat urine sample (diluted 1:1 with Nanopure water) and (b) 10 μl of a rat urine sample (diluted 1:1 with Nanopure water) which has been spiked with homocysteine to give an effective amount of 6.75 μg in the injected sample. Eluting buffer is 0.0212 *M* sodium dihydrogen phosphate, pH 1.8 at a flow-rate of 1.0 ml/min.

required to separate homocysteine from other thiols such as cysteine (Fig. 2), penicillamine and glutathione (the latter two compounds are retained on the column longer than homocysteine). Only a very minor adjustment of pH and molarity of the eluting buffer was required to obtain adequate separation of the homocysteine peak from other components in a urine sample which are electrochemically responsive at the applied potential (Fig. 3a). Presumably the buffer adjustments compensate for effects of urinary material on the cation-exchange column (e.g., such as occupying exchange sites) which alter the separating capability of the column. Co-elution occurred when a known amount of homocysteine was added to a urine sample which contained homocysteine (Fig. 3b).

The reliability and reproducibility of the quantitative HPLC—ED system for homocysteine was assessed by injecting various amounts of homocysteine in 10 μl of buffer and measuring the resulting peak heights. Good linearity was obtained over the range 0—20 ng of homocysteine, with the graph having a slope of $y = 0.4x$. Application of the system for quantitatively determining homocysteine concentrations in urine was also demonstrated by spiking urine samples with known amounts of homocysteine and injecting 10 μl of the resultant solution (after acidification and centrifugation as described previously) into the system. The peak height responses, measured by fitting a tangent across the base of the peak, and measuring to the apex, fell very close to the calibration line, indicating that other urine components did not alter the homocysteine peak height or shape. These responses also showed that other substances contained in urine do not interact with the mercury electrode surface to reduce its sensitivity to homocysteine.

Having demonstrated the reliability of the analytical system, a preliminary study was conducted into effects of synthetic steroids on urinary homocysteine in rats. We initially looked at the effect of a synthetic estrogen on homocysteine excretion. Three mature female rats were kept in separate metabolic cages for seven days. One received a single 45-μg injection of estradiol valerate in oil intramuscularly, the second a methionine-loaded diet (described in the method section), while the third was an untreated control. Table I gives the average value of triplicate determinations performed on each sample collected, and shows obvious differences in the amounts of homocysteine excreted by these rats over the 24 h test periods.

TABLE I

EFFECT OF A SYNTHETIC ESTROGEN ON HOMOCYSTEINE EXCRETION IN 24-h URINE SAMPLES COLLECTED FROM RATS

Excretion rates expressed as ng homocysteine per 24 h.

Periods of collection (h)	Control rat	Rat fed 0.35% methionine in diet	Rat injected with 45 μg estradiol valerate
24—48	5755	1637	2928
96—120	1584	8478	31210
120—144	2450	10041	20859
144—172	6098	14000	15627

The results show that the estrogenic hormone increased the homocysteine excretion rate. Five days after the administration of a single large dose of estradiol valerate (45 μg) the homocysteine content of the urine of the treated rat was five times greater than that of the urine of a control rat. During the following three days the level of homocysteine in the urine of the treated rat dropped. These results may indicate that the mild anabolic effect of estrogen [23] is responsible for elevating the activity of the methionine pathway, resulting in turn in an increase in the excretion of homocysteine which is an intermediate metabolite. The results also suggest that the effect only lasts as long as the elevated estrogen level exists, and as the active form of estrogen is metabolically inactivated, the effect on the methionine metabolic pathway becomes less pronounced, causing the homocysteine content in 24-h urine samples to tend toward control levels.

The treatment of a rat with methionine added to the diet at a level of 0.35% was undertaken because methionine is metabolised partly to homocysteine and we expected homocysteine excretion rates to rise as the methionine level in the diet was raised, increasing the pressure on that metabolic cycle. This hypothesis was supported by the increasing excretion of homocysteine during the seven days of the trial. Malloy et al. [24] have shown that homocysteine may be bound more strongly to plasma proteins than cysteine, and this may be part of the reason that the amount of homocysteine in the urine of the methionine-diet-treated rat is still within the daily range of homocysteine excreted by the control rat up to 48 h after the trial began. As the feeding continues, a new equilibrium between homocysteine, cysteine and the protein binding sites will be established, and the homocysteine excretion rate will rise. The results obtained for this rat also assist in confirming that the chromatographic peak we are observing is homocysteine since it increases during the trial as expected, and is not therefore some other component that has the same retention time as homocysteine when standard solutions of the latter are chromatographed.

The second animal study looked at the effects of a synthetic estrogen, a synthetic progestogen and a combination of the two on homocysteine excretion in 24-h urine samples collected from weanling rats after 21-days treatment as described. All the experimental rats were treated exactly as the controls, except that two received daily subcutaneous injections of 0.2 μg ethynyl estradiol, two were injected with 2 μg levonorgestrel, and another pair had daily injections of 0.2 μg ethynyl estradiol plus 2 μg levonorgestrel. The

TABLE II

EFFECTS OF SYNTHETIC ESTROGEN AND PROGESTOGEN ON HOMOCYSTEINE EXCRETION IN URINE SAMPLES COLLECTED FROM RATS AFTER THE DESCRIBED TREATMENTS FOR 21 DAYS

Excretion rates expressed as ng homocysteine per 24 h.

Control	Daily injection of 0.2 μg/day ethynyl estradiol	Daily injection of 2 μg/day levonorgestrel	Daily injection of both 0.2 μg/day ethynyl estradiol and 2 μg/day levonorgestrel
11,600	33,600	11,500	28,100

dosages of these experiments were designed to resemble the amount per body weight of the synthetic steroids that oral contraceptive users take on a daily basis [25]. Table II shows the differences between the groups, each value being the average homocysteine excretion exhibited by the pair of rats in that group after all collected samples had been analysed in triplicate.

These results confirm that estrogenic substances elevate homocysteine excretion rates, since treatment with daily doses of ethynyl estradiol caused a marked increase in the excretion of homocysteine in the urine of these rats compared to the control rats. Daily administration of levonorgestrel (a synthetic progestogen) caused no apparent change in the level of homocysteine excreted in the 24-h urine samples when compared with the results obtained from the control rats. The combination of estrogen and progestogen administered daily also caused a marked increase in the levels of homocysteine excreted when compared to the control rats, and the similarity in the size of the increase by this group and the estrogen-treated group leads to the conclusion that the increased amount of homocysteine excreted is due to the estrogen in the combination.

We are currently undertaking a study involving a large number of rats to statistically test these observations. We also plan to ascertain whether women who use oral contraceptives have increased urinary levels of homocysteine.

ACKNOWLEDGEMENTS

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Note

Rapid and simple method for quantitative determination of non-protein sulphydryls in mouse liver by reversed-phase high-performance liquid chromatography

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Fractional estimations of the concentrations of endogenous sulphydryls in biological materials have been performed by means of reversed-phase high-performance liquid chromatography (HPLC) based on spectrophotometry with Ellman's reagent [1] and fluorometry with monobromobimane [2]. However, these methods are not convenient for the quantitative separation of specific endogenous cysteine, which is required for the study of glutathione turnover [3].

We have developed a rapid and simple method for the determination of endogenous sulphydryls including cysteine, γ-glutamylcysteine, and glutathione using Ellman's reagent and conventional reversed-phase HPLC. This method should be useful for the estimation of endogenous sulphydryls in the γ-glutamyl cycle [4], in which cysteine plays an important role [3].

MATERIALS AND METHODS

Apparatus

UV absorbance at 412 nm of a sample was recorded on a UVIDEC 610 spectrophotometer (Jasco, Tokyo, Japan), from which the total amount of non-protein sulphydryls was estimated.

High-performance liquid chromatography was performed with a Hitachi Model 655 solvent delivery system (Tokyo, Japan) equipped with a Rheodyne 7125 injection valve (20 μl) and a reversed-phase column (150 mm × 4.0 mm I.D. Hitachi-gel No. 3056 $5C_{18}$) containing C_{18} chemically bonded silica gel (5 μm particle size). The phosphate buffer solution (0.5 *M*, pH 3.0), con-

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taining 15% (v/v) of methanol, was delivered as the mobile phase at a flow-rate of 0.7 ml/min. The eluates were monitored by UV absorption at 330 nm using a Model 1638-41 variable-wavelength detector (Hitachi). Calculation of the eluate concentrations from the UV absorbances was carried out by a Model 833 data processor (Hitachi). All the analyses were performed at room temperature.

Materials

Reduced glutathione was obtained from Sigma (St. Louis, MO, U.S.A.). Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid), DTNB] and cysteine were purchased from Nakarai Chemicals (Kyoto, Japan). γ-Glutamylcysteine was prepared from oxidized glutathione [5]. All the other reagents were of the best available grades. All sample solutions were purged with oxygen-free nitrogen for 5 min prior to use.

Sample preparation

Female, 8-week-old C3H/He mice obtained from the animal centre of Kyoto University were used.

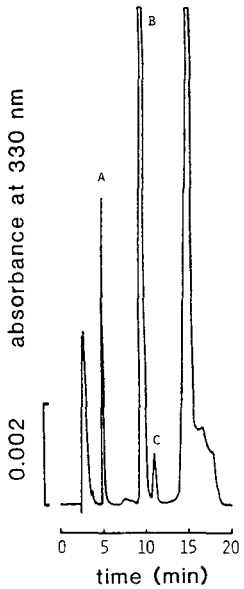
Homogenates (10%) of mouse liver were prepared in 0.02 *M* disodium ethylenediaminetetraacetic acid in an ice bath. Aliquots of 2.5 ml of the homogenates were diluted with 2 ml of water followed by addition of 0.5 ml of 50% trichloroacetic acid. The mixtures were shaken vigorously and permitted to stand for 15 min in the ice bath. The suspensions were centrifuged at 2000 *g* and 4°C for 10 min. To 4 ml of 0.4 *M* Tris buffer solution (hydrochloric acid, pH 8.9) were added 2.0 ml of the supernatant together with 0.1 ml of 10 m*M* DTNB. The sample solution thus obtained was shaken and then filtered through a 0.22-μm Millipore filter (Millipore, Bedford, MA, U.S.A.). After recording the absorbance at 412 nm [6], the sample was subjected to HPLC analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a standard solution of sulphydryls, consisting of 0.3 m*M* cysteine, 5.0 m*M* glutathione, and 0.1 m*M* γ-glutamylcysteine, after treatment with DTNB. Following our procedure, the separation of individual sulphydryl components as their disulphide forms and the baseline assay could be achieved within 20 min. The retention times were highly reproducible. The peak with the retention time of 15.6 min in Fig. 1 was assigned to 2-nitro-5-thiobenzoic acid as a common product from reactions of the sulphydryls with DTNB. The peak of unreacted DTNB failed to be detected under these conditions. The injected sample solution after treatment with DTNB did not contain any detectable amount of unreacted sulphydryls as confirmed by HPLC when eluted with 0.5 *M* phosphate buffer solution (pH 3.0) and UV absorption at 210 nm.

The HPLC peak heights for cysteine, glutathione and γ-glutamylcysteine showed good linear correlations with their concentrations in the physiological range: the correlation coefficient for each sulphydryl was very close to unity (see Table I).

Fig. 2 shows a representative chromatogram observed for endogenous



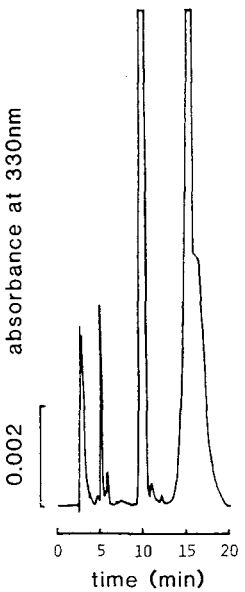


Fig. 1. Chromatogram of a standard solution containing 0.3 m*M* cysteine (A), 5.0 m*M* glutathione (B), and 0.1 m*M* γ-glutamylcysteine (C) after treatment with Ellman's reagent.

Fig. 2. Representative chromatogram of endogenous sulphydryls derived from mouse liver.

TABLE I

CALIBRATION FOR NON-PROTEIN SULPHYDRYLS

The relative HPLC peak height (*H*) calculated by a Model 833 Hitachi data processor was fitted to the sulphydryl concentrations (*C*, μ*M*), using a first-order regression: $C = a + bH$.

Compound	Parameter		Coefficient of correlation	Upper concentration range of the calibration experiment (m*M*)
	a	*b*		
Cysteine	5.895	0.2284	0.9998	1.0
γ-Glutamylcysteine	−4.676	0.8517	0.99997	1.0
Glutathione	−116.2	0.7103	0.9988	7.0

sulphydryls in mouse liver homogenate. By reference to the chromatogram for the standard sample in Fig. 1, we could confirm cysteine (retention time, t_R = 5.02 min), glutathione (t_R = 9.5), and γ-glutamylcysteine (t_R = 11.0). It is particularly important to note that the cysteine originated from the liver could be determined successfully by the present method, owing to the use of an eluent of suitable composition and UV monitoring at 330 nm.

Separate experiments showed that the concentrations of individual sulphydryls did not vary for at least 4 h when the acid extract prepared as described in the experimental section was sealed and cooled in the ice bath. Furthermore, the disulphides as the reaction products with DTNB in Tris buffer were stable for at least 3 h at room temperature, whereas 2-nitro-5-thiobenzoic acid oxidized gradually [6].

TABLE II

DETERMINATION OF ENDOGENOUS NON-PROTEIN SULPHYDRYLS (NPSH) IN MOUSE LIVER (mmol/kg)

Mouse No.	Cysteine	γ-Glutamyl cysteine	Glutathione	Total NPSH concentration	
				HPLC*	UV
1	0.262	0.027	6.37	6.66	7.02
2	0.209	0.025	6.40	6.64	6.90
3	0.258	0.031	6.09	6.38	6.84
4	0.275	0.033	6.06	6.37	6.78
5	0.265	0.030	6.01	6.31	6.92
6	0.216	0.038	6.38	6.64	7.23
7	0.243	0.027	5.94	6.21	6.94
8	0.246	N.D.**	5.60	5.83	6.57
Mean	0.247	0.030	6.11	6.38	6.91
± S.D.	±0.022	±0.004	±0.26	±0.27	±0.20
Coefficient of variation (%)	8.9	13.3	4.3	4.2	2.9

*Sum of three endogenous sulphydryls.
**N.D. = undetectable level.

As shown in Table I, the reproducibility of the present method for the analysis of endogenous sulphydryls was excellent. Table II shows that the total concentration of non-protein sulphydryls measured by UV absorbance was slightly greater than the sum of the individual component sulphydryls determined by HPLC. The γ-glutamylcysteine concentration in mouse liver was estimated as 0.030 mmol/kg. To our knowledge, this is the first determination of reduced γ-glutamylcysteine in mouse liver, though the total amount of γ-glutamylcysteine was reported as 0.70 μmol/g in rat liver [1].

Finally, the recoveries of cysteine (0.1 mmol/kg), γ-glutamylcysteine (0.01 mmol/kg), and glutathione (1.0 mmol/kg) from a liver sample, to which these sulphydryls were previously added, were found to be 96%, 103%, and 98%, respectively.

We have described a simple HPLC method to determine quantitatively endogenous sulphydryls without interference from the biological materials used as samples. This was achieved by the use of Ellman's reagent and a mobile phase of suitable composition.

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Note

High-performance liquid chromatographic analysis for fluorescein and fluorescein monoglucuronide in plasma

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Fluorescein (F) is widely used as a diagnostic aid in medicine, especially in the practice of ophthalmology [1, 2]. Intravenous (i.v.) injection of F for fundus angiography has become an important diagnostic and research tool and has provided an understanding and classification of various diseases of the fundus [3, 4].

Although F has been determined in physiological fluids by measuring its absorbance [5], interference from extraneous absorbing substances has been reduced by the use of its fluorescent properties [6]. Previous investigations with F have assumed that the total fluorescence observed in plasma samples is due entirely to F. However recent studies have suggested that F is quickly converted to the monoglucuronide [7—9]. The fluorescence of the fluorescein monoglucuronide is lower than that of fluorescein, with the fluorescence ratio of the two dependent on pH and the combination of excitation and emission wavelengths [8, 10]. Previous studies have measured free F by the fluorometric determination of native sample, and the conjugate as F after the hydrolysis with either sodium hydroxide or β-glucuronidase [8, 11].

High-performance liquid chromatography (HPLC) with fluorescence detection described in this report provides a fast and sensitive determination of F and its major metabolite FG directly from plasma. In addition, this technique provides a level of sensitivity approaching that of gas chromatography but without the attendant difficulties.

EXPERIMENTAL

Reagents

The sodium salt of fluorescein was purchased from Sigma (St. Louis, MO,

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U.S.A.). Fluorescein monoglucuronide was prepared by Dr. Thomas Guenthner, Department of Pharmacology, University of Illinois Medical Center. Spectrograde methanol and PIC reagent A were supplied by Waters Assoc. (Milford, MA, U.S.A.). All solutions were prepared by using double-distilled water.

Subject and medication

One adult male volunteer was given 14 mg/kg fluorescein by bolus intravenous administration in the vein of the right arm. Venous blood samples were withdrawn from the vein of the left arm at 0, 1, 60, 120 and 240 min following drug administration. The samples were immediately centrifuged and the plasma kept frozen until analysis.

Chromatography

HPLC analyses were conducted using a Waters Assoc. Model 6000A pump coupled with a Waters Model U6K injection system. Separation was performed by ion-pair chromatography using a Waters Assoc. C_{18} bonded reversed-phase column (15 cm × 3.9 mm) with 5 μm particle size. The mobile phase consisted of 47% methanol in a 0.005 *M* aqueous solution of PIC reagent A (tetrabutylammonium phosphate) and was degassed at reduced pressure before use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 1 ml/min.

Fluorescence was measured with a Waters Model 420 fluorescence detector, using a 500-nm long-pass emission filter and a 450-nm band-pass excitation filter in conjunction with the F4T5/D fluorescence lamp.

Ultrafiltration

Plasma ultrafiltrates were prepared using the Amicon (Danvers, MA, U.S.A.) micropartition system with the YMT membrane. Serum samples (0.5 ml) were centrifuged at 2000 *g* for 20 min through the system [12]. Approximately 100 μl of each ultrafiltrate was injected onto the column.

RESULTS AND DISCUSSION

Method development

Ultrafiltration by centrifugation with the micropartition system is a simple, highly reproducible technique ideally suited for the determination of the free fraction of drugs in plasma [13]. In order to investigate the possible binding of F and FG to the YMT filter, standards were prepared in distilled water and centrifuged for 20 min at 2000 *g* through the filter. The YMT filter showed no significant binding of either F or FG (Table I).

Calibration curves were prepared from a series of six triplicate F and FG standards in water, ranging from 12.5 to 500 ng/ml. The samples were processed and the ultrafiltrate was measured using the chromatographic solvent system described previously. Linear regression analyses of peak heights versus the concentration of compounds of interest yielded linear calibration curves with $r^2 \geqslant 0.99$. The detection limit of the assay is approximately 10 ng/ml for both compounds of interest. A listing of these data is provided in Table II.

TABLE I

FLUORESCEIN AND FLUORESCEIN MONOGLUCURONIDE BINDING TO YMT FILTER

Samples were assayed in triplicate. Measured values are means ± standard error.

Drug	Actual (ng/ml)	Measured before filtration (ng/ml)	Measured in ultrafiltrate (ng/ml)
Fluorescein	100	99.6 ± 3.3	103.6 ± 0.3
Fluorescein monoglucuronide	100	102.4 ± 2.3	99.2 ± 3.6

TABLE II

CALIBRATION CURVE PARAMETERS

Linear regression analyses of the given data sets were carried out with y value being peak height and x value being concentration (F, FG); n samples (including duplicate or triplicate determinations) were utilized for each calibration curve with r^2 (correlation coefficient) being a measure of linearity, b representing the y intercept, m indicating the slope of the regression and C.V. the mean coefficient of variation.

Medium	Compound	Range (ng/ml)	n	b	m	r^2	C.V.
Water	F	12.5—500	19	0.018	0.001	0.99	0.1
Water	FG	12.5—500	19	0.005	0.001	0.99	0.6

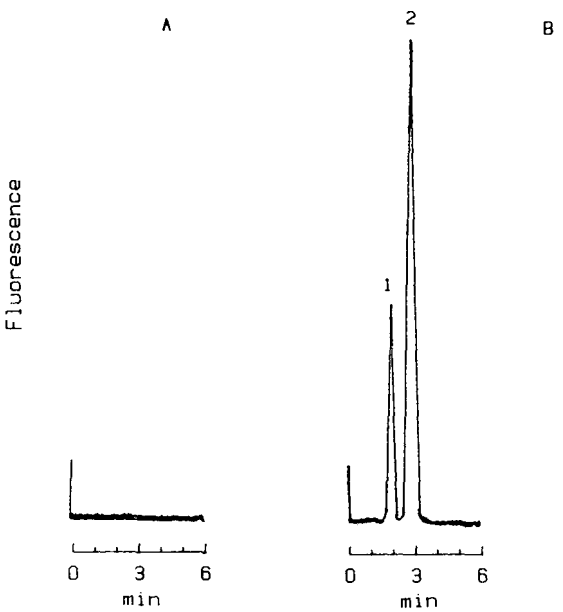

Fig. 1. HPLC chromatograms of (A) blank plasma ultrafiltrate; and (B) ultrafiltrate from a plasma sample containing 100 ng/ml FG (1) and 100 ng/ml F (2).

A calibration curve of F in control plasma was prepared in the concentration range 40—200 ng/ml and the ultrafiltrate analyzed. Linear regression analysis of peak height versus concentration of F in plasma yielded a linear calibration curve with a correlation coefficient $\geqslant 0.99$ and a slope of 0.001 ml/ng. Based on the concentration of free F no significant difference was observed in the chromatograms for standards prepared in water and standards prepared in plasma. The day-to-day precision of the assay based on variation in peak height was less than 3%.

A given chromatogram (Fig. 1A) of plasma ultrafiltrate without added F or FG yielded no peaks over the first 6 min under the chromatographic conditions described earlier. The chromatogram (Fig. 1B) of plasma ultrafiltrate from plasma previously supplemented with an equal concentration (100 ng/ml) of F and FG yielded both peaks of interest. The chromatograms of plasma ultrafiltrate from patients receiving fluorescein appeared to be identical to those of spiked plasma.

Protein binding

Adverse reactions to intravenous fluorescein used in fundus angiography are usually of minimum consequence. However, serious side-effects including anaphylactic reactions, acute pulmonary edema, respiratory obstruction and arrest, and hypotension with a shocklike reaction have been reported [14—16]. It has recently been suggested that the previously reported adverse effects of intravenously injected F may be related to the amount of protein binding of fluorescein [17]. For this reason an investigation of the interaction between human plasma and F and its primary metabolite FG using the micropartition system was performed.

Fluorescein is bound in the blood mainly to protein, particularly to albumin and erythrocytes [18, 19]. Previous investigations have reported the percent F binding to plasma proteins in the range 40—90%, while one recent paper has reported finding no significant protein binding of F using polyacrylamide gel electrophoresis [20].

Protein binding of F to the plasma proteins was investigated at three different F concentrations: 40, 80 and 100 ng/ml (Table III). Plasma was supplemented with F and an aliquot was processed by ultrafiltration. At all

TABLE III

PROTEIN BINDING OF FLUORESCEIN AND FLUORESCEIN MONOGLUCURONIDE

Blank plasma samples were supplemented with F or FG. Values reported are the mean of three separate determinations.

Drug	Actual concentration (ng/ml)	Concentration in ultrafiltrate (ng/ml)	Percentage bound
Fluorescein	40	7.8	80.5
	80	13.4	83.3
	100	15.2	84.8
Fluorescein monoglucuronide	100	57.4	42.6

TABLE IV

EFFECT OF FREEZING ON PLASMA PROTEIN BINDING OF FLUORESCEIN

Blank plasma samples were supplemented with F. Values reported are the mean three to four determinations.

Serum concentration (ng/ml)	Fresh ultrafiltrate concentration (ng/ml, ± S.E.)	Percentage bound	Frozen ultrafiltrate concentration (ng/ml, ± S.E.)	Percentage bound
40	7.8 ± 0.1	80.4	7.7 ± 0.6	80.6
80	13.5 ± 0.1	83.0	13.8 ± 0.5	82.7
100	15.3 ± 0.2	84.5	15.9 ± 0.6	84.1
200	31.2 ± 3.7	84.4	30.3 ± 0.2	84.8

three concentrations F was found to be protein-bound with the average binding calculated to be 82.9%. This corresponds to previous reports of 82.5% and 86.2% plasma protein binding of F [9, 21]. The protein binding of FG at a concentration of 100 ng/ml was found to be 42.6%, approximately one-half that found for an equivalent concentration of F (Table III).

The effect of freezing on protein binding of F was investigated at four different F concentrations (Table IV). Plasma with added F was divided in half, with half kept at room temperature and half frozen overnight. The following day protein binding of F was assessed. No significant change in protein binding was evident between the frozen and non-frozen samples at any of the concentrations used.

Clinical sample

Initial studies were performed on plasma samples from a male volunteer receiving 14 mg/kg F intravenously. Blood samples were withdrawn 0, 1, 60, 120 and 240 min after F administration and assayed as previously described. Detectable concentrations of F and FG were found in plasma ultrafiltrate at the stated times and are presented in Table V. It was observed that free F was

TABLE V

PLASMA ULTRAFILTRATE CONCENTRATIONS OF FLUORESCEIN AND FLUORESCEIN MONOGLUCURONIDE AFTER i.v. ADMINISTRATION OF FLUORESCEIN

Fluorescein (14 mg/kg) was administered by bolus intravenous injection in the right arm. Venous blood samples were removed from the left arm at the stated times. The concentrations reported are the mean of three to four determinations.

Time (min)	Concentration (ng/ml)	
	F	FG
0	0	0
1	35,635	0
60	2661	37,000
120	727	23,982
240	156	14,952

rapidly converted to its glucuronide. At 1 min after i.v. administration free F was at its maximum concentration while there was no detectable free glucuronide. By 1 h there was approximately a fourteen fold increase in free metabolite over drug. This rapid metabolism of F to FG agrees with previously reported work [9].

The present paper describes an assay procedure for the rapid determination of F and its primary metabolite FG in plasma. The described assay is currently being used in this laboratory for clinical study of F and FG pharmacokinetics.

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Note

Simultaneous high-performance liquid chromatographic assay for 5-(2-bromo-*E*-ethenyl)-2′-deoxyuridine and its metabolite, 5-(2-bromo-*E*-ethenyl)uracil, in plasma

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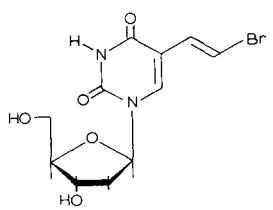
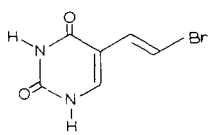
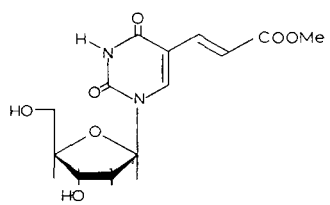
Many modified nucleosides have been synthesised and tested for activity against the *Herpes* viruses. Recently, a derivative of 2′-deoxyuridine, 5-(2-bromo-*E*-ethenyl)-2′-deoxyuridine (Fig. 1, BVDU, SC 38394), has been shown to be a potent anti-herpetic compound [1, 2]. It has shown activity in vitro [1] and in vivo [2] against *Herpes labialis* and *Varicella zoster*, and has proved effective in the treatment of herpetic keratitis and "shingles" in man [3].



(a) BVDU



(b) BVU



(c) Internal standard

Fig. 1. Structures of (a) BVDU, (b) BVU and (c) the internal standard.

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During studies of the disposition of BVDU, 5-(2-bromo-*E*-ethenyl)uracil (Fig. 1, BVU) was identified as a prominent plasma metabolite in animals and man [4]. Further investigation of BVDU required a sensitive assay for the drug and its metabolite, BVU, in the plasma of animals and man. The present report describes the development of the assay and some preliminary results of its application to studies of the pharmacokinetics of BVDU.

EXPERIMENTAL

Materials

BVDU, its metabolite BVU and the internal standard 5-(2-carbomethoxy-*E*-ethenyl)-2′-deoxyuridine (Fig. 1) were supplied by Chemical Development, G.D. Searle (High Wycombe, U.K.). All other reagents and HPLC-grade solvents were purchased from BDH (Poole, U.K.), unless otherwise stated.

Aqueous solutions of potassium dihydrogen orthophosphate (0.2 *M*, 19.5 ml) and disodium hydrogen orthophosphate (0.2 *M*, 30.5 ml) were mixed and diluted to 1500 ml with glass-distilled water. Suitable volumes of this solution were mixed with acetonitrile to give mobile phases containing different proportions of acetonitrile and 0.007 *M* phosphate buffer (pH ~7). The mobile phases were helium-degassed and vacuum-filtered before use.

Stock solutions (0.1 mg/ml) of BVDU, BVU and internal standard were prepared by dissolving each compound (5 mg) in methanol (30 ml) and diluting with glass-distilled water to 50 ml in a volumetric flask. Working standard solutions of these compounds were prepared by suitable dilutions of the stock solutions with glass-distilled water or plasma as required.

Apparatus

Chromatography was carried out with a Series 3B pump, ISS-100 sample processor and LC85 UV detector set at 254 nm (Perkin-Elmer, Beaconsfield, U.K.). The analysis was carried out on a μBondapak C_{18} column (10 μm; 300 mm × 3.9 mm I.D.) fitted with a Bondapak C_{18}/Corasil guard column (37 μm; 20 mm × 3.9 mm I.D.) (Waters Assoc., Hartford, U.K.). The output from the UV detector was linked to a Perkin-Elmer RO56 dual-pen potentiometric recorder and an HP3390A integrator (Hewlett-Packard, Wokingham, U.K.).

Selection of HPLC conditions

The separation of BVDU, BVU and the internal standard from ethyl acetate extractable plasma components was optimised using different proportions of acetonitrile and phosphate buffer. The optimal solvent composition was acetonitrile—0.007 *M* phosphate buffer (pH 7; 12.5:87.5, v/v).

Assay procedure

The internal standard working solution (0.5 ml; 3 μg/ml) was added to plasma (0.5 ml) in an amber glass test tube and mixed. The plasma was extracted with ethyl acetate (2 × 2.5 ml) on a partitioning extractor, centrifuged at 2500 *g* for 10 min, and the combined organic phases were evaporated to dryness under a nitrogen stream at 45°C (SC-3 evaporator, Techne, Cambridge, U.K.).

The extract residue was redissolved in acetonitrile—0.007 *M* phosphate buffer (70 μl; pH ~7; 12.5:87.5, v/v), the mobile phase, and a 40-μl aliquot was analysed on a μBondapak C_{18} column (300 × 3.9 mm I.D.) fitted with a pre-column (20 × 3.9 mm I.D.) containing Bondapak C_{18} Corasil, at a mobile phase flow-rate of 2 ml/min.

The peak height ratios for BVDU and BVU versus internal standard were measured by the integrator. The concentrations of BVDU and BVU in plasma were determined by the integrator using a response factor obtained by the analysis of calibration standards with each batch of samples. The accuracy and precision of the assay were assessed by the analysis of plasma samples containing added BVDU and BVU at concentrations which were unknown by the analyst.

RESULTS AND DISCUSSION

The assay method efficiently resolved BVDU (retention time, t_R 8.5 min), BVU (t_R 7.5 min) and the internal standard (t_R 6 min) from the co-extracted components in the plasma of rat, dog and man (Fig. 2).

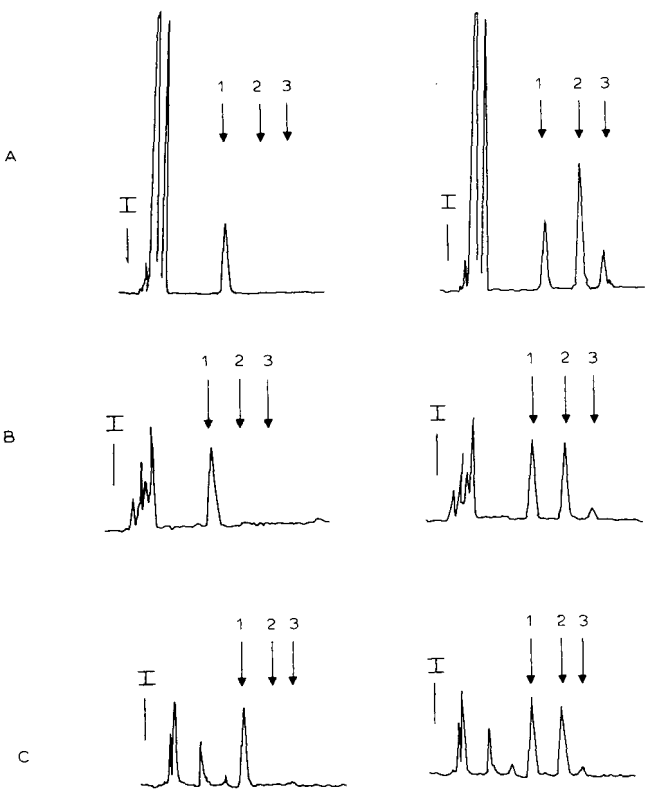

Fig. 2. HPLC profiles of plasma extracts from (A) rat, (B) dog and (C) man before and after oral doses of BVDU. Column: μBondapak C_{18}. Solvent: acetonitrile—0.007 *M* phosphate buffer (12.5:87.5, v/v). I = injection. Peaks: 1 = internal standard; 2 = metabolite (BVU); 3 = BVDU.

There was a linear correlation between peak height ratio and concentration for BVDU and BVU in the plasma of each species over the concentration ranges 0—5 μg/ml and 0—15 μg/ml, respectively. The 95% confidence intervals for the repeated analysis of plasma containing 0.025 μg/ml BVDU and BVU showed that the sensitivity of the assay was < 0.025 μg/ml.

Analysis of quality control plasma samples showed an acceptable level of accuracy and precision of the assay for both compounds (Tables I and II). These results indicated that accurate quantitation was possible down to 0.05 μg/ml for both compounds in rat and dog plasma and 0.125 μg/ml in human plasma, based on achieving a between-assay coefficient of variation ⩽ 15%.

Plasma samples obtained from rat and man following the oral administration of BVDU were analysed by the assay procedure described. The results of these analyses are shown in Fig. 3. Orally administered BVDU appears to be rapidly absorbed and metabolised to BVU in both species, with plasma BVDU levels some three- to ten-fold lower than those of BVU.

In conclusion, the assay method was adequately selective, sensitive, accurate and precise to measure BVDU and BVU in the plasma of rat, dog and man

TABLE I

ACCURACY AND PRECISION FOR THE MEASUREMENT OF 5-(2-BROMO-*E*-ETHENYL)-2′-DEOXYURIDINE IN THE PLASMA OF RAT, DOG AND MAN BY HPLC

Nominal (μg/ml)	Recovery (%)	Coefficient of variation* (%)	
		Within-assay	Between-assay**
Rat plasma			
0.025	90.0	41.6 (5)	41.6 (4)
0.049	82.7	16.4 (5)	16.6 (4)
0.350	108.0	5.2 (5)	7.4 (4)
0.875	113.5	10.3 (5)	11.3 (4)
2.1	105.0	4.0 (5)	4.0 (4)
3.5	104.7	4.3 (5)	4.6 (4)
Dog plasma			
0.05	89.0	14.9 (7)	15.6 (2)
0.10	93.2	7.5 (6)	10.1 (1)
0.25	86.8	1.7 (2)	8.6 (1)
1.0	99.5	9.7 (3)	9.7 (2)
2.5	94.3	5.5 (4)	6.7 (2)
4.5	95.5	3.4 (4)	5.7 (2)
Human plasma			
0.049	107.6	30.8 (5)	50.0 (4)
0.125	102.5	3.5 (4)	4.7 (3)
0.350	103.3	1.6 (5)	7.5 (4)
0.875	95.9	9.8 (5)	12.1 (4)
2.1	98.7	8.3 (5)	8.9 (4)
3.5	98.1	3.1 (5)	4.1 (4)

*Figures in brackets are degrees of freedom.
**Expected precision for singleton analysis.

TABLE II

ACCURACY AND PRECISION FOR THE MEASUREMENT OF 5-(2-BROMO-*E*-ETHENYL)URACIL IN THE PLASMA OF RAT, DOG AND MAN BY HPLC

Nominal (μg/ml)	Recovery (%)	Coefficient of variation* (%)	
		Within-assay	Between-assay**
Rat plasma			
0.046	115.7	6.9 (5)	7.5 (4)
0.288	107.3	8.5 (5)	8.5 (4)
0.575	106.7	5.1 (5)	10.4 (4)
2.875	110.7	9.7 (5)	11.2 (4)
4.60	94.7	4.1 (5)	7.0 (4)
11.5	106.6	3.6 (5)	7.3 (4)
Dog plasma			
0.04	85.0	8.6 (2)	8.6 (1)
0.08	86.3	4.3 (2)	6.9 (1)
0.15	84.0	11.3 (3)	11.3 (2)
0.30	83.5	4.0 (2)	13.3 (1)
0.75	82.5	5.1 (2)	8.2 (1)
3.0	98.7	1.4 (3)	5.3 (2)
7.5	91.1	4.2 (4)	13.1 (2)
15.0	89.8	4.1 (4)	9.6 (2)
Human plasma			
0.046	130.9	37.1 (5)	39.5 (4)
0.125	120.9	1.7 (5)	12.9 (4)
0.288	107.2	8.7 (5)	11.2 (4)
0.575	112.6	4.1 (5)	7.5 (4)
2.875	102.2	8.5 (5)	9.5 (4)
4.6	95.9	8.4 (5)	9.1 (4)
11.5	106.3	4.1 (5)	4.7 (4)

*Figures in brackets are degrees of freedom.
**Expected precision for singleton analysis.

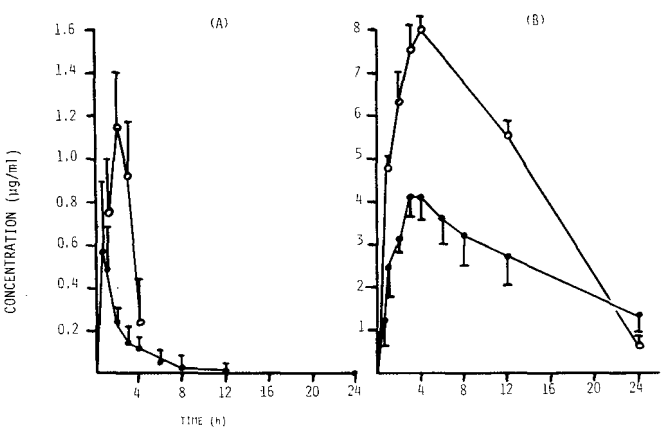

Fig. 3. Plasma levels of (A) BVDU and (B) BVU in the rat (○) after a 30 mg/kg oral dose and in man (●) after a 250-mg oral dose of BVDU.

following oral administration of BVDU. Recent work in these laboratories indicates that a similar assay procedure can be used for the analysis of these compounds in human urine and blood cells.

ACKNOWLEDGEMENTS

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Note

Determination of amoxapine and metabolites in plasma by liquid chromatography with electrochemical detection

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Amoxapine is the N-demethylated metabolite of the neuroleptic dibenzoxazepine loxapine. It has been shown to be a useful antidepressant and a review of its pharmacology and efficacy has been presented [1]. Since there are conflicting reports as to the clinical efficacy versus plasma concentration relationships routine plasma level monitoring of amoxapine and its metabolites appears to be unwarrented at this time [2, 3]. However, concentrations of amoxapine and its metabolites in biofluids may be necessary in various clinical situations as well as in determining their pharmacokinetic profiles.

To date, a number of analytical methods have been reported for amoxapine determinations. Cooper and Kelly [4] reported a gas chromatographic method for loxapine, amoxapine and their respective 7- and 8-hydroxymetabolites in urine and serum. A number of liquid chromatographic procedures have been reported for amoxapine and their major metabolites [5—9]. However, some of these latter procedures do not adequately resolve all the compounds of interest, while others do not have sufficient sensitivity for pharmacokinetic studies or require different chromatographic conditions for the analysis of

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amoxapine and its metabolites. All procedures used an ultraviolet detector at 254 nm or 214 nm to monitor the peaks.

We have developed an alternative approach to the detection of amoxapine and its metabolites using liquid chromatography coupled with an electrochemical detector. The method affords adequate sensitivity for single-dose pharmacokinetic studies as well as simultaneously resolving all compounds of interest in a single chromatogram.

EXPERIMENTAL

Reagents and standards

Phosphoric acid, potassium phosphate monobasic, *n*-butylamine, 1-heptanesulfonic acid, sodium salt, were all reagent or HPLC grade (Fisher Scientific, Fairlawn, NJ, U.S.A.). Acetonitrile, UV grade, and methyl *tert.*-butyl ether were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Stock solutions (1 mg/ml) of amoxapine, 8-hydroxyamoxapine, 7-hydroxyamoxapine and 8-hydroxyloxapine were prepared in 0.01 *M* hydrochloric acid. Working solutions were prepared by dilution of the above to 1 μg/ml. All standards were supplied by American Cyanamid, Lederle Labs. (Pearl River, NY, U.S.A.).

Apparatus

Chromatography was performed using a Model 6000A solvent delivery pump, and a WISP 710B injector (Waters Assoc., Milford, MA, U.S.A.). Separations were achieved with a 25 cm × 4.6 mm I.D. column packed with trimethylsilyl bonded silica (LC-1, particle size 5 μm; Supelco, Bellafonte, PA, U.S.A.) and detected by a Model 5100A coulometric analyzer having a Model 5011 dual porous graphite electrode cell (ESA, Bedford, MA, U.S.A.). Chromatograms were recorded on a Model B5217-5 omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.).

Extraction

To 1 ml of plasma standard or unknown sample, 50 μl (50 ng) of internal standard 8-hydroxyloxapine and 1.0 ml of 0.6 *M* carbonate buffer (pH 9.8) were added in a 15-ml round-bottom screw cap culture tube. Methyl *tert.*-butyl ether (7 ml) was added and the mixture shaken for 15 min and centrifuged at 1500 *g* for 15 min. The organic layer was then transferred to a 15-ml tapered centrifuged tube containing 250 μl of 0.1 *M* acidic phosphate buffer (pH 2.0). After mixing for 10 min and centrifuging at 1500 *g* for 10 min, the organic layer was aspirated and discarded and the aqueous contents transferred to small glass vials suitable for automatic injection by the WISP 710B.

Chromatographic conditions

The mobile phase consisted of 0.05 *M* potassium dihydrogen phosphate—acetonitrile (75:25) with 1.2 ml/l *n*-butylamine, 1.0 ml/l orthophosphoric acid (85%) and 0.005 *M* heptanesulfonic acid added. The flow-rate was 1.8 ml/min and the temperature ambient. The detector potential was set at +0.75 V versus

the proprietary reference electrode. A Model 5020 guard cell (placed between the pump and injector) was also set at a potential of +0.75 V versus the proprietary reference electrode.

Quantitation

All determinations of plasma samples were calculated based upon peak height ratios using the internal standard method.

RESULTS AND DISCUSSION

Analyses of amoxapine, 7-hydroxyamoxapine and 8-hydroxyamoxapine in plasma were performed by liquid chromatography with electrochemical detection. This method is able to separate and quantitate the 7- and 8-hydroxy metabolites as well as amoxapine in a single chromatogram. A sample chromatogram appears in Fig. 1A. The use of a trimethylsilyl column with the addition

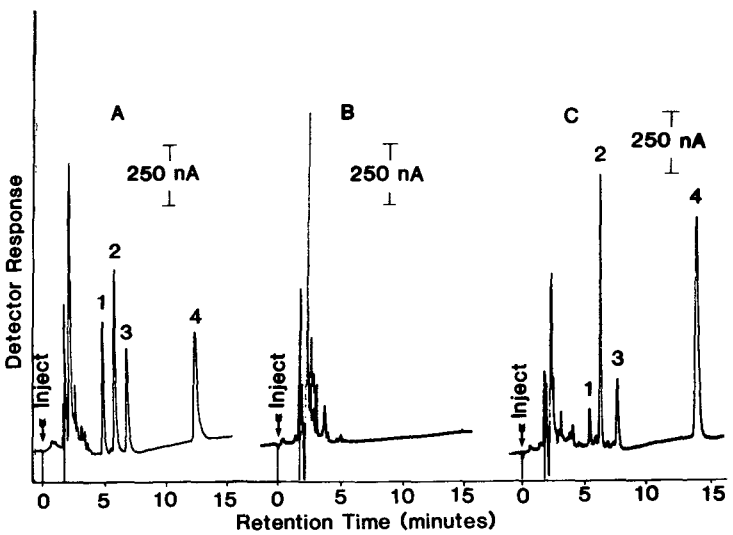

Fig. 1. Sample chromatogram of: (A) a spiked 1-ml plasma sample containing 75 ng of 7-hydroxyamoxapine (1), 8-hydroxyamoxapine (2) and amoxapine (4), with 50 ng of internal standard 8-hydroxyloxapine (3); (B) a drug-free plasma blank; and (C) a 1-ml plasma sample from a patient receiving amoxapine: 30 ng of 7-hydroxyamoxapine (1), 137 ng of 8-hydroxyamoxapine (2) and 194 ng of amoxapine (4).

TABLE I

RECOVERY OF AMOXAPINE AND METABOLITES (50 ng/ml) FROM 1 ml OF PLASMA (n = 8)

Compound	Recovery (%)	C.V. (%)
Amoxapine	88	5.0
8-Hydroxyamoxapine	85	4.4
7-Hydroxyamoxapine	81	4.6

of *n*-butylamine in the mobile phase enhanced peak symmetry and affords resolution between the peaks. No interfering endogenous peaks were observed (Fig. 1B).

The absolute recovery of amoxapine and metabolites from plasma was determined by spiking 1-ml blank plasma samples with 50 ng of amoxapine and metabolites, and processing as described, except for the addition of the internal standard which was added to the final extract just before injection on column. The peak height ratios of these compounds to the internal standard were compared to those recorded when the same concentration of unextracted amoxapine and metabolites and internal standard were injected. The results appear in Table I.

The precision of the procedures was determined by spiking 1-ml aliquots of drug-free plasma with various concentrations of amoxapine and its metabolites together with the internal standard. The samples were processed as described. The results appear in Table II. Day-to-day reproduciblity was assessed by analysis of the slopes of the calibration curve for each day (Table III).

The lower limit of detection of this assay is about 5 ng/ml for all compounds. This assay is therefore suitable for single-dose pharmacokinetic studies if 2—3 ml of sample are used.

The use of an electrochemical detector for this method yielded an increase in sensitivity over an ultraviolet absorbance detector (254 nm). Furthermore, in this application the coulometric type of electrochemical detector was observed to be more sensitive than the thin-layer amperometric transducer. This

TABLE II

WITHIN-RUN PRECISION OF ASSAY BASED UPON PEAK HEIGHT RATIOS AT VARIOUS CONCENTRATIONS (n = 8)

Compound	Concentration (ng/ml)					
	10		30		100	
	Peak height ratio	C.V. (%)	Peak height ratio	C.V. (%)	Peak height ratio	C.V. (%)
Amoxapine	0.12	4.5	0.35	3.4	1.21	1.2
8-Hydroxyamoxapine	0.21	7.4	0.67	1.3	2.25	0.8
7-Hydroxyamoxapine	0.11	13.4	0.38	5.3	1.51	2.0

TABLE III

DAY-TO-DAY PRECISION OF THE ASSAY BASED UPON LINEAR REGRESSION SLOPES (n = 5)

Compound	Slope (ng/ml)	C.V. (%)
Amoxapine	69.3	8.8
8-Hydroxyamoxapine	43.4	3.5
7-Hydroxyamoxapine	58.0	5.0

TABLE IV

STEADY-STATE PLASMA LEVELS OF AMOXAPINE AND METABOLITES IN NINE PATIENTS RECEIVING AMOXAPINE

Subject	Dose (mg per day)	Amoxapine (ng/ml)	8-Hydroxyamoxapine (ng/ml)	7-Hydroxyamoxapine (ng/ml)
1	200	71	157	8
2	250	150	226	5
3	150	74	133	T*
4	250	88	170	8
5	150	104	239	5
6	150	195	137	30
7	150	26	70	T
8	350	222	582	28
9	300	92	204	11

*T, Level below 5 ng/ml.

may be due in part to the "pre-conditioning" (pre-oxidation) of the mobile phase by the guard cell prior to the actual sample detection, thus eliminating the potential interference from any impurities in the mobile phase and increasing signal-to-noise ratio.

Table IV illustrates the plasma levels of amoxapine and metabolites of several depressed patients receiving amoxapine. The concentration of the 8-hydroxy metabolite was usually found to be equal to or greater than the parent compound. The 7-hydroxy metabolite was found in concentrations near the limit of detection for most patients. These data agree with those previously reported [2–6, 9].

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Note

Determination of apomorphine in rat plasma and brain by high-performance liquid chromatography with electrochemical detection

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Several analytical methods for the determination of apomorphine in biological samples, including gas chromatographic [1, 2], spectrophotometric [3], fluorimetric [4], radioenzymatic [5] and radiochemical [6] assay, have been described.

In recent years high-performance liquid chromatographic (HPLC) methods for apomorphine have been employed using ultraviolet [7—9] or fluorimetric detection [10]. HPLC with electrochemical detection (ED) has also been employed for measuring brain levels of apomorphine [11]. This procedure shows greater sensitivity but has not been extended to biological fluids.

This paper describes a simple and sensitive HPLC—ED method for the detection of apomorphine in plasma and brain (striatum) with detection limits of 0.3 ng/ml and 3 ng/g, respectively. The method has been used to investigate apomorphine pharmacokinetics in rats.

MATERIALS AND METHODS

Reagents and materials

Apomorphine hydrochloride was kindly supplied by Sandoz (Basel, Switzerland). All solvents, buffer components and other chemicals were analytical-reagent grade or better and used as received. Aqueous standard solutions of apomorphine (1 μg/ml) and ascorbic acid (2 mg/ml) were freshly prepared every day. The EDTA solution was 100 g/l in 0.25 *M* sodium hydroxide (final pH 7).

High-performance liquid chromatography

The HPLC system, composed of a Hewlett-Packard 1084 B liquid

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chromatograph and controlled by a computer terminal (HP 7985 B LC model), was used in conjunction with an electrochemical detector. The electrochemical cell (TL-5, Bioanalytical System, Lafayette, IN, U.S.A.) contained a glassy carbon working electrode, a stainless-steel auxiliary electrode and an Ag/AgCl reference electrode. The working electrode was maintained at an applied potential of +0.77 V versus Ag/AgCl. Separation was achieved with a μBondapak CN reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc.) and the mobile phase was acetonitrile—0.02 *M* potassium dihydrogen phosphate (11:89) containing 0.5 m*M* EDTA adjusted to pH 3 with phosphoric acid. The mobile phase was filtered through a Millipore filter (0.4 μm) and degassed before use. The flow-rate was set at 1 ml/min.

Animals

Male CD-COBS rats (Charles River, Como, Italy), average weight 200 g, were used.

Collection of samples

Blood samples were collected in heparinized tubes; plasma was obtained by centrifugation. Ascorbic acid solution was added (0.05 ml/ml of plasma) and the samples were stored at —25° C until assayed. Rat brains were dissected by the method of Glowinski and Iversen [12]; striatum was removed, immediately frozen on solid carbon dioxide and stored as for plasma.

Extraction procedure

Plasma. To 1 ml of plasma in a plastic centrifuge tube 0.1 ml of EDTA solution was added; the pH was controlled or adjusted to between 7 and 7.5 using a pH meter. Apomorphine was successively extracted with 2 ml of ethyl acetate.

After centrifugation (1500 *g*, 5 min) 1.6 ml of the organic layer were transferred into another tube and apomorphine was back-extracted with 0.4 ml of 0.1 *M* hydrochloric acid. After shaking and centrifugation, 0.37 ml of the aqueous phase was transferred into an eppendorf vial and 10—200 μl were injected into the HPLC system.

Brain. After addition of 0.15 ml of EDTA and ascorbic acid solution, tissue (about 100 mg) was homogenized in 0.7 ml of 0.1 *M* hydrochloric acid using an Ultra Turrax. The Ultra Turrax was then washed with 0.7 ml of 0.1 *M* hydrochloric acid which was combined with the homogenate. After adjustment of the pH to 7.0—7.5 with 1 *M* tripotassium phosphate, the sample was processed as indicated for plasma. All operations described were performed at about 5° C.

Calibration and quantification

Calibration was effected by adding known amounts of apomorphine to plasma and brain tissue, and processing the samples as described above.

The peak heights for apomorphine in the chromatograms were measured and a calibration graph was plotted which was used to calculate the drug concentration in unknown samples.

RESULTS AND DISCUSSION

Detector response

Fig. 1 shows the change in response current against applied potential (current—potential curve) for apomorphine. The onset potential of apomorphine oxidation was about 0.4 V and the detector response increased up to (at least) 0.85 V.

The applied potential selected in this study was 0.77 V because a higher detector potential resulted in a greater noise level and also in lower specificity of the detector [13].

The glassy carbon electrode produces a linear response using a range of 0.1—100 ng of apomorphine injected.

The electrochemical detector shows a slight loss of sensitivity over a day's chromatographic runs ($\leqslant$ 10%), probably due to build-up of oxidation products of apomorphine on the working electrode surface [14]. The performance of the detector was partially restored by overnight delivery of the mobile phase through the chromatographic system. However, in our conditions the electrode was used for about 200 determinations of apomorphine in tissue extracts or body fluids, after which it was polished with alumina.

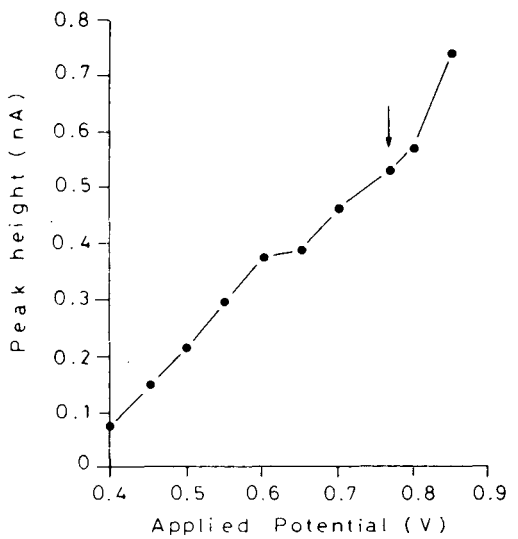

Fig. 1. Current—potential curve for apomorphine (3.5 ng injection). The arrow shows the applied potential used in the present study (0.77 V).

Chromatography

Apomorphine is usually analysed using mobile phases containing a high percentage of organic solvents [7, 10]. However, a high concentration of organic solvent is not, in general, suitable for electrochemical reactions [13]. The use of a relatively polar column, such as a CN column, permits reduction of the organic component in the mobile phase without increasing retention time. Apomorphine separation on a CN reversed-phase column with 11% acetonitrile in pH 3 phosphate buffer made for rapid analysis with symmetrical peaks.

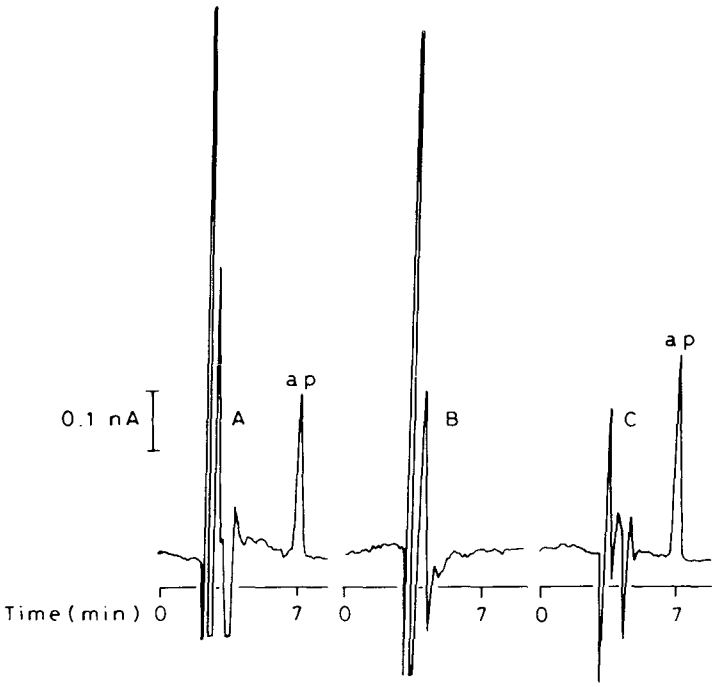

Fig. 2. Chromatograms from a striatum sample containing 10 ng of apomorphine (ap) (A), from drug-free striatum (B) and from striatum of rats treated with apomorphine (C).

Fig. 2 shows typical chromatograms of extracts from (A) striatum to which 10 ng of apomorphine were added, (B) drug-free striatum, and (C) striatum of a rat treated with apomorphine. The extract from untreated striatum shows no peaks that could interfere with drug analysis.

Under the chromatographic conditions specified, apomorphine eluted at 7 min; thus, a sample can be injected every 9 min.

TABLE I

RECOVERY AND LINEARITY OF APOMORPHINE FROM PLASMA (1 ml) AND BRAIN TISSUE (100 mg WEIGHT)

Each value is the mean of four determinations.

Sample	Amount added	Recovery (%) ± S.D.	Apomorphine peak height (nA per 100-μl injection) ± S.E.M.
Plasma	1	74.8 ± 7.2	0.025 ± 0.003
	3	77.8 ± 3.4	0.076 ± 0.003
	10	80.4 ± 1.5	0.261 ± 0.006
	30	84.8 ± 3.9	0.815 ± 0.028
	100	81.8 ± 2.8	2.654 ± 0.089
Brain	1	79.8 ± 2.5	0.026 ± 0.001
	3	85.6 ± 6.3	0.077 ± 0.005
	10	78.2 ± 2.9	0.249 ± 0.010
	30	84.2 ± 1.6	0.806 ± 0.004
	100	82.4 ± 2.0	2.680 ± 0.050

Recovery, linearity and sensitivity of the method

Apomorphine was well extracted from plasma or brain tissue at pH 7—7.5 with ethyl acetate [9], and the back-extraction into a small volume of hydrochloric acid gave a clean chromatogram with good recovery.

The linearity and recoveries (corrected for solvent losses) of apomorphine obtained by this method are summarized in Table I.

The calibration curves of peak height versus amount of apomorphine added to the samples were linear over the concentration range 1—100 ng of apomorphine base ($y = 0.0266x + 0.0008$, $r = 0.99996$ for plasma; $y = 0.0269x - 0.0062$, $r = 0.99997$ for brain tissue). The mean recovery was 80.3% with a coefficient of variation (C.V.) between 1.9% and 9.6% for plasma, and 82% with a C.V. between 1.9% and 7.4% for brain tissue.

The detection limit (signal-to-noise ratio ca. 4) was 0.3 ng/ml for plasma and 3 ng/g for striatum (about 100 mg weight).

Animal studies

In order to obtain information on the kinetic profile of apomorphine, male rats were given an intraperitoneal injection of apomorphine hydrochloride (5 mg/kg), and plasma and striatum were analysed as described.

Fig. 3 shows the time course of plasma and striatum concentrations for the drug. Apomorphine in plasma rose rapidly to a peak (0.31 μg/ml) within 10 min, after which the peak disappeared with an apparent half-life ($t_{1/2}$) of 9.65 min. Apomorphine levels in striatum were higher than in plasma, the maximum concentration being reached after 10 min (1.92 μg/g) declining thereafter with a $t_{1/2}$ comparable to that of plasma. The area under the curve (AUC) for apomorphine in striatum was approximately eight times that of the plasma AUC (see Table II), confirming that apomorphine concentrates in this tissue [5, 11, 15].

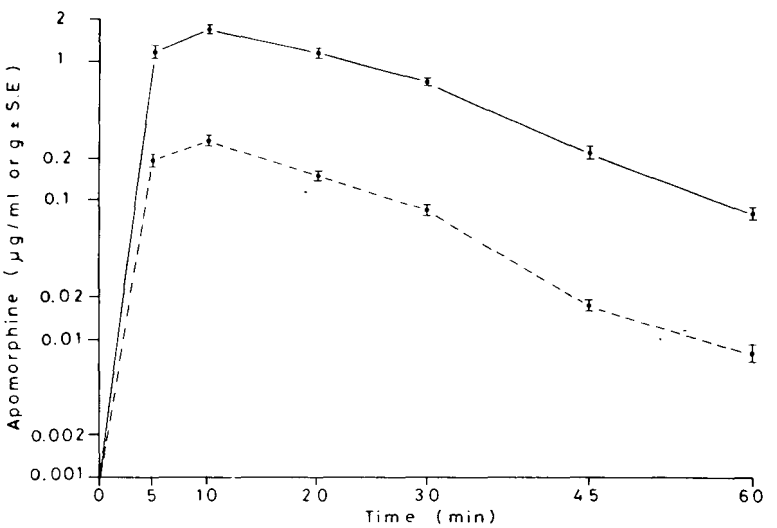

Fig. 3. Plasma (- - -) and striatum (——) concentration—time curves for apomorphine after administration of apomorphine hydrochloride (5 mg/kg, intraperitoneally) to rats. Each point is the mean ± S.E. of four animals.

TABLE II

PEAK CONCENTRATIONS (C_{max}), HALF-LIVES ($t_{½}$) AND AREA UNDER THE CURVE (AUC) FOR APOMORPHINE IN PLASMA AND STRIATUM OF RATS TREATED WITH APOMORPHINE HYDROCHLORIDE (5 mg/kg INTRAPERITONEALLY)

Compartment	C_{max} (μg/ml or μg/g)* ± S.E.M.	$t_{½}$** (min)	AUC*** (μg/ml or μg/g × min)
Plasma	0.31 ± 0.02	9.65	6.15
Striatum	1.92 ± 0.09	11.07	51.51

*Observed values.
**Plasma and striatal $t_{½}$ values were calculated assuming a one-compartment open model.
***AUC was calculated by the trapezoidal rule and extrapolated to infinity.

The $t_{½}$ values were obtained assuming a one-compartment model, but our data do not exclude the presence of a β-phase after the time considered, as reported in mice by Burkman et al. [16].

CONCLUSION

The present HPLC—ED method appears specific and sensitive; it shows good recovery and precision and thus does not necessarily require the use of an internal standard. Chromatographic analysis is relatively rapid (9 min) and the extraction procedure does not involve a time-consuming evaporation step (too risky because apomorphine has limited stability).

The procedure has proved particularly useful for pharmacokinetic studies of apomorphine in laboratory animals. A trained technician can analyse 40 samples of biological material per day.

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Note

Rapid and simple purification of the ^{125}I-labeled α-1 adrenergic radioligand 2-[β-(4-hydroxylphenylethyl)aminomethyl] tetralone (BE 2254) using reversed-phase high-performance liquid chromatography

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The ^{125}I-labeled α-1 adrenergic antagonist HEAT {2-[β-(4-hydroxylphenylethyl)aminomethyl] tetralone, BE 2254} has been found to be a useful radioligand for the quantitation of α-1 adrenoceptors in a variety of tissues [1—3] because of its high affinity and high specific activity. However, iodinated HEAT (I-HEAT) is somewhat labile, and undergoes decomposition under alkaline conditions at room temperature to form iodotyramine and non-iodinated residues. The resulting iodotyramine interferes with binding assays by causing a high background of non-specifically bound radioactivity. Thus, we find that repurification at regular intervals is necessary to maintain the usefulness of this ligand.

The usual method utilized for the purification of I-HEAT is paper chromatography [4, 5]. We have found that this technique results in a low recovery of purified I-HEAT, involves an undesirable exposure hazard owing to the need for large paper sheets and relatively large volumes of solvents, and requires long chromatographic times. We now report the development of a method for the rapid single-step purification of I-HEAT using high-performance liquid chromatography (HPLC). This method resolves I-HEAT from non-iodinated HEAT, its decomposition products, and by-products of its radioiodination.

EXPERIMENTAL

The conditions used for iodination of HEAT are the same as those reported by Engel and Hoyer [4], except that the amount of Na^{125}I was reduced by

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half. The iodination product (and non-iodinated HEAT) is extracted from the reaction mixture with three portions (300 μl each) of ethyl acetate. A pipette tip is used to facilitate separation of the layers, and the organic (upper) layers were recovered and pooled. The volume is reduced by evaporation under a fine stream of anhydrous nitrogen in a fume hood to approximately 30 μl. Distilled water (30 μl) is added and the residual ethyl acetate driven off with the nitrogen stream. Methanol (70 μl) containing 1% (v/v) 50 m*M* Tris—HCl (pH 6.5) is then added to the aqueous residue, producing a solution which is approximately 70% methanol, 30% water, and which is suitable for HPLC.

HPLC purification of HEAT

A Waters Model 204 liquid chromatograph consisting of two delivery systems (a Model 6000A and a Model M45), a Model U6K injector, and a Model 680 automated gradient controller were used. HEAT was monitored by absorbance at 254 nm using a Waters Model 450 variable-wavelength detector. An isocratic reversed-phase HPLC system (C_{18}, 10 × 0.5 cm, 10-μm column, Alltech) was employed for all separations. The mobile phase was methanol—water—diethylamine, pH 6.5 (70:30:0.04) and a flow-rate of 0.5 ml/min was used. Between runs, the column was washed with 100 ml water followed by 100 ml of 100% methanol containing 0.04% diethylamine (pH 6.5) to remove any remaining free iodine and decomposition products.

[^{125}]HEAT binding to myometrial membrane particulates

A membrane particulate fraction was prepared from rabbit myometrium as described by Roberts et al. [6]. Protein was quantitated by the method of Bradford [7] as modified for membrane proteins [6] using bovine serum albumin as standard.

Aliquots of membrane particulate fraction (40 μl) in 50 m*M* Tris, 4 m*M* magnesium chloride, pH 7.4 containing 50—70 μg of protein are added to 20 μl [^{125}I]HEAT (25—1600 p*M* final concentration in saturation studies, 80 p*M* in competition studies) in 50 m*M* Tris, 4 m*M* magnesium chloride, pH 7.4 and 20 μl of 1 m*M* hydrochloric acid to start the binding reaction. Non-specific binding is estimated by substituting Prazosin · HCl (Pfizer), 40 μ*M* in 1 m*M* hydrochloric acid for the 1 m*M* hydrochloric acid in the reaction. Incubation is carried out for 30 min at 30°C and terminated by the addition of 5 ml of ice cold 50 m*M* Tris, 4 m*M* magnesium chloride, pH 7.4 containing 10% (v/v) polyethylene glycol (average MW 400) followed by filtration under low vacuum on Whatman GF/C filters. Washing of filters is completed by the addition of three 5-ml vols. of the same cold buffer solution. Radioactivity is quantitated by γ-emission counting (Packard Instruments) at 72% efficiency.

RESULTS

The separation of ^{125}I-labeled HEAT from non-iodinated HEAT is shown in Fig. 1A. At the flow-rate employed (0.5 ml/min), native HEAT had a retention time of approximately 8 min while I-HEAT was eluted 14 min after injection. Thus unlabeled HEAT, which is present in great excess in the extract of the iodination mixture, can be easily and completely resolved from I-HEAT. The

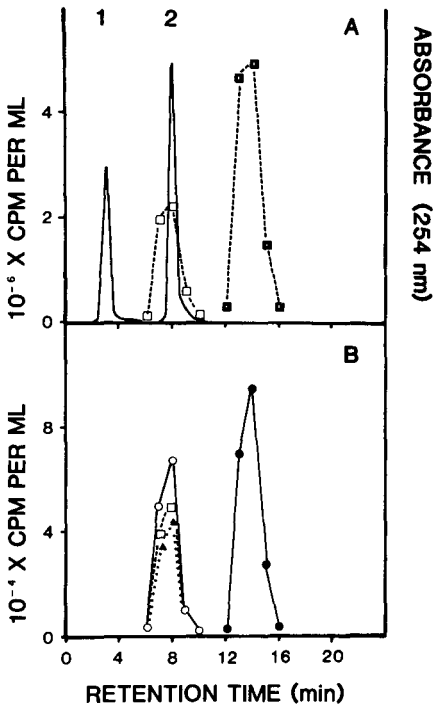

Fig. 1. HPLC separation of HEAT and related compounds. Separatons were performed using a C_{18} reversed-phase column (10 × 0.5 cm, 10 μm) eluted isocratically at a flow-rate of 0.5 ml/min using a solvent consisting of methanol—water—diethylamine, pH 6.5 (70:30:0.04). Panel A: the solid line represents the absorbance profile for tyramine (1) and HEAT (2). The dashed line represents the radioactive profile obtained with ^{125}I-labeled tyramine (open squares) and ^{125}I-labeled HEAT (closed squares). Panel B: ^{125}I-labeled tyramine and ^{125}I-labeled HEAT were isolated by HPLC as in panel A. Aliquots of each were treated under mild acidic or basic conditions as described in the experimental section prior to rechromatography. The samples are as follows: (□) I-tyramine after base treatment; (▲) I-tyramine after acid treatment; (○) I-HEAT after base treatment; and (●) I-HEAT after acid treatment.

major radioactive product formed upon the decomposition of ^{125}I-labeled HEAT is ^{125}I-labeled tyramine. Fig. 1A also shows the elution profiles for tyramine and ^{125}I-labeled tyramine. ^{125}I-labeled tyramine elutes in a position concurrent with native HEAT at a retention time of about 8 min. Thus, ^{125}I-labeled HEAT is readily resolved from both the non-iodinated compound and its major decomposition product.

In order to confirm the above results, the following experiment was performed. I-HEAT is unstable under mildly alkaline conditions but is stable under mildly acid conditions [3]. In contrast, I-tyramine is apparently stable under either of these conditions. I-HEAT and I-tyramine were isolated as described above and the solvent was removed under a stream of nitrogen. Aliquots of each were treated for 1 h in the presence of either dilute ammonium hydroxide (pH 9) at 50°C or dilute hydrochloric acid (pH 3) at −20°C (the recommended storage temperature for I-HEAT). Following this treatment, the pH of each solution was adjusted to 6.5 and the samples were run again on the HPLC system described above. As shown in Fig. 1B, treatment

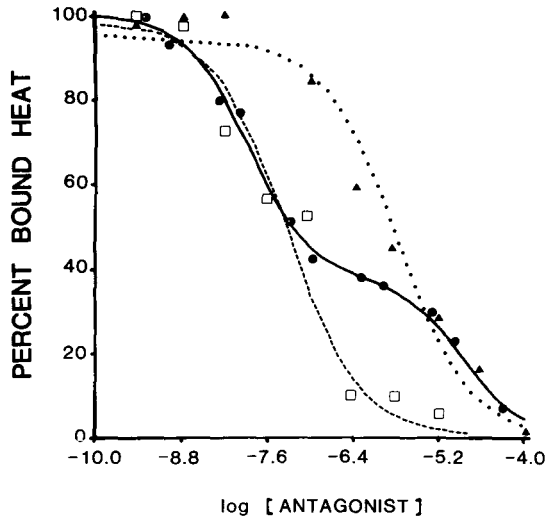

Fig. 2. Competition curves for I-HEAT binding to myometrial membranes. Myometrial membrane particulate fractions were prepared as described in Experimental and incubated with I-HEAT (80 p*M*) in the presence of the unlabeled antagonists. Phentolamine • HCl (•): equilibrium binding affinity constant of the inhibitor (K_i) 1—6.9 n*M*, 40.4%; K_i 2—2400 n*M*, 40%. Prazosin • HCl (□): K_i 1.0 n*M*. Yohimbine • HCl (▲): K_i 1100 n*M*.

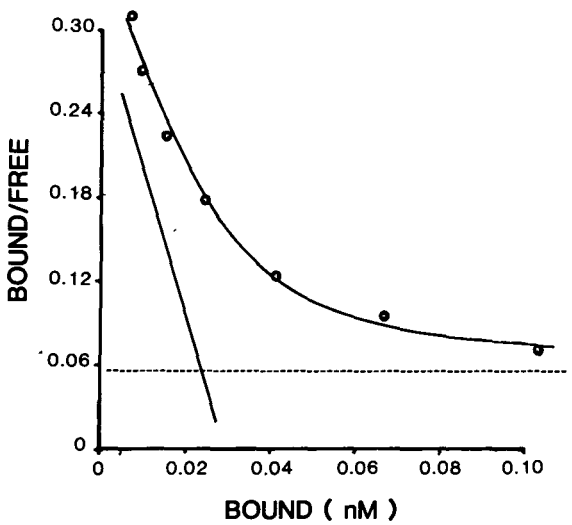

Fig. 3. Saturation curve for I-HEAT binding to myometrial membranes. Myometrial membranes were incubated with I-HEAT as described in Experimental. Total binding data was then analyzed with the following results: equilibrium binding affinity constant (K_d) = 96.6 ± 17.3 p*M* (S.E.M.); equilibrium binding site concentration (B_{max}) = 74.2 ± 10.3 fmol/mg of membrane protein. Non-specific binding is indicated by the dashed line parallel to the abscissa (bound/free = 0.0055 ± 0.0005).

of I-tyramine with either acid or base has no effect on its relative retention time. Similarly, treatment of I-HEAT with acid did not alter its retention time. Treatment of I-HEAT with mild base, however, resulted in the generation of a radioactive compound which cochromatographed with I-tyramine. Further

treatment of this degradation product with either acid or base had no effect on its elution position.

To confirm the usefulness of the ligand isolated by the described procedure, competition studies of the binding of I-HEAT to myometrial membrane preparations were conducted and are shown in Fig. 2. I-HEAT binds to these preparations in a manner consistent with α-1 adrenergic potencies. Fig. 3 shows a typical saturation isotherm for I-HEAT binding arrayed according to Scatchard [8]. The best-fit curve was calculated from untransformed data by fitting highest-affinity binding using a computer-based iterative non-linear curve fitting program [9]. I-HEAT binds with high affinity (equilibrium binding affinity constant, K_d = 97 pM) and identifies approximately 70 fmol of α-1 binding sites per mg of myometrial membrane protein.

DISCUSSION

We have described a simple one-step HPLC method for the rapid purification of I-HEAT. This method is especially useful for the initial purification of I-HEAT following iodination, since it readily resolves labeled and unlabeled HEAT. I-HEAT is a high-affinity and high-specific-activity radioligand which has been found to be quite useful for the quantitation of low concentrations of α-1 adrenergic receptors in brain [1], pituitary [3], and smooth muscles such as vas deferens [2] and, as shown here, for myometrium.

The usefulness of this ligand can be compromized by the accumulation of the decomposition product iodotyramine, which increases the level of non-specific binding in the assay. The purification method we have described is simple and rapid enough for the routine repurification of I-HEAT at regular intervals, thereby extending the useful life of this ligand. While storage of I-HEAT at −20°C or −80°C under acidic conditions retards the formation of iodotyramine, we find that some decomposition still occurs.

Although I-HEAT is available commercially, its cost and lack of stability may limit its usefulness for the detection of low concentrations of α-1 adrenoceptors. The procedure described in this communication facilitates the preparation of this ligand at a very low cost. Furthermore, the ability to rapidly repurify the ligand from its degradation products will both extend its useful life and maintain the high signal-to-noise ratio needed for the accurate measurement of low levels of α-1 adrenoceptors.

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Note

Analysis of the toxicologically relevant metabolites of phenytoin in biological samples by high-performance liquid chromatography

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Phenytoin (5,5-diphenylhydantoin, Dilantin®) is one of the most efficacious and widely prescribed anticonvulsants for the treatment of epilepsy. The association of phenytoin with teratological sequelae in animals and humans has been reviewed along with the proposed mechanisms [1]. Phenytoin teratogenicity has been established and characterised in animals, and strongly implicated in humans. Teratologic sequelae are thought to result from the cytochrome P-450-mediated bioactivation of phenytoin to a reactive arene oxide intermediate which, if not detoxified, can bind covalently to essential fetal cellular macromolecules, thereby causing developmental aberrations. The arene oxide can be detoxified by rearrangement via an NIH shift to 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), or can be hydrated by epoxide hydrolase to a *trans*-dihydrodiol and subsequently to a catechol and then a methoxycatechol metabolite. Mechanistic studies of phenytoin teratogenicity require the ability to measure all these metabolites to evaluate the balance of phenytoin bioactivation and detoxification. Several assays have been reported using high-performance liquid chromatography (HPLC) [2—6]; however, they fail to detect or separate all toxicologically relevant metabolites, and generally require long analysis time to complete. We describe herein a sensitive HPLC method for the detection within 15 min of phenytoin and its metabolites. Results from a number of HPLC columns and solvent systems are compared.

METHODS

Materials and reagents

Sodium phenytoin (5,5-diphenylhydantoin, sodium salt), 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), and 5-(3-hydroxyphenyl)-5-phenyl-

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hydantoin (*m*-HPPH) were obtained from Sigma (St. Louis, MO, U.S.A.). The α,α'-diphenylglycine was obtained from Aldrich (Milwaukee, WI, U.S.A.). The 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (dihydrodiol) and 5-(3-4-dihydroxyphenyl)-5-phenylhydantoin (catechol) were gifts from Dr. J.H. Maguire (University of North Carolina), and 5-(3-methoxy-4-hydroxyphenyl)-5-phenylhydantoin (methoxycatechol) were gifts from Dr. T. Chang of Warner-Lambert Labs. (Ann Arbor, MI, U.S.A.). Metabolites were dissolved in HPLC-grade methanol (Fisher Scientific, Toronto, Canada). The 5,5-[phenyl-4-^{3}H(N)-diphenylhydantoin, 51.5 Ci/mmol, was obtained from New England Nuclear (Lachine, Quebec, Canada). Mephenytoin was obtained as a gift from Sandoz Pharmaceutical (East Hanover, NJ, U.S.A.). Purity of the radiolabeled phenytoin was > 99% as determined by HPLC. Solvents used were HPLC-grade 2-propanol, methanol, isopropyl alcohol and acetonitrile (Fisher Scientific); HPLC-grade water was prepared using a purification system (Milli-Q, Millipore, Mississauga, Canada). Prior to use, solvents were passed through a 0.45-μm filter (type HA® for aqueous solvents and type FH® for organic solvents, Millipore).

High-performance liquid chromatography

HPLC was employed using an automated system (Perkin-Elmer Canada, Toronto, Canada). Samples were injected from microvials using an autosampler (Model ISS-100). The pumping system (Model Series 4) consisted of a quaternary gradient system with on-line helium degassing and a controller. The detector was a double-beam, variable-wavelength, UV—VIS spectrophotometer (Model LC 85) equipped with a 2.4-μl flow cell, automatic baseline compensation (Autocontrol®) for gradient conditions, and stop-flow scanning accessories. The detector signal was integrated as the chromatographic peak area-under-the-curve by a 16-bit microcomputer (Model Sigma 15) and stored on-line on floppy disks using an 8-bit microcomputer (Model 3600).

Spectra for the HPLC peaks of phenytoin and the authentic standards for its metabolites were analyzed individually by stop-flow scanning from 190 to 300 nm under chromatographic solvent conditions to determine the respective optimal wavelengths for maximal UV absorbance.

A number of columns and solvent conditions were evaluated. The columns included a 15-cm reversed-phase (RP) C_{18} column with 5 μm particle size (Perkin-Elmer), three similar columns from Beckman Instruments (Toronto, Canada), a 5-cm RP C_{18} (3 μm) column, a 7.5-cm RP C_3 (5 μm) column, a 25-cm RP C_{18} (5 μm) microbore column, and a 3-cm RP C_{18} (3 μm) column (Perkin-Elmer). The internal diameter was 4.6 mm for all columns except the microbore column, which was 2.0 mm.

The criteria for optimal HPLC separation include rapid analysis time and reproducible baseline resolution of all metabolites. These criteria are dependent on the efficiency and selectivity of the column, and on solvent conditions. These characteristics determine the ability of an HPLC system to resolve similar chemical species, giving narrow, symmetrical peaks with baseline separation. For phenytoin, the methoxycatechol and *p*-HPPH metabolites were particularly difficult to resolve under conditions favourable to all chemical species. The resolution of these two metabolites was a major factor in the final choice of column and solvent conditions.

Preparation of biological samples for HPLC analysis

Using CD-1 mice (Charles River Canada, St. Constant, Canada), phenytoin and its metabolites were measured in 100-μl blood and urine samples obtained respectively by tail-vein sampling and the use of a metabolic cage. The extraction procedure involved mixing plasma and urine samples with an equal volume of β-glucuronidase (Sigma), 20 000 U/ml in 0.2 *M* sodium acetate buffer, pH 4.9. This suspension was incubated at 37° C for 20 h, at which time 20 μl of a 100 μg/ml solution of mephenytoin, internal standard, was added. The mixture was extracted five times with 2 vols. of ethyl acetate. After each extraction the sample was centrifuged at 1000 *g* for 5 min (centrifuge Model TJ-6, Beckman) and the organic layer was removed. The ethyl acetate layers were pooled and blown to dryness under nitrogen. The sample was then reconstituted in 20 μl of methanol and 5 μl of this solution were injected into the HPLC system equipped with a 15-cm RP C_{18} (5 μm) column (Beckman).

RESULTS

High-performance liquid chromatography

Stop-flow spectral scans from the HPLC detector under optimal chromato-

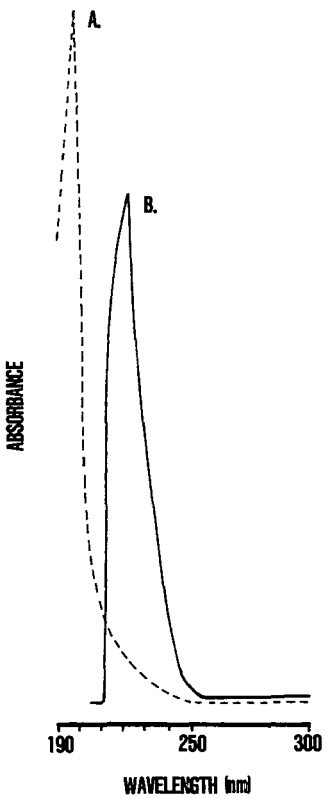

Fig. 1. Stop-flow UV spectral scans of phenytoin. Scans were obtained under different chromatographic conditions using a variable-wavelength, spectrophotometric HPLC detector. (A) water—isopropyl alcohol (80:20). This was the optimal solvent system used in gradient mode for subsequent analyses. (B) Methanol—1% acetic acid (30:70).

graphic conditions indicated a maximal UV absorbance for phenytoin at 195 nm (Fig. 1A), and within ± 2 nm for its metabolites. Subsequent analyses were performed at 225 nm to minimize the confounding absorbance of endogenous compounds. Changes in solvent system produced a marked displacement in the absorbance maximum (Fig. 1B).

The 15-cm RP C_{18} (5 μm) column from Perkin-Elmer was evaluated using two solvent systems; 70—76% phosphoric acid (1%) and 30—24% methanol, or 20—30% acetic acid (1%) and 80—70% methanol. Under isocratic elution the methoxycatechol and *p*-HPPH metabolites were unresolved. Peaks were broad with considerable tailing, and retention times were long.

The 5-cm RP C_{18} (3 μm) column from Beckman provided adequate metabolite resolution with sharp peaks and short retention times using a stepwise linear gradient at 2 ml/min, with a ternary solvent system which consisted of methanol (15—25%), acetonitrile (0—10%) and 1% acetic acid (85—65%). Binary solvent conditions caused drifting baselines with fair resolution.

The 7.5-cm RP C_3 (5 μm) column from Beckman provided fair resolution of all metabolites with fast retention times (less than 10 min). The solvent system used was 17% methanol and 83% water with a flow-rate of 1.5 ml/min.

The 25-cm RP C_{18} (5 μm) microbore column from Beckman could not resolve the methoxycatechol and *p*-HPPH metabolites to baseline, but there was a dramatic increase in sensitivity. A ternary solvent system was used, ranging from 70% to 77% phosphoric acid (0.5%), 5% to 15% acetonitrile and 15% to 18% isopropyl alcohol. Retention times were long, with phenytoin eluting at 26 min, and peak shapes were broad even under optimal conditions. Gradient methods proved to be impractical owing to the very low flow-rates (0.2 ml/min) and long equilibration times.

Gradient Solvent Conditions

Time (min)	Water (%)	2-Propanol (%)	Gradient Curve
3	80	20	-
7	75	25	1
3	80	20	1

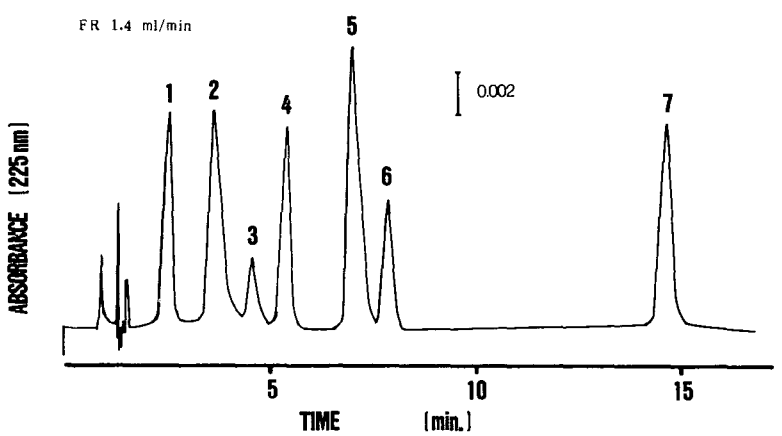

Fig. 2. HPLC chromatogram of metabolite standards. Dihydrodiol (1); catechol (2); methoxycatechol (3); *p*-HPPH (4); *m*-HPPH (5); mephenytoin, internal standard (6); phenytoin (7). The α,α'-diphenylglycine metabolite (not shown) elutes with retention time of 2.60 min. The injection volume was 5 μl and UV detection was performed at 225 nm for this and all subsequent HPLC analyses. The HPLC column was a 15-cm RP C_{18} column with a particle size of 5 μm (Beckman).

The 3-cm RP C_{18} (3 μm) column from Perkin-Elmer could not completely resolve the methoxycatechol and *p*-HPPH peaks, however, peak retention times were rapid and metabolite peak shapes were excellent; only the phenytoin peak was broad. The optimal solvent system under isocratic conditions was 88% phosphoric acid (1%) and 12% isopropyl alcohol at a flow-rate of 1.0 ml/min. This column was too short for gradient techniques. The sensitivity for metabolites was superior to all other columns except the microbore column.

The three 15-cm RP C_{18} (5 μm) columns from Beckman provided the best results, with baseline resolution of all metabolites, sharp peak shapes, good sensitivity and short retention times. A total analysis time of 15 min was obtained using a stepwise gradient elution (Fig. 2). Column pressures varied between 24 and 27 MPa at the beginning and end of the gradient. The variability observed with these three columns over four months was low, as demonstrated by the retention time for phenytoin. The intra-day retention time was 14.68 ± 0.13 min (mean ± S.D., $n = 19$), and the inter-day time was 14.50 ± 0.54 min ($n = 144$).

Biological samples

The extraction efficiency from urine and plasma was 94.7% with a 4.8% coefficient of variation. The limit of UV detection sensitivity, taken as two times the baseline excursion, was 5 ng, and the standard curve was linear up to at least 5 μg. However, the use of radiolabeled phenytoin extends the lower limit of sensitivity by 10 000-fold, to 0.1 pg. Fig. 3 and 4 show the UV chromatograms of urine and plasma samples taken from mice treated with native phenytoin together with radiolabeled phenytoin. Analysis of the fractions of the column eluent by liquid scintillation spectrometry revealed several critical metabolite peaks below the limit of sensitivity by UV detection, or peaks masked or obliterated by endogenous components. Table I shows the percent urinary recovery of phenytoin and its metabolites following each successive ethyl acetate extraction. The total recovery of each metabolite represents the

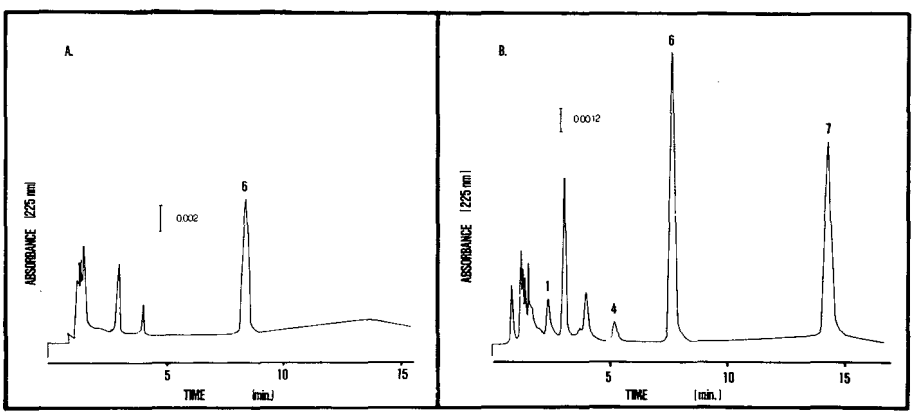

Fig. 3. HPLC chromatogram of mouse plasma. (A) Control plasma. (B) Plasma sample taken 17 h after administration of phenytoin, 55 mg/kg intraperitoneally. The volume injected into the chromatograph was 5 μl and the metabolite concentrations were 11.28 μg/ml dihydrodiol (1), 9.68 μg/ml *p*-HPPH (4), 200 μg/ml mephenytoin internal standard (6), and 181.6 μg/ml phenytoin (7).

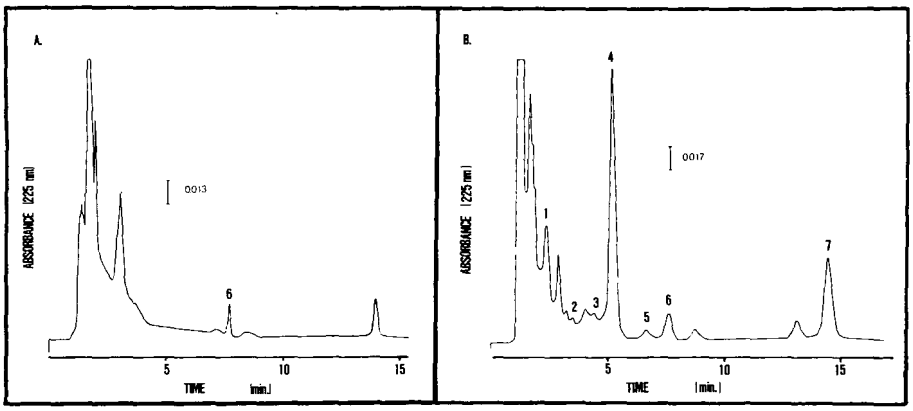

Fig. 4. HPLC chromatogram of mouse urine. (A) Control urine. (B) Urine sample collected over 16 h following administration of phenytoin, 55 mg/kg intraperitoneally. The volume injected into the chromatograph was 5 μl and the metabolite concentrations were 260 μg/ml dihydrodiol (1), 2.7 μg/ml catechol (2), 3.9 μg/ml methoxycatechol (3), 741 μg/ml *p*-HPPH (4), 84 μg/ml *m*-HPPH (5), 200 μg/ml mephenytoin, internal standard (6), and 369 μg/ml phenytoin (7).

TABLE I

URINARY RECOVERY (%) OF PHENYTOIN AND ITS METABOLITES FOLLOWING SUCCESSIVE EXTRACTIONS

Radiolabeled phenytoin (DPH), 2 μCi/g, was administered intraperitoneally to pregnant CD-1 mice on gestational day 12 and urine was collected over 48 h. Phenytoin and its metabolites were extracted from the urine with ethyl acetate.

Metabolite*	Extraction No.					Total
	1	2	3	4	5	
DHD	6.28	4.81	4.10	0.95	0.96	17.10
CAT	—	—	—	—	—	—
MET	0.29	0.13	0.03	—	—	0.45
p-HPPH	32.97	9.50	2.55	0.70	0.18	45.90
m-HPPH	—	0.56	0.28	0.07	—	0.91
DPH	20.25	5.81	1.82	0.83	0.45	29.16
						93.52

*Abbreviations: DHD, dihydrodiol; CAT, catechol; MET, methoxycatechol; *p*-HPPH, *para*-hydroxyphenyl-5-phenylhydantoin; *m*-HPPH, *meta*-hydroxyphenyl-5-phenylhydantoin; DPH, unmetabolized phenytoin.

percent contribution of each route of phenytoin's biotransformation. The use of radiolabeled phenytoin was essential for quantification of toxicologically relevant metabolites generated as products of minor metabolic pathways undetectable by UV absorbance.

DISCUSSION

The optimal column for the resolution and detection of phenytoin and its

toxicologically relevant metabolites was a 15-cm RP C_{18} column with a particle size of 5 μm. It is worth noting that even this type of column with identical specifications from several suppliers provided inadequate metabolite resolution or peak shape. In addition, there is some variation in the performance of different lots of the same column from the same supplier. For a given assay, columns from the same production lot will ensure optimal column reproducibility. The optimal solvent conditions are given in Fig. 2. While the columns with a particle size of 3 μm resulted in sharper peaks, increased detection sensitivity, and shortened retention times, the methoxycatechol and *p*-HPPH were not completely resolved, and this is a disadvantage if effluent peak fractions are to be collected for further analysis. The 3-μm columns also incur higher back-pressures and degenerate more rapidly than 5-μm columns with repeated injections of biological samples. For detection of the toxicologically relevant trace metabolites (dihydrodiol, catechol and methoxycatechol) which reflect the arene oxide pathway, sensitivity is increased 10 000-fold with the use of radiolabeled phenytoin and HPLC-fraction collection followed by scintillation spectrometric analysis.

The assay reported herein will permit more complete quantification of the critical pathways of phenytoin biotransformation and should prove useful to investigators concerned with the toxicology of phenytoin.

ACKNOWLEDGEMENTS

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Note

Timolol determination in plasma and urine by high-performance liquid chromatography with ultraviolet detection

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The β-adrenoceptor antagonist, timolol, is a potent antihypertensive and antianginal drug which has also proved to be effective for the secondary prevention of myocardial infarction [1].

The analysis of timolol in human plasma following therapeutic doses (10—20 mg) requires methods capable of measuring the drug in the lower ng/ml range. Gas—liquid chromatography (GLC) with electron-capture or alkali-flame-ionization detection have been used most commonly for this purpose and the methods have lower limits of assay of 1—5 ng/ml [2—4]. Greater sensitivity (0.5 ng/ml) has been obtained using GLC—mass spectrometry [5, 6]. However, this technique is not available in many laboratories. Furthermore, all the above GLC procedures involve derivatisation of the drug and in some cases they incorporate tedious sample and reagent clean-up steps.

High-performance liquid chromatography (HPLC) allows analysis of underivatised β-adrenoceptor antagonists. However, a lack of suitably sensitive detectors has been a limiting factor in the use of this technique to analyse timolol concentrations in plasma after therapeutic doses. Thus, the method of Lefebvre et al. [7] can only detect peak plasma concentrations of the drug. Recently, however, Gregg and Jack [8] have reported a limit of sensitivity of 2 ng/ml in plasma and breast milk, using an electrochemical detector.

We now describe an alternative HPLC method for the determination of timolol in plasma and urine which is comparable in sensitivity to that of Gregg and Jack [8] but which uses a very stable UV detector. Although the capital cost of this UV detector is about twice that of most electrochemical detectors, the latter tend to require more attention during routine use.

This method was developed to explore the relationship between oxidation phenotype and the metabolism of timolol in man [9]. Plasma concentration

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and urine excretion data from one extensive and one poor metaboliser of debrisoquine are presented.

MATERIALS AND METHOD

Chemicals and drugs

Timolol maleate was a gift from Merck Sharpe & Dohme (Hoddesden, U.K.). The internal standard, phenacetin, was obtained from BDH (Poole, U.K.). HPLC-grade acetonitrile and glass-distilled methyl *tert.*-butyl ether were purchased from Rathburn Chemicals (Walkerburn, U.K.). All other chemicals were of analytical grade.

Stock solutions of timolol (1 μg base per ml) prepared in distilled water were found to be stable at 4°C for at least three months.

HPLC instrumentation and conditions

The chromatograph consisted of a Model 3000 Applied Chromatography Systems pump, a Model 773 Kratos Spectroflow UV—VIS absorbance detector and a Model 7125 Rheodyne injector (0.5-ml loop), all purchased from HPLC Technology (Macclesfield, U.K.). The detector wavelength was set at 295 nm. The stainless-steel column (10 cm × 5 mm I.D.) used was packed with Hypersil 5-ODS reversed-phase material (5 μm particle size) (HPLC Technology). A standard pre-column (5 cm × 5 mm I.D.) containing reversed-phase guard column material (40 μm; Waters Assoc., Northwich, U.K.) was fitted between the injector and the analytical column.

Water—acetonitrile (87:13) containing 1% triethylamine and adjusted to pH 3 with orthophosphoric acid was used as the mobile phase. Chromatography was performed isocratically at a flow-rate of 2 ml/min and at ambient temperature.

Extraction procedure

Heparinised plasma or diluted urine (1.0 ml of a ten-fold dilution in water) was shaken gently with sodium hydroxide (4 *M*, 0.1 ml), internal standard (0.25 and 1.0 μg for plasma and urine, respectively) and methyl *tert.*-butyl ether (5 ml) for 10 min. After centrifugation (900 *g*, 5 min), as much of the upper layer as possible was transferred to a 10-ml conical centrifuge tube and evaporated to dryness at 40°C on a Buchler Vortex Evaporator (Baird and Tatlock, Romford, U.K.). The residue was reconstituted in mobile phase (100 μl) and an aliquot (50—100 μl) was injected into the chromatograph.

RESULTS AND DISCUSSION

Owing to limitations in the sensitivity of their method, Lefebvre et al. [7] concluded that timolol could not be monitored effectively in plasma by HPLC with UV detection. The UV monitor used in the present work, however, was operated routinely at very high sensitivity settings allowing detection of as little as 0.5 ng of the drug on column.

As in previous work with metoprolol [10], addition of a triethylamine—phosphoric acid modifier to the HPLC mobile phase resulted in sharp, sym-

metrical peaks for timolol (Fig. 1). Under the chromatographic conditions used retention times for timolol and the internal standard were 3.7 and 8.6 min, respectively.

Dichloromethane, the solvent most frequently employed in this laboratory for the extraction of β-blocking drugs from biological fluids, gave unexpectedly poor recoveries for timolol. Replacement of dichloromethane with methyl *tert.*-butyl ether [11] overcame this problem giving yields of > 90%.

Neglible interference was observed in extracts of samples which did not contain timolol (Fig. 1). In addition the following drugs and metabolites when injected directly were either undetectable or had substantially different retention times from those of timolol and phenacetin: atenolol, acebutolol, sotalol,

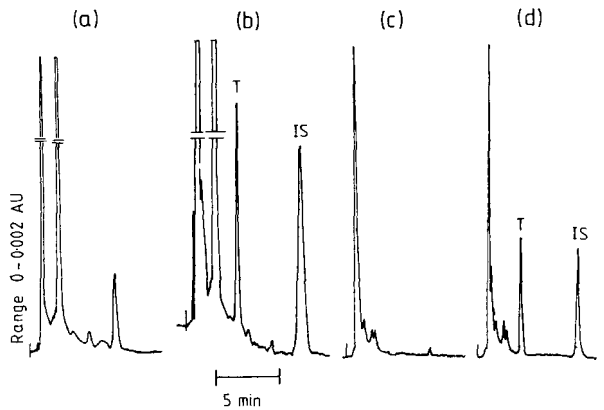

Fig. 1. Chromatograms of extracted plasma and urine. Plasma containing no drug (a); 20 ng/ml timolol base (b). Urine containing no drug (c); 700 ng/ml timolol base (d). Peaks: T = timolol; IS = internal standard.

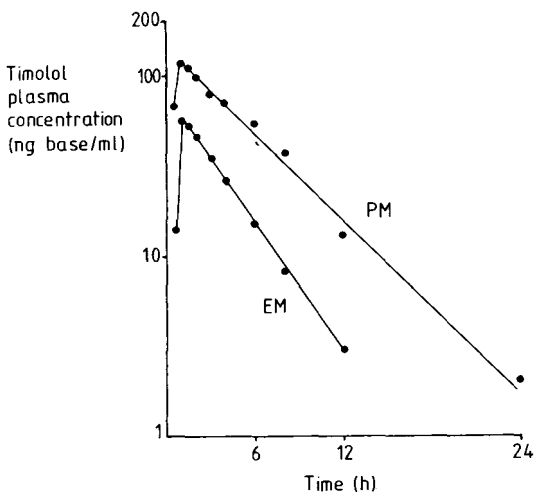

Fig. 2. Plasma concentrations of timolol in a poor (PM) and an extensive metaboliser (EM) of debrisoquine following a single oral dose of 20 mg timolol maleate. The PM and EM subjects excreted 20.6% and 6.6%, respectively, of the dose unchanged in the urine after 24 h.

nadolol, propranolol, labetalol, verapamil, norverapamil, paracetamol, warfarin, disopyramide, canrenone, nifedipine, lignocaine, hydralazine and three of its metabolites (methyltriazolophthalazine, 3-hydroxymethyltriazolophthalazine and phthalazine). A fourth metabolite of hydralazine, triazolophthalazine, had a retention time sufficiently close to that of timolol to be a possible source of interference.

Calibration plots of the peak height of timolol to that of the internal standard versus timolol concentration were linear over the range 5—200 ng/ml for plasma ($r^2 > 0.99$) and 50—2000 ng/ml for urine ($r^2 > 0.99$) and passed through the origin. Calibration standards were included in each batch of analyses. The intra-assay coefficients of variation for plasma were 5.9% and 5.7% at 5 and 50 ng/ml, respectively, and for urine were 5.0% and 9.3% at 500 and 1000 ng/ml, respectively. The lowest assayable concentration of timolol in plasma was about 2 ng/ml.

Plasma concentrations of timolol were found to be significantly higher in poor metabolisers than in extensive metabolisers of debrisoquine, indicating a close link between the metabolism of the two drugs [9]. The plasma drug concentration versus time profiles of one subject from each phenotype group are shown in Fig. 2. Timolol was detectable up to 24 h in the plasma of most poor metabolisers but could not be followed beyond 12 h in the extensive metabolisers. The 24-h urine concentrations of the drug were approximately ten times higher than peak plasma concentrations.

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Note

High-performance liquid chromatographic determination of 3-hydroxymethyl-dibenzo[*b*,*f*]thiepin 5,5-dioxide and its acid metabolite in human plasma and urine

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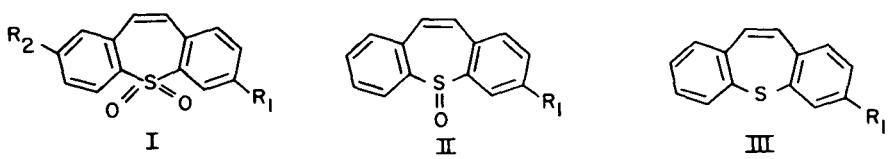
Prostaglandins are potent, biologically active agents with a wide spectrum of activity depending on species, organ, and prostaglandin involved. Activation of prostaglandin biosynthesis results in the production of unstable prostaglandin endoperoxides, prostaglandins G_2 and H_2. These in turn degrade to prostaglandin I_2 and thromboxane A_2 which are also unstable. Thromboxane A_2 is a strong contractor of vascular smooth muscle and induces platelet aggregation [1, 2].



COMPOUND	STRUCTURE	R_2	R_1
1	I	H	CH_2OH
2	I	H	COOH
3	I	F	CH_2OH
4	I	H	CHO
5	I	H	$CONHCH_2COOH$
6	II		COOH
7	III		COOH

Fig. 1. Chemical formulae of compounds 1—7.

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3-Hydroxymethyl-dibenzo[*b*,*f*]thiepin 5,5-dioxide, 1 (see Fig. 1), has been found to antagonize the actions of thromboxane A_2 and other contractile prostaglandins in in vitro and in vivo studies [3]. Compound 2, the acid metabolite of 1, was also found to have a similar profile of pharmacological activity and may be approximately twice as potent in in vitro studies of platelet function [3].

A new high-performance liquid chromatographic (HPLC) method for the concurrent determination of compounds 1 and 2 in plasma and urine is reported. The method has been developed to determine the pharmacokinetic profile of 1 and 2 in man. Compounds 1 and 2 can be reliably quantitated with detection limits of 5 ng/ml in plasma and 1 μg/ml in urine. Only free 2 was found in plasma and urine from patients given 1. Free, Glusulase® and base-hydrolyzable concentrations of 2 were determined in urine from two subjects given different doses of 1.

MATERIALS AND METHODS

All reagents were analytical grade or better. Chromatographic solvents were HPLC grade. An Analytichem Vac-Elut® system (Harbor City, CA, U.S.A.) equipped with a Bond-Elut® 200-mg octadecylsilane cartridge was used for sample extraction. Glusulase was obtained from Endo Labs. (Garden City, NY, U.S.A.).

All standards were supplied by Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.). Standards were dissolved in a mixture of acetonitrile—water (1:4, v/v) with the exception of compound 2, which was soluble in acetonitrile—1 *M* sodium acetate (pH = 6)—water (4:1:15, v/v/v). Stock standard solutions of 1 and 2 were 500 μg/ml and stock internal standard, 3, was 100 μg/ml.

Apparatus

The chromatographic separation was attained using a Rainin Gradient HPLC system (Woburn, MA, U.S.A.). Injections were made with a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, U.S.A.). The column system was held at 50°C and included a Brownlee octadecylsilane, 5-μm, 3 cm × 4.6 mm precolumn (Santa Clara, CA, U.S.A.) and an Analytical Sciences octadecylsilane, 10-μm 30 cm × 4.6 mm analytical column (Santa Clara, CA, U.S.A.). Peaks were detected at excitation wavelength 315 nm and emission wavelength 390 nm with a Perkin-Elmer 650-10S fluorescence detector. The mobile phase consisted of 0.85% phosphoric acid solution (pH = 3.5)—acetonitrile (60:40), and was pumped at 2 ml/min. Data were generated by a Spectra-Physics SP4270 integrator (Santa Clara, CA, U.S.A.).

Clinical samples

Healthy male volunteers received single rising oral doses of compound 1 over the range of 250—2000 mg. Plasma was collected at 0, 1, 4, 10 and 24 h and urine was collected and pooled for time intervals of −1—0, 0—4, 4—10, 10—24 and 24—48 h. Plasma and urine samples were stored at −15°C until time of analysis.

Sample preparation and extraction of plasma

Plasma analyzed for free 1 and 2 was prepared by placing 0.25 ml of plasma, 0.1 ml of 1 *M* hydrochloric acid, 0.1 ml of working internal standard (10 μg/ml) and 0.75 ml of 0.85% phosphoric acid solution in a polypropylene tube.

After vortexing, the plasma sample was applied to a Bond-Elut octadecylsilane cartridge. The cartridge was pre-wet with 3 ml of methanol followed by 3 ml of water. The sample was passed through the cartridge and washed with 1 ml of water. Excess water was removed from the cartridge using the house vacuum. Methanol (1 ml) was passed through the cartridge and the eluate was collected into a tube containing 0.15 ml of 0.85% phosphoric acid solution. After vortexing and centrifugation, the sample was injected on the HPLC system.

Sample preparation and extraction of urine

Free concentrations of compounds 1 and 2 were determined in urine by placing 0.1 ml of urine, 0.1 ml of working internal standard (100 μg/ml), 0.1 ml of water and 0.75 ml of acetonitrile in a test tube. After vortexing and centrifugation, 0.10 ml was removed and diluted with 0.75 ml of mobile phase. The sample was then injected on the HPLC system.

Urine hydrolyzed by Glusulase was prepared by placing 0.1 ml of urine, 0.1 ml of internal standard (100 μg/ml), 0.1 ml of diluted Glusulase (activity ≈10000 U/ml) and 0.70 ml of 0.5 *M* sodium acetate (pH = 4.5) in a test tube and capping. Urine was incubated for 90 min at 40°C. After incubation, 0.1 ml of urine was diluted with 0.5 ml of acetonitrile and 0.25 ml of 0.85% phosphoric acid solution. The sample was then vortexed, centrifuged and injected on the HPLC system.

Urine for base hydrolysis was prepared by adding 0.1 ml of urine, 0.1 ml of working internal standard (100 μg/ml), 0.3 ml of 5 *M* sodium hydroxide solution and 0.5 ml of water to a test tube and capping. The sample was incubated for 90 min at 40°C. The sample was then diluted by placing 0.1 ml of hydrolyzed urine, 0.2 ml of hydrochloric acid and 0.75 ml of acetonitrile in a sample vial. After vortexing and centrifugation, the sample was injected on the HPLC system.

RESULTS AND DISCUSSION

An HPLC method using fluorescence detection has been developed for the concurrent determination of compounds 1 and 2 in plasma and urine. Owing to the natural fluorescing properties of structure I (see Fig. 1), the method was found to be more sensitive and selective than using UV detection at 254 nm [3]. Under the chromatographic conditions utilized, compound 1 was completely separated from its carboxylic acid metabolite (2), the internal standard (3) and other structurally related analogues (compounds 4—7) (Table I and Fig. 2A). For 1 and 2, the detection limit was 5 ng/ml in plasma and 1.0 μg/ml in urine.

Representative chromatograms of control and subject plasma and urine samples are shown in Fig. 2. No interfering peaks at the retention times of compounds 1, 2 or 3 were seen in control samples of plasma and urine.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF COMPOUNDS 1—7 UNDER HPLC CONDITIONS

Compound	Retention time (min)
1	4.1
2	3.6
3	4.8
4	7.2
5	2.8
6	NF*
7	NF

*NF = no fluorescence at 100 μg/ml.

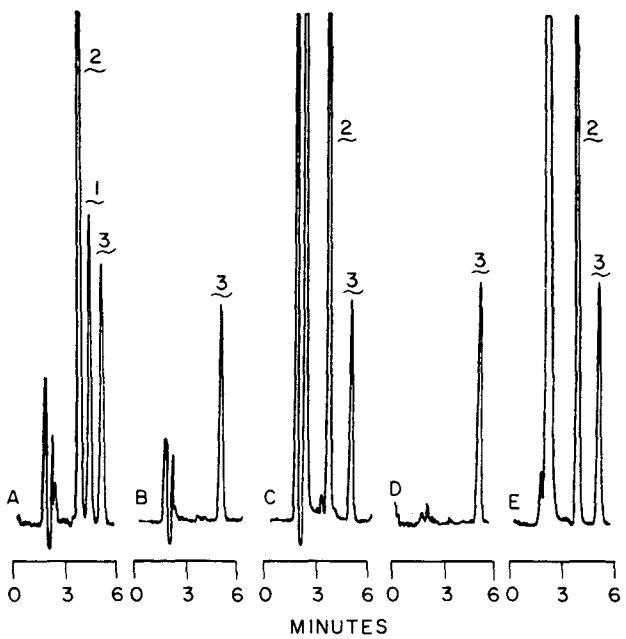

Fig. 2. Representative chromatograms of (A) standards; (B) predose plasma spiked with 0.1 ml of 10 μg/ml internal standard; (C) plasma collected after administration of compound 1 to human subject; (D) predose urine spiked with 0.1 ml of 100 μg/ml internal standard; and (E) urine collected after administration of compound 1 to human subject.

Drug-related peaks observed in high-dose urine were also separable from compound 2 under the conditions described. However, these peaks interfered with 2 when samples were analyzed under fast HPLC conditions using a Regis octadecylsilane, 3-μm, 3 cm × 4.6 mm Little Champ column (Morton Grove, IL, U.S.A.) and a mobile phase of 0.85% phosphoric acid (pH = 4.5)—acetonitrile (70:30).

Since compound 1 was not identified in any samples analyzed, 2 could feasibly be moved from interfering peaks and closer to the internal standard by altering the pH of the mobile phase. This would make the fast HPLC method suitable for analysis of samples, thus reducing the run time by 50%. Future work may include validation of this fast HPLC method.

Plasma was extracted using a Bond-Elut cartridge with octadecylsilane packing. Extraction with cartridges was found to be more efficient than protein precipitation methods. The small precipitate particles not removed by centrifugation required microfiltration of samples. This step was found to be more time-consuming than sample extraction with cartridges. Recovery using extraction cartridges was greater than 90% for compounds 1 and 2 spiked in control plasma and was comparable to that observed using protein precipitation methods.

Methods for determining free 1 and 2 in plasma and urine were evaluated for intra-day and inter-day variation. Standard curves in plasma ranged from 5 to 500 ng/ml. Coefficients of variation were less than 4% for each point of the curve. The inter-day correlation (n = 6) calculated using linear regression was r^2 = 0.9999 for both 1 and 2. In urine, standard curves ranged from 1 to 500 μg/ml with an inter-day correlation (n = 6) of r^2 = 1.0000 for both 1 and 2. Coefficients of variation (n = 6) were less than 3.5% for every value on the standard curve.

Quality control samples were prepared at the high and low ends of all standard curves. In plasma and urine, coefficients of variation (n = 6) were less than 2.7% for quality control samples. Analysis of quality control samples over a period of one month showed compounds 1 and 2 to be stable when stored at −15°C in plasma or urine.

Retention times of unidentified drug-related peaks observed in chromatograms of urine from high-dose treatments did not match with retention times of standards 4—7 (Table I). Glusulase, an enzyme preparation specific for hydrolysis of β-glucuronide and sulfate conjugates, was used to evaluate β-

TABLE II

URINE RECOVERY (mg) OF COMPOUND 2 FOLLOWING NO, GLUSULASE, OR BASE HYDROLYSIS

Dose (mg)	Subject	No hydrolysis	Glusulase hydrolysis	Base hydrolysis
500	No. 7	51.14	219.66	383.80
1000	No. 12	77.13	190.62	454.98
Low quality control		47.71	46.89	47.97
High quality control		256.18	248.71	253.28

TABLE III

PLASMA CONCENTRATION AND URINE RECOVERY OF COMPOUND 2 FOLLOWING SINGLE DOSES OF COMPOUND 1

Dose (mg)	Subject No.	Plasma concentration (μg/ml)					Urine recovery (mg)					
		Time (h)					Time interval (h)					
		0	1	4	10	24	0	0—4	4—10	10—24	24—48	Total
250	3	0	0.41	0.10	0.11	0.05	0	12.48	1.52	2.33	NC*	16.33
500	1	0	0.17	0.61	0.14	0.11	0	19.57	12.98	5.01	NC	37.56
1000	9	0	2.48	0.29	0.22	0.32	0	5.32	11.97	41.50	34.06	92.85
2000	16	0	4.11	0.65	0.57	0.46	0	86.77	17.85	27.65	73.12	205.39

*NC = samples were not collected.

glucuronide conjugation. Sodium hydroxide was used to evaluate base-hydrolyzable conjugates including β-glucuronide.

Both chemical and enzymatic hydrolysis of subject urine resulted in an increase of compound 2 (Table II). Treatment of quality control samples under each of these conditions resulted in no change in concentration of 2. The increase in 2 observed following Glusulase treatment is assumed to be due to hydrolysis of the β-glucuronide conjugate since sulfate conjugation is improbable. Base hydrolysis resulted in a greater increase in 2 than Glusulase hydrolysis and complete removal of drug-related peaks observed near the solvent front. This suggests that base-hydrolyzable metabolites in addition to the β-glucuronide exist. Further work is needed to identify and characterize these metabolites.

The methods developed for analysis of compounds 1 and 2 in plasma and urine have been shown to be reproducible and reliable. Table III shows levels of 2 in plasma and urine from selected subjects participating in an active rising oral dose study of 1.

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Note

Determination of piperine in biological tissues by thin-layer chromatography and ultraviolet absorption densitometry

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Piperine is the active principle of black pepper (*Piper nigrum* L.), which is one of the most commonly used spices and which is valued for its pungency and aroma. Methods available for the determination of piperine in pepper have been reviewed recently [1, 2]. These are based on: (1) formation of colour with chromotropic acid [3], (2) spectrophotometry [4, 5], (3) estimation of nitrogen and multiplying the nitrogen content by the appropriate factor [6], and (4) the colorimetric determination of piperidine after alkaline hydrolysis of piperine [7]. Examination of the above methods revealed that they were not suitable for tissues because of high blanks and/or low sensitivity. As a part of metabolic studies on black pepper and piperine in progress in this laboratory, it became necessary to standardize a method for the determination of piperine in biological tissues. In the suggested procedure, the tissue extract is subjected to thin-layer chromatography (TLC) in 35% acetone in petroleum ether (60—80°C) by which piperine is separated from interfering material. It is then quantitated by UV absorption densitometry.

EXPERIMENTAL

Materials

Piperine (Sigma, St. Louis, MO, U.S.A.) was used as the authentic sample at a concentration of 2 mg/ml in chloroform. Solvents were distilled before use. Rat serum, liver, stomach, small intestine, caecum and large intestine were used as important representative tissues for spiking with piperine.

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Animals

Male albino rats of Wistar strain (120—140 g body weight) were maintained on a 18% casein diet [8] for two weeks. After an overnight fast, but with no restriction on water, six rats were administered, by gavage, 15 mg per 100 g body weight of piperine in 1.0 ml of refined peanut oil. Another six rats were administered 1.0 ml of oil only and served as controls. Rats were sacrificed 2 h later under ether anaesthesia; the gastrointestinal tract was removed and the contents were thoroughly washed out with 0.9% sodium chloride.

Extraction

Various concentrations of piperine were added to 1 g of each tissue, homogenized and then extracted according to the method of Folch et al. [9]. Portions of 1 g of the intestinal tissue from control and piperine-administered rats were similarly extracted. All tubes were covered with black paper since piperine in solution isomerizes to isopiperine, chavicine and isochavicine on exposure to light [10]. Each extract was made up to 20 ml, concentrated by flash evaporation under nitrogen, and suitable aliquots were taken for piperine determination. Total lipid in the extracts was determined by gravimetry.

Spectrophotometric method

Extracts were suitably diluted with chloroform—methanol (2:1) and covered with black paper. Estimation of piperine in extracts was made as described by Fagen et al. [4], by determining the absorbance at 345 nm.

Thin-layer chromatography

TLC plates were prepared by coating 20 × 20 cm glass plates with a 0.3-mm layer of silica gel G (Glaxo Labs., Bombay, India) using a Camag automatic TLC coater. The plates were air-dried and activated at 100°C for 1 h before use. Authentic piperine and different tissue extracts containing piperine were applied to the TLC plates and run in a solvent system of petroleum ether (60—80°C)—acetone (65:35, v/v) in the dark at 24 ± 1°C. After the plates were air-dried, the piperine spots were visulaized under UV light and R_F values were determined.

UV absorption densitometry

The chromatoplates were scanned in the direction of solvent development using an automatic Camag TLC scanner Model 2, mounted on a fluorometer Model III (Turner Assoc.), attached to a W + W Recorder 1100 (Scientific Instruments, Switzerland). Scanning conditions were: lamp, No. 110-850 (emission 310—390 nm); primary filter, 110-811 (can pass 365 nm); secondary filter, 110-823 (10% neutral density); plate speed 2 cm/min; chart speed, 1 cm/min. Quantitation was by calculation of areas after triangulation.

RESULTS AND DISCUSSION

Chromatography

Attempts at quantitative determination of piperine in tissue extracts using available methods were not successful because of high blanks and low sensitivi-

ty in the chromotropic—sulphuric acid [3] and phosphoric acid [11] procedures and low recovery of added piperine in the spectrophotometric procedure [4]. TLC followed by chromotropic—sulphuric acid spray followed by densitometry as described by Gunner [12] was also not suitable because of the coloured background of lipid spots. In order to eliminate interference from tissue components, TLC separation of piperine was resorted to. TLC on silica gel G using the mobile phase 35% acetone in petroleum ether (60—80°C) yielded the best separation of piperine with an R_F value of 0.60 ± 0.009 (S.E.M., n = 15). Quantitation was by UV absorption densitometry.

Sensitivity, linearity and reproducibility

Linear calibration curves were obtained when the concentration of piperine spotted was in the 1—4 μg range. The reproducibility was evaluated by making a series of eight chromtograms of a given concentration of piperine and determining the relative standard deviation of peak areas. The relative standard deviation ranged from 2.0% to 7.3% for different concentrations of piperine (Table I).

TABLE I

LINEARITY, SENSITIVITY AND REPRODUCIBILITY OF THE SUGGESTED METHOD

	Peak area (cm^2) corresponding to piperine			
	1 μg	2 μg	3 μg	4 μg
1	2.88	5.20	7.45	9.94
2	2.56	4.95	7.50	9.31
3	2.76	4.86	7.30	9.99
4	2.48	4.95	6.00	9.80
5	2.80	5.01	7.20	9.00
6	2.60	5.01	7.44	9.84
7	2.52	5.00	7.68	9.95
8	2.48	5.00	7.48	—
Average	2.64	5.00	7.26	9.69
S.E.M.	±0.055	±0.034	±0.186	±0.144
Coefficient of variation (%)	5.9	2.0	7.36	3.98

Non-interference by high lipid content

Fig. 1 represents the densitometric scans of chromatograms of pure piperine, tissue extract containing piperine (piperine administered and spiked) and a control tissue extract. Under these experimental conditions, the intestinal tissue contained 45 ± 12 μg/g piperine.

Data on the effect of adding different amounts of tissue lipids to known amounts of piperine are given in Table II for the spectrophotometric method as well as for the TLC—UV absorption densitometric method. From the above it is seen that tissue lipids did not interfere in the suggested procedure even when their concentration was 60 times that of piperine.

In all tissues except the caecum, the proposed method was comparable or

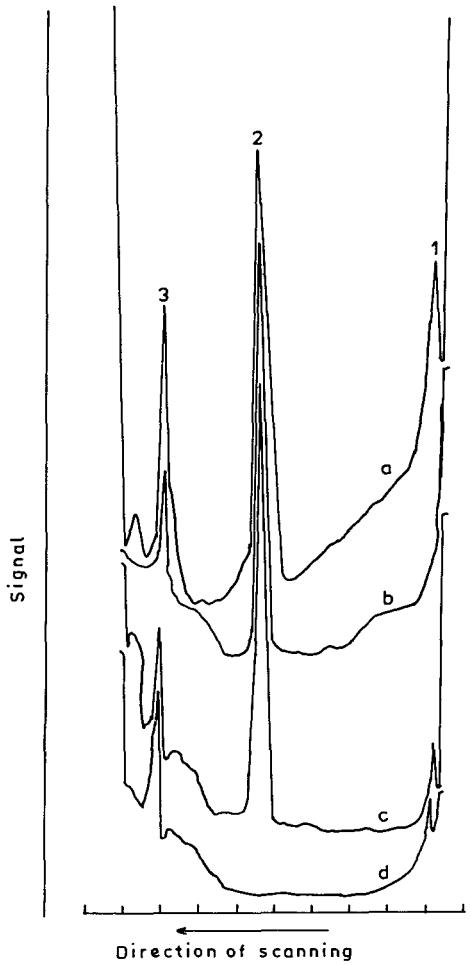

Fig. 1. Densitometric scans of chromatoplates spotted with (a) tissue extract (small intestine) containing piperine 2 h after administration, (b) piperine, (c) tissue extract spiked with piperine, and (d) control tissue extract. Peaks: 1 = origin, 2 = piperine, 3 = solvent front.

TABLE II

NON-INTERFERENCE BY TISSUE LIPIDS IN THE DETERMINATION OF PIPERINE

Tissue	Lipid/ piperine	Recovery* (%)	
		Spectrophotometric method	Suggested method
Serum	3	97	99
Liver	27	149	99
Stomach	23	92	99
Small intestine	58	90	97
Caecum	34	98	96
Large intestine	43	93	95

*Average of duplicates.

superior to the spectrophotometric method. The interference by non-piperine-absorbing materials in the spectrophotometric procedure is clearly seen in the case of liver. Since, in metabolic studies, it would not be possible to have a tissue blank, the suitability of the proposed method is evident.

Recovery

Known amounts of piperine were added to tissues which were then extracted by the method of Folch et al. [9]. These extracts were then taken through the procedure for piperine determination as mentioned above. As indicated in Table III, the recovery of added piperine was in the range 95—100%.

The method worked out has the advantage that the chromatoplate may be scanned directly, is more sensitive than other published procedures [3, 4, 7, 11, 12] and 0.01 μmol can be detected per gram tissue.

TABLE III

RECOVERY OF PIPERINE ADDED TO DIFFERENT TISSUES

Piperine added (mg)	Recovery (%) in tissues*					
	Serum	Liver	Stomach	Small intestine	Caecum	Large intestine
0.5	100	97	97	99	100	99
1.0	99	99	99	97	96	95
2.0	100	98	100	98	100	96
5.0	100	98	97	100	98	95

*Average of duplicates.

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