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THE USE OF GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC–COMPUTER SYSTEMS IN PHARMACOKINETIC STUDIES

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SUMMARY

Pharmacokinetic studies involving plasma, urine, breast milk, saliva and liver homogenates have been carried out by selective ion detection with a gas chromatographic–mass spectrometric–computer system operated in the chemical ionization mode. Stable isotope labeled drugs were used as internal standards for quantification. The half-lives, the concentration at zero time, the slope (regression coefficient), the maximum velocity of the reaction and the apparent Michaelis constant of the reaction were determined by regression analysis, and also by graphic means.

INTRODUCTION

Earlier studies from our laboratory have described methods for measuring the concentration of drugs in plasma, urine, breast milk and amniotic fluid[1–4]. Measuring the concentration of drugs in a single sample, however, provides very little information compared to that obtained from multiple samples. With a series of timed samples or timed-paired samples (for example, plasma and breast milk), it is possible to obtain information on the binding of drugs to plasma proteins, the biological half-life of the drugs, the plasma clearance rate, and the length of time various drugs remain in the body.

Spectrophotometric methods are usually employed for the determination of these pharmacokinetic parameters but analytical procedures based on selective ion detection (SID) with a gas chromatographic–mass spectrometric–computer (GC–MS–COM) system can also be used[5,6]. When SID is used for quantification the absolute intensities of one or more characteristic ions in the mass spectrum are recorded and compared to the absolute intensity of the internal standard. This analytical approach was used initially with magnetic instruments operated in the electron impact mode[7,8]. However, chemical ionization (CI) offers several advantages, and in our studies a quadrupole GC–MS–COM system operated in the CI mode is employed for most of the quantitative analyses.

In order to evaluate the usefulness of GC–MS–COM systems in pharmacokinetic studies, we have carried out analyses of urine, plasma, breast milk and saliva. All of the biological samples were collected after oral administration of the drugs. In

addition, the methods have been applied to *in vitro* studies of drug metabolism using liver homogenates.

EXPERIMENTAL

Kinetic studies of drug metabolism *in vitro* were performed using a 10,000 *g* liver homogenate supernatant fraction and an NADPH (nicotinamide–adenine dinucleotide phosphate, reduced) generating system. The supernatant fraction was prepared from freshly excised livers dispersed in three volumes of a 1.15% potassium chloride +0.05 *M* potassium phosphate, pH 7.4 solution. The drug incubation system consisted of 2 ml of the 10,000 *g* supernatant fraction, 25 m*M* NADP (nicotinamide–adenine dinucleotide phosphate, oxidized), 45 m*M* glucose-6-phosphate, 20 m*M* magnesium chloride, 0–15 units of glucose-6-phosphate dehydrogenase, and 0.05 *M* potassium phosphate, pH 7.4 to a final volume of 3 ml. Metabolism was initiated by addition of 1–50 μg of drug, followed by oxygenation and incubation at 37 °C. Reactions were terminated at appropriate time intervals by the addition of ammonium carbonate and ethyl acetate.

The drugs and their metabolites were extracted from plasma, urine, breast milk, saliva and tissue homogenates using ammonium carbonate–ethyl acetate as the salt–solvent pair[1]. The usual sample size was 0.1–1.0 ml of plasma, saliva or breast milk, 1–5 ml of urine and 3 ml of homogenate. Stable isotope labeled drugs were added as internal standards to the biological samples before isolation was begun. Both carbon-13 and deuterium labeled standards were used and included 2,4,5-^{13}C-phenobarbital, 2,4,5-^{13}C-diphenylhydantoin, 2,4,5-^{13}C-pentobarbital, N-C^{2}H$_3$-antipyrine and N-C^{2}H$_3$-caffeine. The isolated drugs and their metabolites were converted to derivatives for analysis; methylated, ethylated and silylated derivatives were prepared[2]. An aliquot, usually 1–2 μl out of a final volume of 25 μl, was analyzed by the GC–MS–COM system.

Analyses were carried out by SID using a GC–MS–COM (Finnigan 1015-PDP 8/I) system operated in the CI mode. Glass coil columns (9 ft. × 2 mm) packed with either 1% SE-30 on 80–100 mesh Gas-Chrom Q or 3% PZ-176 (ref. 9) on 80–100 mesh Gas-Chrom Q were used. Methane was used as the carrier gas and the GC separations were carried out isothermally or by temperature programming. Calculations of concentrations were performed automatically by the computer program[2] and were based on either peak height or peak area after determining which type of measurement provided the more precise results.

For the calculation of the pharmacokinetic parameters, regression analysis of the concentrations of drugs in the timed biological samples was used to obtain the concentration at zero time (C_0; y intercept) and the slope (regression coefficient). The RASS (remote access statistical service) program was used for the calculation of C_0 and the slope, and the RASSER program for plotting the data. The half-lives ($t_{1/2}$) were calculated using eqn. 1.

$$t_{1/2} = \frac{0.693}{k} \tag{1}$$

where k is the regression coefficient.

The apparent volume of distribution V_{D_a}, the elimination constant K_{el} and plasma clearance of the drug were calculated using eqns. 2–4 (ref. 10).

$$V_{Da} = \frac{\text{dose of drug}}{\text{conc. at zero time}} = \frac{D\ (\text{mg})}{C_0\ (\mu\text{g/ml})}\ (\text{liters}) \tag{2}$$

$$K_{el} = \frac{0.693}{t_{1/2}\ (\text{h})} \tag{3}$$

$$\text{Plasma clearance} = V_{Da} \times K_{el}\ (\text{liters/h}) \tag{4}$$

Kinetic parameters for *in vitro* drug metabolism were determined from graphic analysis of initial velocity calculations and Lineweaver–Burk reciprocal values[11]. The RASS program was used for regression analysis ($V_{max.}$, intercept; K_m, slope or regression coefficient) and the RASSER program for graphic display.

RESULTS AND DISCUSSION

Both *in vivo* and *in vitro* kinetic studies of drug metabolism have been carried out by SID with a GC–MS–COM system operated in the CI mode. For the *in vitro* studies homogenates prepared from rat liver were employed; the rates of disappearance of drugs and the appearance of metabolites during incubation were followed. In the *in vivo* studies the clearance of drugs from plasma, urine, saliva and breast milk was followed in human subjects. Although the *in vivo* and *in vitro* studies appear to be quite different, the same analytical procedures including isolation, quantification with a GC–MS–COM system, and computer analysis of data were employed in both investigations.

In the *in vivo* studies the half-lives of several drugs were determined following oral ingestion of one or more drugs. The half-lives were obtained either by plotting the logarithm of the concentrations of the drug against time using semilogarithmic paper or by regression analysis of the data to obtain C_0 (y intercept) and the slope (regression coefficient). The calculations were based on the assumption that single compartment kinetics was involved.

Some of the results obtained from the analysis of plasma following oral administration of caffeine, mephobarbital and antipyrine are shown in Figs. 1–3.

The elimination of caffeine from plasma was followed in an adult subject who had consumed 1420 ml of coffee over a period of 4.5 h (Fig. 1). The ions at m/e 195 (MH^+, caffeine), 209 (MH^+, ethylated theobromine), and 198 (MH^+, N-C^2H_3-caffeine, the internal standard) were monitored by SID.

The total amount of caffeine and theobromine ingested was 730 mg and 3.3 mg respectively. The half-life of caffeine obtained by regression analysis was 4.6 h. This value is in agreement with the half-life of 3.5–10.5 h reported by Grab and Reinstein[12] for the oral administration of caffeine. Theobromine is a major metabolite of caffeine and the elimination of theobromine was also followed. From Fig. 1 it is evident that during the first 2.5 h, theobromine is being formed from caffeine more rapidly than it is being cleared from plasma and that theobromine is eliminated from plasma more slowly than caffeine in this subject. The apparent half-life of theobromine, using only three points for the regression analysis, was 13 h. The ratio of caffeine to theobromine in coffee was approximately 200:1 while the ratio in plasma varied from

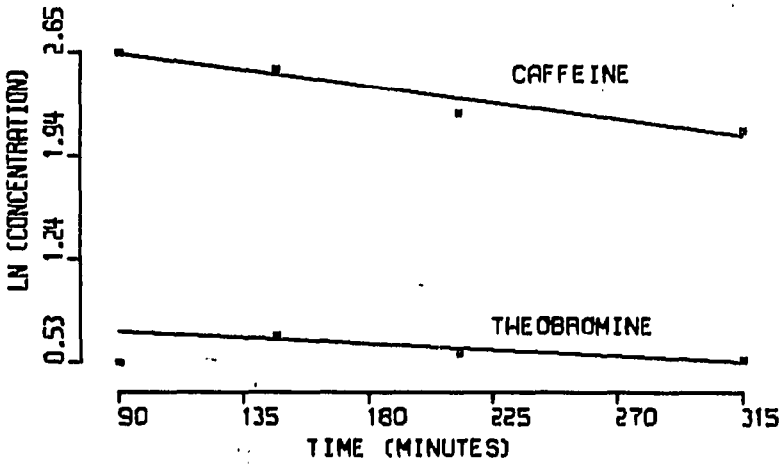

Fig. 1. Comparison of the caffeine and theobromine levels in plasma. The analyses were carried out by SID using ammonium carbonate–ethyl acetate extracts of 100 μl of plasma. The ions at *m/e* 195 (MH^+, caffeine), 209 (MH^+, ethylated theobromine) and 198 (MH^+, N-C^2H_3-caffeine) were monitored. The curves were obtained by regression analysis and were plotted by the computer. Half-lives: caffeine, 4.6 h; theobromine, 13 h.

8:1 to 5:1, indicating that theobromine in coffee was not the sole source of theobromine and that theobromine was being formed from caffeine.

Antipyrine is frequently used in pharmacokinetic studies because the drug is rapidly absorbed after oral administration. Only 10% of the drug is bound to plasma protein[13] and clearance of the drug from plasma corresponds to metabolism by the liver.

The clearance of antipyrine from plasma was followed in several young healthy

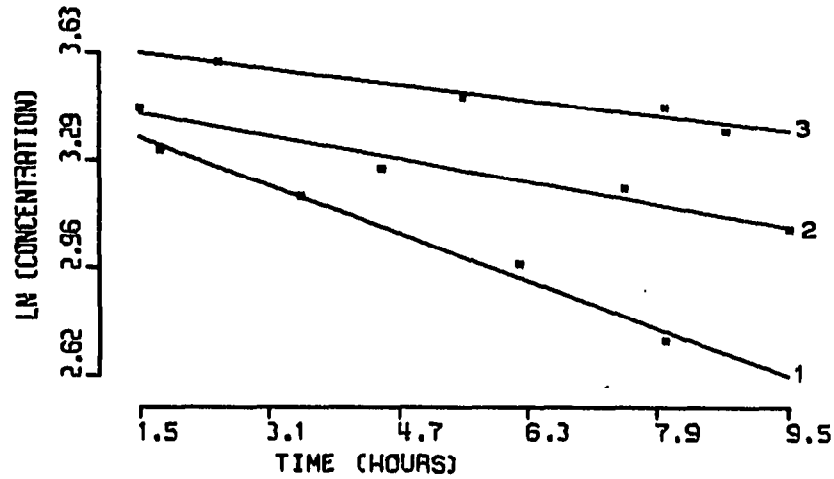

Fig. 2. Comparison of the analyses of ammonium carbonate–ethyl acetate extracts of 100 μl samples of plasma in three subjects by SID. The ions at *m/e* 189 (MH^+, antipyrine) and 192 (MH^+, N-C^2H_3-antipyrine) were monitored. The curves were obtained by regression analysis and were plotted by the computer. Antipyrine half-lives: 1, 7.5 h; 2, 15.6 h; 3, 22.2 h.

TABLE I

CALCULATED VALUES FOR THE APPARENT VOLUME OF DISTRIBUTION, THE ELIMINATION CONSTANT AND THE PLASMA CLEARANCE RATE

Subject	*Dose (mg)*	$t_{1/2}$ *(h)*	C_0 *(μg/ml)*	V_{Da} *(l)*	K_{el}	*Plasma clearance (l/h)*
A	1224	7.5	33.1	37.0	0.092	3.4
B	1260	15.6	33.2	38.0	0.044	1.67
C	1008	22.2	39.4	25.6	0.031	0.79

subjects after an oral dose of 18 mg/kg. The ions at *m/e* 189 (MH^+, antipyrine) and 192 (MH^+, N-C^2H_3-antipyrine, the internal standard) were monitored. The half-lives varied from 6–28 h. In Fig. 2, curves are shown for three of the subjects. The half-lives calculated by regression analysis were 7.5, 15.6 and 22.2 h. The half-life of antipyrine, reported by Vesell and Page[14] was 10–15 h following a single oral dose of 18 mg/kg. The apparent volume of distribution (V_{Da}), the elimination constant (K_{el}) and the plasma clearance rate were calculated[10] for the three individuals using the data obtained during the log–linear phase of elimination from plasma. The results are listed in Table I. Single compartment kinetics was assumed.

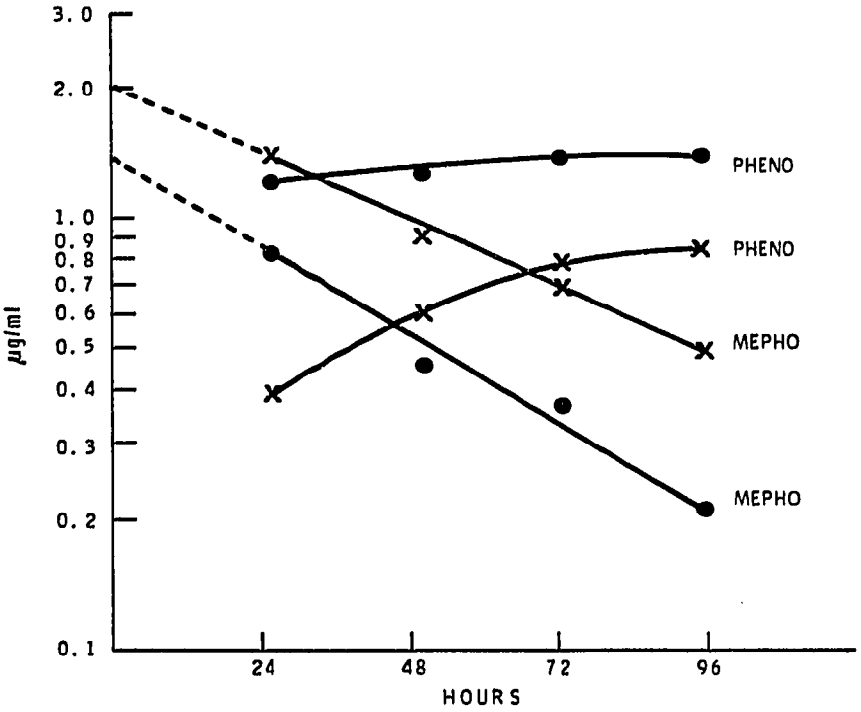

Fig. 3. Comparison of the mephobarbital and phenobarbital levels in plasma of two subjects (×—× and ●—●). The analyses were carried out as described in Fig. 1. The ions at *m/e* 275 (MH^+, N-ethyl-mephobarbital), 289 (N,N-diethylphenobarbital) and 292 (N,N-diethyl-2,4,5-^{13}C-phenobarbital) were monitored. The results were plotted on semi-logarithmic paper and the mephobarbital half-lives, obtained by regression analysis, were 46.5 h (×—×) and 37.6 h (●—●).

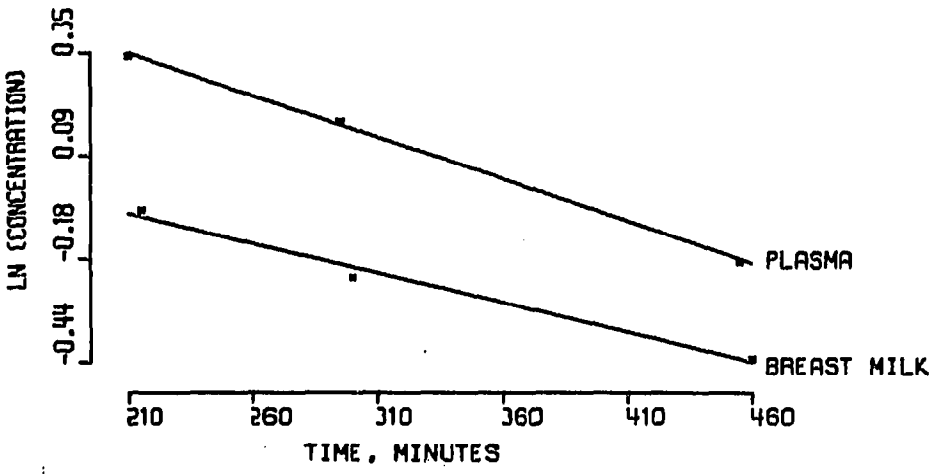

Fig. 4. Comparison of the analyses of ammonium carbonate-ethyl acetate extracts of 200 μl samples of plasma and 500 μl samples of breast milk by SID. The ions at *m/e* 267 (N-methyldiphenylhydantoin) and 270 (N-methyl-2,4,5-^{13}C-diphenylhydantoin) were monitored. The curves were obtained by regression analysis and were plotted by the computer. Diphenylhydantoin half-lives: plasma, 5 h 19 min; breast milk, 7 h 36 min.

These analytical procedures can also be used to follow the rates of formation of metabolites as well as the clearance of the parent drug from biological fluids. An example is shown in Fig. 3; mephobarbital and its metabolite phenobarbital were quantified by monitoring the ions at *m/e* 275 (MH^+, N-ethylmephobarbital), 289 (MH^+, N,N-diethylphenobarbital) and 292 (MH^+, N,N-diethyl-2,4,5-^{13}C-phenobarbital, the internal standard). The half-lives of mephobarbital, determined for two individuals who had ingested 300 mg of the drug were 37.6 and 46.5 h. The concentration of phenobarbital, the major metabolite of mephobarbital, was also measured in plasma. From the data (Fig. 3) it was not possible to calculate the half-life of phenobarbital, but a half-life of 3.5 days has been reported[15]. During the period studied, phenobarbital was being formed from mephobarbital more rapidly than it was being cleared from plasma; that is, the half-life of phenobarbital was longer than the half-life of mephobarbital. The plasma concentration of phenobarbital is determined by the rate of metabolism (hydroxylation and conjugation) and the rate of elimination of phenobarbital and its metabolites in urine as well as by the rate of formation from mephobarbital.

A study of the half-life of drugs in human breast milk was also undertaken since studies carried out in our laboratory have confirmed that drugs ingested by the lactating female are transferred to breast milk. About twenty drugs have been investigated and all were detected in breast milk as well as in plasma[3]. Timed samples of plasma and breast milk were obtained from several mothers who had ingested a single dose of a drug after breast feeding of their infants had terminated. An analysis of paired samples of breast milk and plasma is shown in Fig. 4. The first samples were collected 3.5 h after oral administration of 100 mg of diphenylhydantoin because the drug is absorbed rather slowly from the digestive tract. The ions monitored were at *m/e* 267 (MH^+, N-methyl-diphenylhydantoin) and 270 (MH^+, N-methyl-2,4,5-

^{13}C-diphenylhydantoin, the internal standard). The half-life of diphenylhydantoin, obtained by regression analysis, was longer (7 h 36 min) in breast milk than in plasma (5 h 19 min). This is shorter than the reported half-life[16] of 9 h. In similar studies, it was found that the half-lives of secobarbital and acetaminophen were also significantly longer in breast milk than plasma. Additional studies are being carried out to ascertain if the half-lives of drugs are generally longer in breast milk than in plasma for most women. The longer half-lives may be due to a slower rate of metabolism in breast tissue or to binding of the drug to some component (lipid) in breast tissue.

If plasma samples are not available, or if the plasma concentrations are very low, the biological half-life of drugs can be determined by measuring the concentrations in timed urine samples. Results obtained with urine samples are based on the assumption that the renal elimination rate is proportional to the plasma concentration.

Urine samples were obtained from a newborn infant whose mother (an epileptic) had ingested mephobarbital daily throughout the gestational period. The excretion of mephobarbital and its metabolite, phenobarbital, was followed in neonatal urine for 22 days (Fig. 5). The excretion of creatinine (μg/ml) was essentially constant during this period. The infant had not been breast fed, and the drugs excreted in urine after birth had been acquired by the fetus by placental transfer from the maternal circulation.

The urine collections were timed and the average excretion (μg/h) was deter-

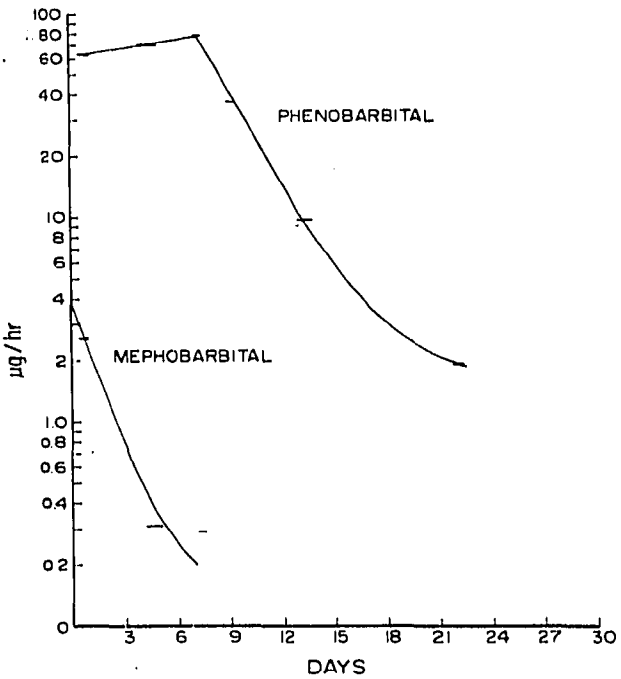

Fig. 5. Comparison of the mephobarbital and phenobarbital levels in neonatal urine. The analyses including the ions monitored were carried out as described for Fig. 3; 1–5 ml of urine were used. The results were plotted on semi-logarithmic paper and the half-lives were: phenobarbital, 48 h; mephobarbital, 30 h.

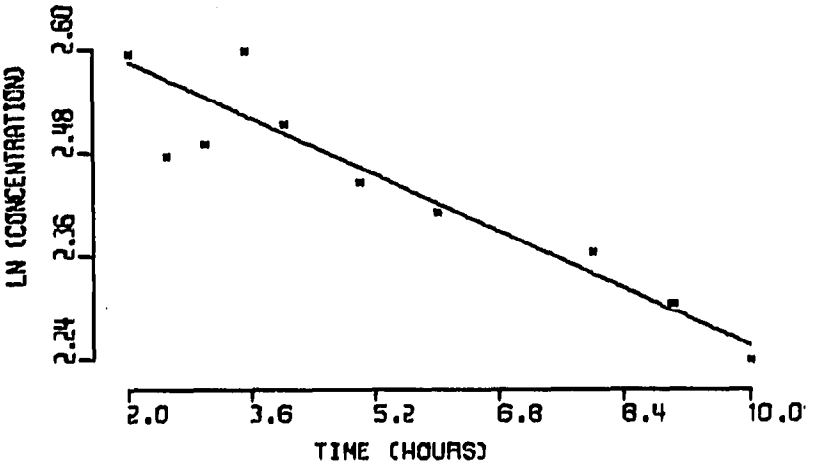

Fig. 6. Analysis of antipyrine levels in saliva by SID using ammonium carbonate–ethyl acetate extracts of 100 μl of saliva. The ions at *m/e* 189 (MH^+, antipyrine) and 192 (MH^+, N-C^2H_3-antipyrine) were monitored. The curve was obtained by regression analysis and plotted by the computer. Half-life: 16.7 h.

mined for each sample and plotted against time (days) on semilogarithmic paper. The excretion of the metabolite, phenobarbital (expressed as μg/h), increased as long as mephobarbital was present in urine. When mephobarbital could no longer be detected (day 10), the concentration of phenobarbital in urine began to fall. The half-life of mephobarbital was approximately 30 h (compare with Fig. 3). Unfortunately, mephobarbital was detected in only three urine samples, and as a result, the half-life can only be estimated. The half-life of phenobarbital from day 8 to day 13 was 48 h. Between day 13 and day 22, the rate of clearance changed, and a half-life of 6–7 days can be projected if the curve is extended. This infant exhibited withdrawal symptoms for 5–6 months after birth.

It is also possible to determine the half-life of many drugs by measuring the concentration of the drugs in saliva[17–19]. There are several advantages in the use of saliva. In order to carry out a complete pharmacokinetic study following oral administration of a drug, it may be necessary to draw 15–20 samples of blood. In most investigations, it is not possible to collect such a large number of samples of blood. However, the collection of saliva is non-invasive and multiple timed samples can be obtained without difficulty. Furthermore, the concentration of the drug in saliva is reported to correspond to the level of unbound drug in plasma[19].

An example is shown in Fig. 6. Seventeen saliva samples were collected over a 10-h period from a subject who had ingested 600 mg of antipyrine. The concentration in saliva reached a maximum 30 min after ingestion. A rapid elimination of the drug from saliva was observed from 30–120 min and this was followed by a much slower rate of elimination over the following 8 h.

The elimination curve suggests that the drug was rapidly transferred from plasma to a tissue compartment during the period of rapid clearance (30–120 min) and that the slower phase (2–10 h) represents the elimination of the drug from plasma. The half-life during this second phase, obtained by regression analysis, was 16.7 h

when the last six values (4–10 h) were used and 17.9 h when ten values (2–10 h) were used.

Two plasma samples were obtained and the concentrations of antipyrine in plasma and saliva were compared. The levels in plasma (15.5 μg/ml) were slightly higher than those in saliva (13.4 μg/ml), indicating that about 10–15% of the drug is bound to protein. This value agrees with that reported by Soberman *et al.*[13].

In the *in vitro* studies using rat liver homogenates, the kinetic parameters of secobarbital metabolism were determined. These studies were carried out using very small quantities (1.5–50 μg) of the drug. Although analyses using GC–MS–COM procedures require considerably more time to carry out than spectrophotometric assays, direct measurements of the disappearance of substrate (drug) and appearance of one or more products (metabolites) are obtained since the protonated molecular ions (MH^+) and one or more additional characteristic ions can be monitored. Thus, the specificity of the measurements can be considerably greater than that provided by other procedures in which coupled reactions are employed and the concentration of the substrate is measured indirectly.

The kinetic parameters of secobarbital metabolism *in vitro* were determined by following the disappearance of the ions at *m/e* 267 (MH^+, secobarbital, N,N-dimethyl derivative) and the change in concentration of the ions at *m/e* 355 (MH^+, hydroxysecobarbital, N,N-dimethyl trimethylsilyl derivative). Pentobarbital-2,4,5-^{13}C (N,N-dimethyl derivative) was added as the internal standard and the ions at *m/e* 258 (MH^+) were monitored.

A graphic analysis of (*RS*)-secobarbital (1.5 μg) metabolism is shown in Fig. 7. The disappearance of secobarbital and corresponding appearance of hydroxysecobarbital are linear for the first 2 min of reaction, while disappearance of hydroxysecobarbital is linear for 5 min. The initial velocity of secobarbital metabolism can be calculated from the linear portion of this curve. A family of such initial velocity calculations generated by varying the initial concentration of secobarbital was used to determine the maximum velocity ($V_{max.}$) and apparent Michaelis constant (K_m) of the reaction. A reciprocal plot (velocity *vs.* velocity/secobarbital concentration) is shown in Fig. 8. The plot indicates that $V_{max.}$ (intercept) is 4.75 μg/min and the apparent K_m (−slope) is 19.93.

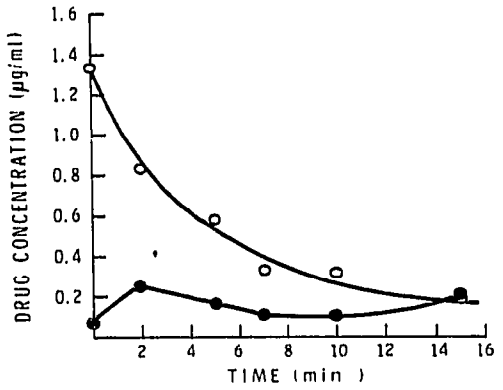

Fig. 7. Graphic analysis of (*RS*)-secobarbital metabolism. Each value was determined from duplicate samples obtained and analyzed as described in the text from two separate experiments. ○—○, (*RS*)-secobarbital disappearance; ●—●, (*RS*)-hydroxysecobarbital dynamics. 1 μg/ml = 3 n*M*.

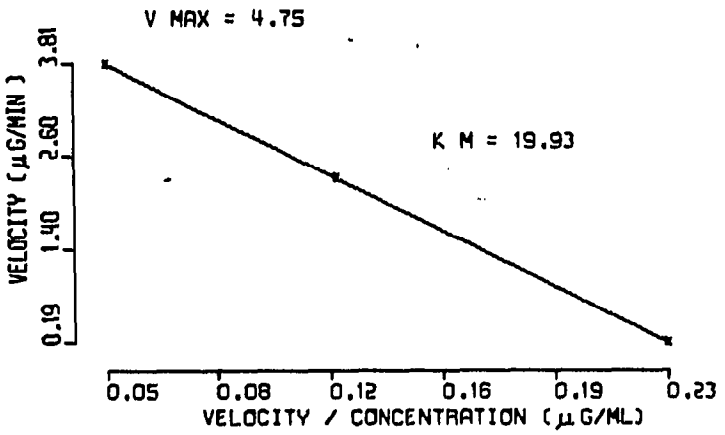

Fig. 8. Lineweaver–Burk reciprocal plot for the determination of kinetic parameters. Initial velocities were calculated from graphic analysis of (*RS*)-secobarbital metabolism at several concentrations of drug. $V_{max.}$ = *y* intercept = 4.75 µg/min; apparent K_m = (−slope), determined by regression analysis = 19.93; 1 µg/ml = 3 n*M*.

Kinetic parameters of metabolites as well as the parent drug can be determined using this method. Since very small quantities of substrate are required, the use of this system is particularly advantageous when studying the kinetics of metabolites which are difficult to synthesize but which can be isolated in very limited quantities from the urine of animals treated with the parent drug. For example, hydroxysecobarbital [5-allyl-5-(3-hydroxy-1-methylbutyl)-barbituric acid] is difficult to synthesize, but 1–2 mg can be readily isolated by thin-layer chromatography from a single 24-h urine collection following administration of 10 mg of secobarbital to a rat. Less than 10% of this amount is required for the *in vitro* incubation mixture. Thus, the $V_{max.}$ and the apparent K_m can be determined for secobarbital metabolites as well as secobarbital. The accumulation of kinetic parameters of a parent drug and multiple metabolites would be useful in determining the rate limiting step in specific drug metabolic pathways.

From the examples cited, it is evident that GC–MS–COM systems can be used for both *in vivo* and *in vitro* kinetic studies. There are several advantages to this system. First, a single analysis with a GC–MS–COM system takes 10–15 min. Secondly, since as many as eight ions can be monitored at the same time, a total of eight drugs and/or metabolites and internal standards can be quantified simultaneously. Finally, because of the specificity and sensitivity of detection, very simple rapid isolation procedures can be employed; for most biological samples no additional purification is required before analysis.

The overall procedure, including isolation, quantification and computer analysis of data, is a general procedure that can be applied to the analysis of a large number of drugs present in biological fluids and tissues. At the present time about thirty drugs are being monitored in our laboratory by this technique. Resulting pharmacokinetic data will be useful in the elucidation of a variety of drug metabolic pathways and mechanisms of drug action.

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