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# COMPARISON OF GAS CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAYS FOR THE DETERMINATION OF DEBRISOQUINE AND ITS 4-HYDROXY METABOLITE IN HUMAN FLUIDS

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## SUMMARY

A comparison of an established gas chromatographic assay for 4-hydroxydebrisoquine and debrisoquine and a modified high-performance liquid chromatographic assay was made. Both assays used guanoxan as the internal standard and required derivatization of all three compounds with acetylacetone at 96°C for 2.5 h and subsequent ethereal extraction and cleaning steps before chromatographic analysis. For detailed pharmacokinetic studies the gas chromatographic assay was more sensitive in the measurement of low concentrations in plasma, but the liquid chromatographic assay was adequate for phenotyping the 4-hydroxylation of debrisoquine in a population. In the latter assay a mobile phase consisting of 70% methanol in water at pH 3.5 (adjusted with orthophosphoric acid containing 10 m*M* 1-pentanesulphonic acid was employed (flow-rate 1.5 ml/min) with a pre-column ($C_8$) linked to a reversed-phase μBondapak $C_{18}$ cartridge in a Z-module. The eluate was detected at 248 nm. With this assay it was observed that the buccal absorption of debrisoquine and 4-hydroxydebrisoquine was affected by the pH of the buccal medium. This indicates that urinary pH may influence the excretion of both substances at high pH. The debrisoquine-to-4-hydroxydebrisoquine ratio may be dose-dependent.

## INTRODUCTION

Debrisoquine (D), 3,4-dihydro-2-(1*H*)-isoquinoline-carboxamidine, was first introduced in 1966 as an orally active antihypertensive agent [1]. It is seldom used nowadays because newer and more efficient antihypertensives, such as the β-adrenergic receptor blockers and calcium antagonists, are preferred. Studies have shown that the metabolism of D is under genetic control [2] and that the responsiveness to the drug is influenced by metabolic status [3]. Thus, subjects who poorly metabolize the drug are more sensitive to the hypotensive effects than

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those who are extensive metabolizers. More importantly, it is now established that the hydroxylation of D to 4-hydroxydebrisoquine (4OHD) can be used for the study of polymorphic oxidation in various populations. Gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods have been reported [4,5] for the measurement of D and 4OHD, using guanoxan (G) as the internal standard, in plasma and urine. We are interested to investigate the possible existence of D hydroxylation phenotypes in Chinese people. For this purpose we compared the two procedures, and subsequently refined an HPLC assay based on in situ derivatization with acetylacetone before analysis of D and 4OHD in plasma, saliva and urine samples. Simple extraction of 4OHD and G has been unsatisfactory.

## EXPERIMENTAL

### *Reagents and apparatus*

Declinax (10-mg tablets), debrisoquine hemisulphate and 4-hydroxydebrisoquine hemisulphate were gifts from Roche (Welwyn Garden City, U.K.). Guanoxan hemisulphate was obtained from Pfizer (Sandwich, (U.K.). 1-Pentanesulphonic acid sodium salt was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile (HPLC grade), orthophosphoric acid, diethyl ether, acetylacetone and methanol (all of analytical grade) were obtained from Merck (Darmstadt, F.R.G.). The following glassware was used: 15-ml capacity centrifuge tubes with well fitted screw caps containing PTFE lining, 15-ml capacity Quick-fit glass tubes with tapered base of 50 $\mu$l. All glassware was cleaned and silanized with 3% hexamethyldisilizane (HMDS) in chloroform before use [6]. Stock solutions containing 1 mg/ml D, 4OHD and G were prepared in methanol and stored at 4 °C until use. Standard of drug-free biological fluids (plasma, saliva and urine) were made by dilution of stock solutions. A methanolic solution of G (10 $\mu$l, equivalent to 10 $\mu$g) was added to each sample as internal standard.

### *Gas chromatography*

The apparatus used was a Varian Model 6000 gas chromatograph, equipped with a nitrogen-specific detector. The coiled-glass column used (2.0 m × 2 mm I.D., 6 mm O.D.) was packed with 3% OV-225 on Chromosorb W, 80–100 mesh. The temperature of the oven was maintained at 200 °C, and those of the detector and injector at 220 and 250 °C, respectively. The carrier gas was nitrogen at a flow-rate of 30 ml/min; the flow-rates for hydrogen and air were 4.5 and 175 ml/min, respectively.

### *High-performance liquid chromatography*

The liquid chromatograph consisted of a Waters 6000A pump, a U6K continuous-flow injector with a 25 to 200-$\mu$l loop (Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength Hitachi 220-S UV detector with a chart recorder (Hitachi, Tokyo, Japan). Analyses were performed on a reversed-phase $\mu$Bondapak $C_{18}$ cartridge (100 mm × 8 mm I.D.) in a Z-module (Waters Assoc.), which was linked to a $C_8$ pre-column (Merck). The operating conditions of the HPLC sys-

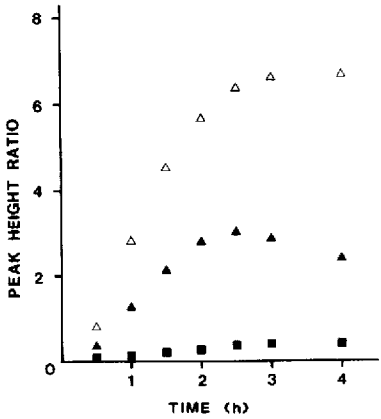


Fig. 1. Effect of duration on the formation of pyrimidine derivatives of D (▲), 4OHD (△) and G (■) at 96°C.

tem were as follows: the mobile phase (consisting of 70% methanol in water at pH 3.5, which was adjusted by orthophosphoric acid, with 10 m*M* 1-pentanesulphonic acid sodium salt) was run at 1.5 ml/min at room temperature (25 ± 1°C). The UV detector was set at 248 nm.

*Analytical procedures*

Derivatization of D, 4OHD and G with acetylacetone was achieved in a shorter period than previously reported [1]. The incubation time for complete condensation of acetylacetone with the guanidino groups of D, 4OHD and G was 2.5 h at 96°C (Fig. 1). Subsequent assays were carried out using these conditions for the in situ derivatization before extraction of the pyrimidine products into organic solvent.

*Assay I (for the determination of D and 4OHD in urine samples).* To a 1-ml aliquot of urine containing D and 4OHD in a well fitted screw-cap (lined with PTFE) tube the following reagents were added: G (10 μg in methanol, as the internal standard), saturated sodium bicarbonate solution (0.5 ml), methanol (0.5 ml) and acetylacetone (0.5 ml). The reaction mixture was placed in a incubator at 96°C for 2.5 h. To this mixture, after cooling to room temperature (25°C), 5 *M* sodium hydroxide solution (3 ml) was added and extracted with diethyl ether (8 ml), and the aqueous layer was discarded. The derivatives of D, 4OHD and G were then back-extracted (mixing by an automatic shaker for 15 min and followed by centrifugation) into 2 *M* hydrochloric acid (0.5 ml). Then 5 *M* sodium hydroxide (0.5 ml) was added, and the derivatives were finally extracted into 8 ml of diethyl ether. The ethereal extract was evaporated to dryness at 45°C. The residue was redissolved in methanol (20 μl) and analysed by both GC and HPLC systems.

*Assay II (for the determination of D and 4OHD in saliva samples).* As for assay I, except that 2 μg of G were used as internal standard to study the effect of pH on the buccal absorption of D and 4OHD.

*Assay III (for the determination of D and 4OHD in blood or plasma samples).*

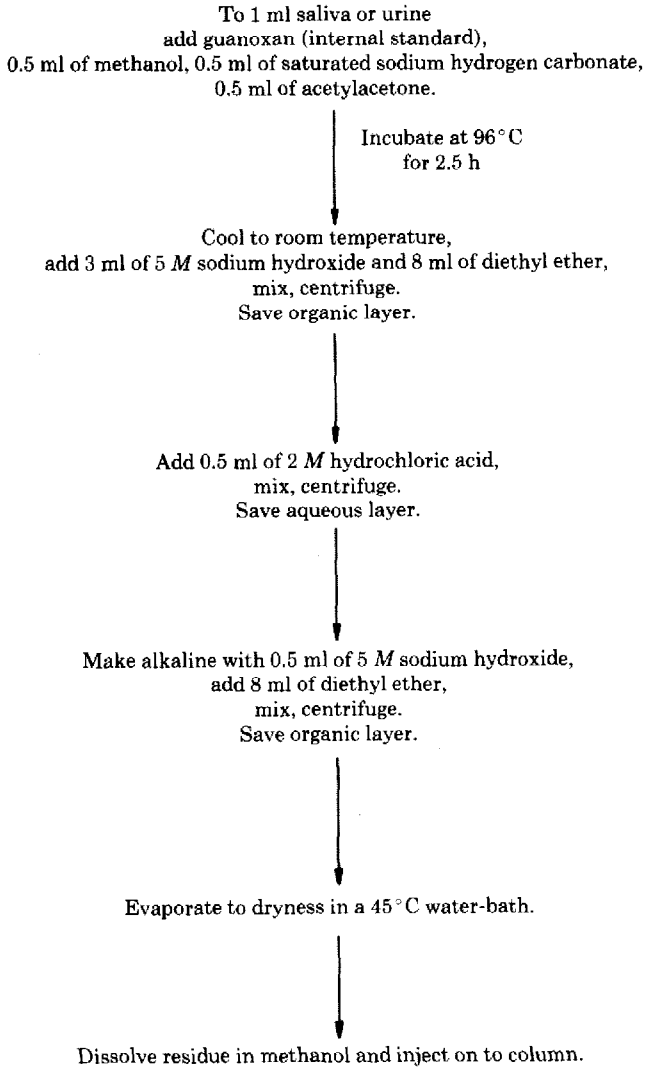


Fig. 2. Derivatization and extraction procedures for saliva and urine samples.

A clean-up procedure was included before the derivatization step. Blood or plasma (1 ml) and internal standard in a screw-cap centrifuge tube were mixed with methanol (1 ml) and 2 *M* hydrochloric acid (1 ml). The precipitated proteins were separated by centrifugation. The supernatant was transferred to a clean centrifuge tube containing 2 *M* hydrochloric acid (1 ml) and washed with diethyl ether (8 ml). The aqueous layer was alkalinized with 5 *M* sodium hydroxide (1 ml). Subsequent steps were the same as described in assay I.

Figs. 2 and 3 summarize the whole derivatization and extraction procedures for the analysis of D and 4OHD in biological samples.

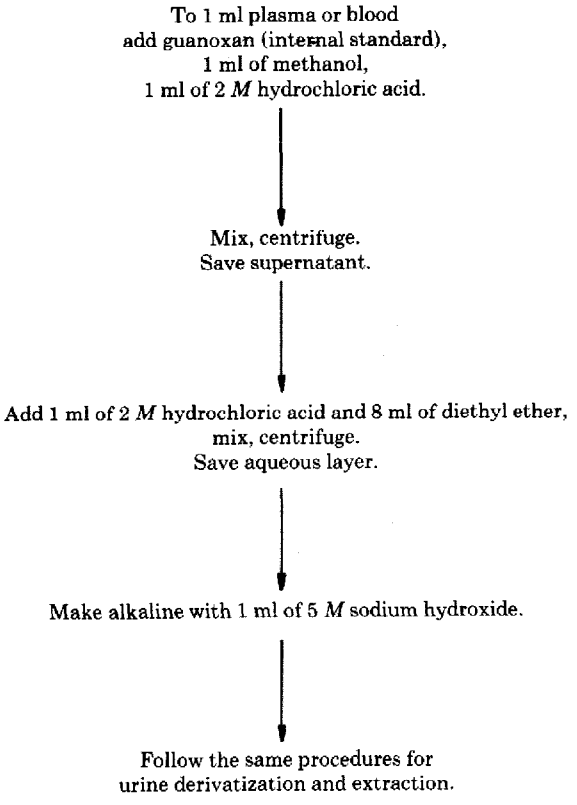


Fig. 3. Derivatization and extraction procedures for blood and plasma.

## RESULTS AND DISCUSSION

### *Derivatization and extraction of D, 4OHD and G*

Preliminary attempts made to extract D, 4OHD and G using various combinations of organic solvents under alkaline conditions resulted in unsatisfactorily low recovery of 4OHD and G. These compounds were successfully extracted from urine samples with the aid of solid-phase extraction using Bond-Elut columns containing silica modified with carboxylic acid ion-exchange functional groups [5]. The use of this type of extraction requires expensive accessories, such as a Vac-Elut chamber. The authors did not include an internal standard for the assay, thus a precise procedure of solvent additions was necessary for accurate reproducibility, and the recoveries of D and 4OHD were 76–87% [5].

On the other hand, derivatization of the guanidino groups of D, 4OHD and G with acetylacetone produced pyrimidines with good GC properties [4], as confirmed by the present study, which were easily extracted into diethyl ether under alkaline conditions. Using a similar procedure, Róna et al. [7] analysed the pyrimidines by HPLC after extraction of the derivatives with the aid of an Extrelut column. The present derivatization step shortened the reaction time for the condensation of acetylacetone with the guanidino groups from 16 to 2.5 h by increas-

ing the reaction temperature from 50 to 96°C (Fig. 1). Similarly, Peart et al. [8] decreased the reaction time to 1 h by increasing the reaction temperature to 93°C. The structures of the pyrimidines of D and 4OHD were previously identified by Allen et al. [9] using mass spectrometry and nuclear magnetic resonance spectroscopy. The reaction products were identified by a GC procedure similar to that reported by Lennard et al. [4]. Similar GC properties of the pyrimidine derivatives of D, 4OHD and G were observed, and further spectroscopic analyses of these compounds were not carried out.

Subsequent extraction of the pyrimidines from the reaction mixture was carried out by simple acid/alkaline back-extraction with diethyl ether [4,8]. Although the procedure involved additional solvent-extraction steps, the present assay did not require additional and expensive solid-phase extraction column and Vac-Elut accessories. The assay can be repeated easily in other laboratories. Additional pre-derivatization clean-up steps were found necessary for the assay of D and 4OHD in blood and plasma samples, because the presence of proteins in the reaction mixture either gave interfering substances in the chromatograms during GC or HPLC analyses or interfered with the reaction. Thus the cleaned supernatant from plasma or blood samples, after precipitation with methanol and 2 *M* hydrochloric acid to remove proteins, was used in the derivatization step. No difficulty was encountered during the derivatization of D, 4OHD and G in urine and saliva samples.

*Comparison of the GC and HPLC analyses*

The residues of extracts from biological samples containing D, 4OHD and G were analysed by both GC and HPLC. Methanolic concentrates of the samples were injected into the corresponding chromatographs. Fig. 4 shows typical chromatograms obtained by GC of plasma and urine extracts from a subject who had taken an oral dose of 10 mg of D. Fig. 5 illustrates the chromatograms of the same extract obtained by HPLC. In both analyses, there were no interfering peaks that would mask the analytical peaks of D, 4OHD and G. Tables I and II give data to indicate that both assays were reliable, accurate and reproducible for the measurement of D and 4OHD in biological samples. The respective retention times for 4OHD, G and D were 10.4, 8.8 and 3.6 min, respectively, in the GC analysis (Fig. 4), and 4.6, 5.6 and 7.4 min, respectively, in the HPLC assay (Fig. 5). 4OHD was not detected in the 3-h plasma sample by either method. Results from the analysis of various samples using the two chromatographic techniques were in good agreement, as shown by the very high coefficients of linear regression analysis (Figs. 6 and 7). In the present method, the use of an ion-pair agent, 1-pentanesulphonic acid, in the mobile phase improved the peak shapes and resolution of the analytical peaks compared with the results reported in the literature [5,7].

*Applications*

The buccal absorption of D and 4OHD at various buffer pH was carried out according to procedure previously described for pethidine and its basic metabolites [10]. A quantity of D and 4OHD (equivalent to 25 μmol) was dissolved in buffer solutions (25 ml) at pH 4, 5, 6, 7, 7.4, 8 and 9. The subject held the 25-ml

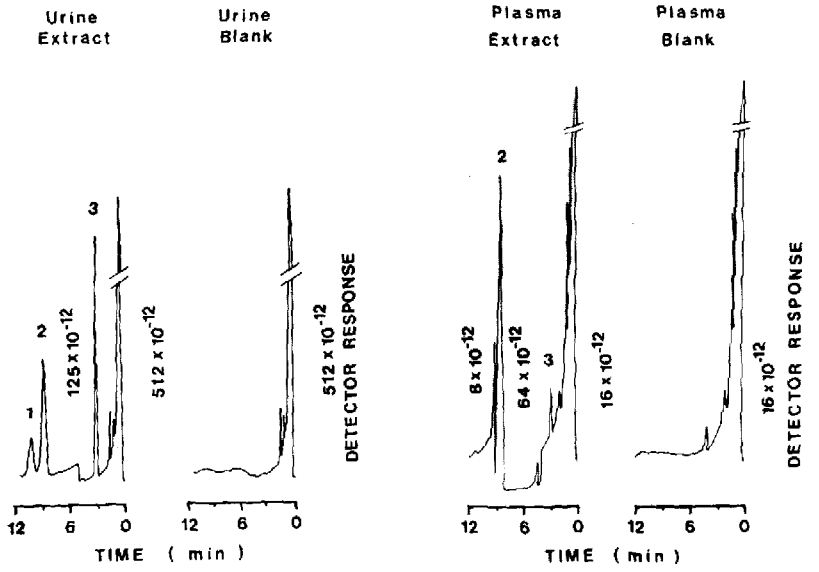


Fig. 4. Chromatograms obtained by GC of extracts of plasma and urine from a subject after an oral dose of one Declinax tablet (10 mg of D). Plasma sample was taken at 3 h post-dose, and urine at 3–4 h collection. Note that 4OHD was not detected in plasma at 3 h. Peaks: 1 = 4OHD; 2 = G; 3 = D.

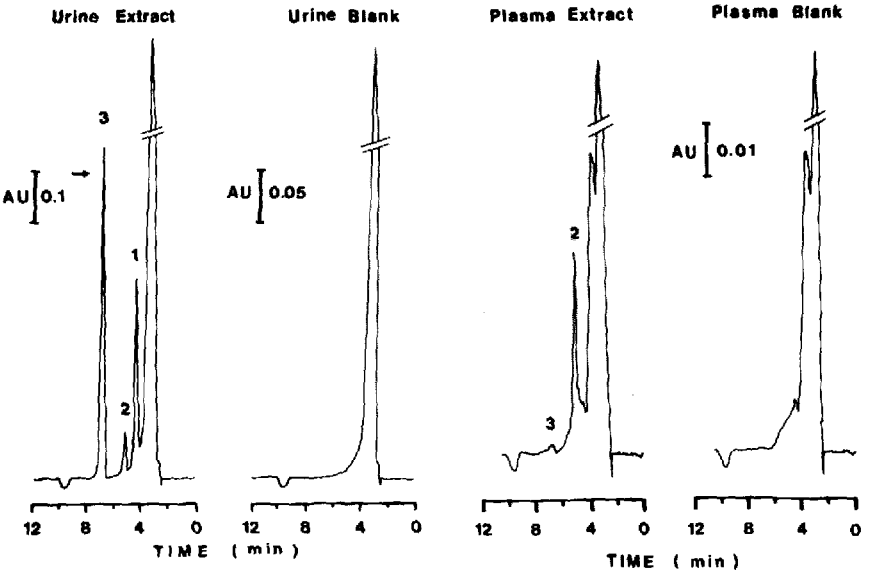


Fig. 5. Chromatograms obtained by HPLC of plasma and urine from a subject after an oral dose of one Declinax tablet (10 mg of D). Plasma sample was taken at 3 h post-dose, and urine at 3–4 h collection. Note that 4-OHD was not detected in plasma at 3 h. Peaks: 1 = 4OHD; 2 = G; 3 = D.

mouth-wash in the buccal cavity for 6 min while moving cheek and tongue to facilitate absorption. The expelled mouth-wash, plus 10 ml of water-rinse (in 10 s), were mixed together for analysis by the HPLC assay. Fig. 8 illustrates that at pH 4–6 (acidic environment), the absorption of D and 4OHD through the buccal

TABLE I

CALIBRATION AND PRECISION OF THE GC ASSAY

C.V. = coefficient of variation.

| Concentration ($\mu$g/ml) | D/G peak-height ratio (mean ± S.D.) | C.V. (%) ($n$=6) | 4OHD/G peak-height ratio (mean ± S.D.) | C.V. (%) ($n$=6) |
|---|---|---|---|---|
| 0.5 | 0.141 ± 0.008 | 6.0 | 0.028 ± 0.001 | 4.3 |
| 1.0 | 0.279 ± 0.009 | 3.3 | 0.066 ± 0.003 | 5.3 |
| 5.0 | 1.455 ± 0.098 | 6.7 | 0.474 ± 0.031 | 6.5 |
| 10.0 | 3.042 ± 0.183 | 6.0 | 1.039 ± 0.055 | 5.3 |
| 20.0 | 6.134 ± 0.258 | 4.2 | 2.204 ± 0.101 | 4.6 |
| Batch standards* at 5.0 $\mu$g/ml ($n$=10) | 1.438 ± 0.105 | 7.3 | 0.453 ± 0.036 | 7.9 |
| Calibration curve | $y = 0.128 + 3.245x$, $r = 0.9999$ | | $y = 0.501 + 8.92x$, $r = 0.9995$ | |

*An indication of between-day precision.

mucosa into the circulation was fairly constant. An increase in absorption was noted at higher pH with a subsequent fall at pH 9. This observation suggests that the excretion of D and 4OHD might be influenced by an alkaline urinary pH, under which conditions there might be re-absorption of D and possible 4OHD

TABLE II

CALIBRATION AND PRECISION OF THE HPLC ASSAY

C.V. = coefficient of variation.

| Concentration ($\mu$g/ml) | D/G peak height ratio (mean ± S.D.) | C.V. (%) ($n$=6) | 4OHD/G peak-height ratio (mean ± S.D.) | C.V. (%) ($n$=6) |
|---|---|---|---|---|
| 0.5 | 0.648 ± 0.038 | 5.8 | 0.187 ± 0.010 | 5.6 |
| 1.0 | 1.210 ± 0.040 | 3.3 | 0.438 ± 0.012 | 2.9 |
| 5.0 | 5.230 ± 0.421 | 8.1 | 2.220 ± 0.136 | 6.1 |
| 10.0 | 11.262 ± 0.457 | 4.1 | 5.060 ± 0.321 | 6.3 |
| 20.0 | 22.190 ± 1.313 | 5.9 | 11.287 ± 0.544 | 4.8 |
| Batch standards* at 5.0 $\mu$g/ml ($n$=9) | 5.525 ± 0.381 | 6.9 | 2.374 ± 0.208 | 8.7 |
| Calibration curve | $y = 1.109x + 0.011$, $r = 0.9998$ | | $y = 0.569x - 0.317$, $r = 0.9981$ | |

*An indication of between-day variation.

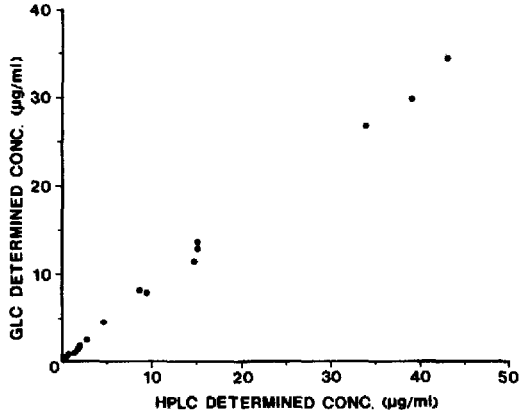


Fig. 6. Correlation of GC and HPLC assayed concentrations of D from analyses of plasma, saliva and urine samples: $y = 1.258x - 0.563$; $r = 0.9987$.

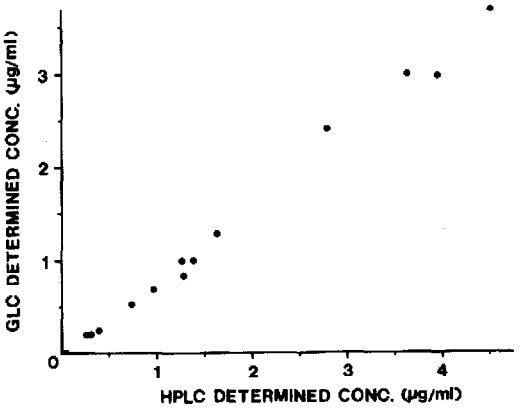


Fig. 7. Correlation of GC and HPLC assayed concentrations of 4OHD from analyses of saliva and urine samples: $y = 1.208x + 0.085$; $r = 0.9969$.

(which is more polar and water-soluble) through the renal tubules into the circulation.

The urinary excretion of D and 4OHD was followed at intervals after two separate oral doses of 10 and 20 mg of D to one subject. Figs. 9 and 10 illustrate, respectively, the urinary rates of excretion of D and 4OHD and the cumulative recoveries in the urine of D and 4OHD over 48 h after oral doses of D. Relatively higher D/4OHD ratios were obtained after a 20-mg dose of D. The well established D/4OHD ratio at 0–8 h for this subject of Chinese origin (who was a non-smoker) was 9.88 and 6.34 at 20- and 10-mg dose, respectively. This suggests that 4-hydroxylation of D in this subject may be dose-dependent. However, the estimated elimination half-life (Fig. 9) of D was identical (10.5 h) after either dose. More studies on the phenotyping of D 4-hydroxylation are being carried out in the Chinese population [11].

In conclusion, the well established GC procedure [4] was useful for detailed pharmacokinetic study of D, as the nitrogen-selective detector can measure ca. 3

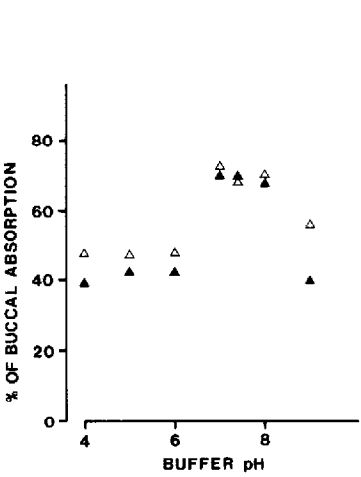


Fig. 8. Effect of pH on the buccal absorption of 4OHD (△) and D (▲). Data were generated from HPLC.

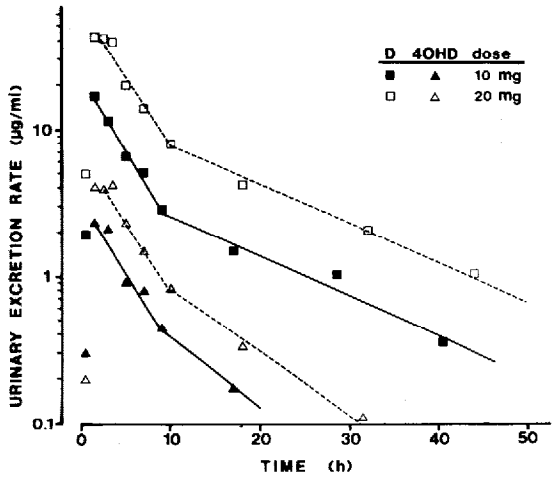


Fig. 9. Urinary excretion rates of D and 4OHD plotted against mid-points of collection after two separate oral doses of 10 and 20 mg of D. Data were generated from HPLC.

ng/ml in a 1.0-ml sample of plasma. For phenotyping purposes, the HPLC method is adequate and accurate for the determination of 4OHD and D in urine. The simple solvent clean-up procedure after derivatization, though repetitive, can be reproduced in any laboratory without the use of expensive accessories for solid-phase extraction. The shortening of the reaction time in derivatization may enable ready phenotyping of a subject within 24 h. Many centres throughout the world are now interested in phenotyping D 4-hydroxylation in populations. The present study, for the first time, compares the well established GC analysis with

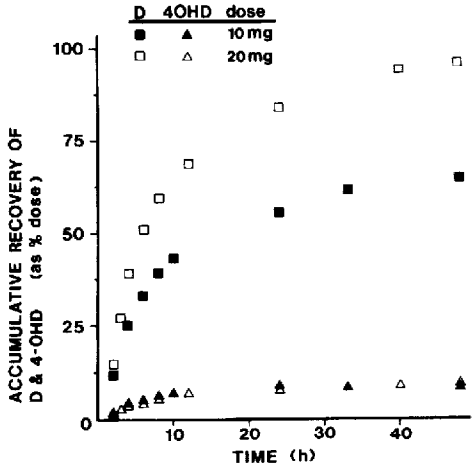


Fig. 10. Cumulative recoveries of D and 4OHD over 48 h after two separate oral doses of 10 and 20 mg of D. Data were generated from HPLC.

the more recently introduced HPLC assay. Some centres do not have access to GC equipment, and this comparison indicates that the modified HPLC assay with the inclusion of an ion pair in the mobile phase is an adequate, reliable and alternative assay for the measurement of 4OHD and D in urine for phenotyping studies.

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