*Journal of Chromatography*, 493 (1989) 313–324
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4815

# DIRECT DETERMINATION OF CODEINE-6-GLUCURONIDE IN PLASMA AND URINE USING SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

ZHAO RONG CHEN*

*Department of Clinical and Experimental Pharmacology, University of Adelaide, G.P.O. Box 498, Adelaide 5001 (Australia)*

GEOFFREY REYNOLDS

*School of Chemical Technology, South Australian Institute of Technology, Adelaide (Australia)*

and

FELIX BOCHNER and ANDREW SOMOGYI

*Department of Clinical and Experimental Pharmacology, University of Adelaide, and Department of Clinical Pharmacology, Royal Adelaide Hospital, Adelaide (Australia)*

(First received January 16th, 1989; revised manuscript received April 4th, 1989)

## SUMMARY

A sensitive and selective method was developed for the direct determination of codeine-6-glucuronide in plasma and urine using high-performance liquid chromatography (HPLC) with fluorescence detection. Codeine-6-glucuronide was synthesised and its purity estimated using acid and enzyme hydrolysis. The hydrolysis of codeine-6-glucuronide by β-glucuronidase was incomplete and urine reduced the extent of hydrolysis. Codeine-6-glucuronide was recovered from plasma using a solid-phase extraction column and separated on a reversed-phase $C_{18}$ HPLC column. The assay showed good reproducibility and accuracy (within 10%), and standard curves were linear between 32 and 1600 ng/ml in plasma and between 0.32 and 160 μg/ml in urine. The assay has been applied to the study of the pharmacokinetics and metabolism of codeine in patients.

## INTRODUCTION

Codeine is one of the most widely used analgesic and antitussive drugs. Its disposition and metabolism in man have been studied, often using analytical

0378-4347/89/$03.50 

methods which lack specificity. The major metabolites of codeine are morphine (via O-demethylation), norcodeine (via N-demethylation) and codeine-6-glucuronide [1–4]. The percentage of the dose recovered from urine has been reported to be between 60 and 90%, of which 'conjugated codeine' accounted for up to 50% [1,2]. Plasma concentrations of conjugated codeine have been reported to be approximately ten times higher than those of codeine in healthy volunteers receiving 60 mg codeine orally [5,6]. In these studies, the conjugate was not directly determined, but had been estimated after release of codeine base by acid or β-glucuronidase hydrolysis. The interpretation of these results [2,5,6] is difficult, because it has been shown that β-glucuronidase does not completely hydrolyse codeine-6-glucuronide [7,8].

Codeine-6-glucuronide may be a clinically important metabolite, by analogy with the emerging importance of morphine-6-glucuronide as an active analgesic substance in man [9–11]. The pharmacology and disposition of codeine-6-glucuronide in man have not been evaluated because of the lack of a specific assay, whose development has been limited because of the absence of a commercial source of pure reference substance. Our objectives were to synthesize pure codeine-6-glucuronide and to develop a specific and sensitive high-performance liquid chromatographic (HPLC) assay for its direct determination in plasma and in urine.

## EXPERIMENTAL

### *Reagents*

Hydrochloric acid, orthophosphoric acid, sodium bisulfite (BDH, Port Fairy, Australia), sodium carbonate, benzene, methanol, dichloromethane (Univar), triethylamine, ammonium sulphate, citric acid, acetic acid, sodium acetate, sodium dihydrogenphosphate and sodium hydroxide (Ajax, Sydney, Australia) were all of analytical grade. Acetonitrile (Mallinckrodt, South Oakleigh, Australia) was of HPLC grade. Silver nitrate was obtained from ACE (Beverley, Australia), silica 35–70 μm from Amicon (Danvers, MA, U.S.A.) and methyl-(2,3,4-tri-O-acetyl-*D*-glucopyranosylbromide)uronate from Koch-Light (Haverhill, U.K.). Dihydrocodeine bitartrate (Knoll, Ludwigshafen, F.R.G.) and codeine phosphate (F.H. Faulding, Adelaide, Australia) were of British Pharmacopoeial grade quality. β-Glucuronidase (*Helix pomatia* type H-1) was obtained from Sigma (St. Louis, MO, U.S.A.).

### *Codeine-6-glucuronide synthesis*

This was performed following a procedure described by Yoshimura et al. [7] with the following modifications. The Koenigs–Knorr reaction was performed over 48 h to ensure complete disappearance of the starting material. The crude methyl[codein-6-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid]uronate was chromatographed on silica and the purified product eluted with 3% methanol in

dichloromethane. The conversion to codeine-6-glucuronide was carried out as described by Yoshimura et al. [7].

*Purity of codeine-6-glucuronide sample*

The purity of the synthesized codeine-6-glucuronide sample was investigated by two independent methods as follows.

*β-Glucuronidase hydrolysis.* Codeine-6-glucuronide solutions (0.5 ml) diluted in 0.2 *M* acetate buffer pH 5 to achieve concentrations of 3.2, 16 and 160 μg/ml, $n=5$) were mixed with 0.5 ml (8000 U/ml in the 0.2 *M* acetate buffer) β-glucuronidase and incubated in a shaking water bath at 37°C for 14 h (overnight). The protein was removed by ultrafiltration using Centrifree™ (Amicon) ultrafiltration units at 2000 *g* for 15 min. Aliquots of the ultrafiltrate were analysed for codeine-6-glucuronide and codeine as described below for urine by direct injection onto the column. Purity was calculated as:

$$\frac{\text{percentage of original sample recovered as codeine}}{\text{percentage of original sample released of codeine-6-glucuronide}}$$

The numerator in the above equation was calculated as the codeine concentration in the ultrafiltrate divided by the notional codeine concentration in the original codeine-6-glucuronide sample and the denominator was calculated as the codeine-6-glucuronide concentration in the original sample minus the codeine-6-glucuronide concentration in the ultrafiltrate divided by the codeine-6-glucuronide concentration in the original sample. The dilution of the ultrafiltrates was considered. The dilution of the incubation samples was taken into account in the above calculations.

The intrinsic influence of urine on the hydrolytic capacity of β-glucuronidase under the above conditions was investigated in two ways. Firstly, 16 μg codeine-6-glucuronide ($n=2$) in 0.5 ml drug-free urine or in 0.5 ml acetate buffer were added to tubes containing 0, 800, 2400, 4000 and 4800 U β-glucuronidase in 0.5 ml acetate buffer. Secondly, 16 μg of codeine-6-glucuronide in 10 μl distilled water were incubated with a constant amount (4000 U in 0.5 ml of 0.2 *M* acetate buffer) of β-glucuronidase, and varying volumes of urine (0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 ml, $n=2$) were added. The final volume was adjusted to 1.0 ml with acetate buffer, except for the 1.0-ml urine sample in which 4000 U of β-glucuronidase were added as powder. The samples were processed and analysed as described above.

*Hydrochloric acid hydrolysis.* The acid hydrolysis of codeine-6-glucuronide was performed following a procedure described by Yoshimura et al. [12] with modifications: to 2.5 ml of codeine-6-glucuronide (concentrations of 3.2, 16 and 80 μg/ml, $n=5$) were added 0.05 ml of 40% $NaHSO_3$ solution and 3 ml of 37% HCl to bring the final acid concentration to 20%. The mixture was then heated in a boiling water-bath for 60 min. To this hydrolysate, 0.05 ml of 40% $NaHSO_3$ and 3 ml of 10 *M* NaOH solution were added to achieve a pH of 5.0.

The mixture was diluted with 0.2 *M* pH 5.0 acetate buffer to a final volume of 10 ml. Aliquots of each sample were analysed for the liberated codeine as described below for urine. Codeine solutions in the concentration range 2–50 μg/ml ($n=2$) were treated in the same manner.

*Chromatography*

The HPLC system consisted of an M-6000A pump (Waters Assoc., Milford, MA, U.S.A.), an LS-5 luminescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.), a WISP 710B automatic injector (Waters Assoc.) and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm × 4.6 mm I.D. stainless-steel column was packed with Spherisorb 5-μm ODS-2 packing material (Phase Separations, Queensferry, U.K.). The composition of the mobile phase was 10% acetonitrile–0.03% triethylamine in 8 m*M* citric acid–phosphate buffer, adjusted to pH 3 with orthophosphoric acid. The flow-rate through the column at ambient temperature was 1 ml/min which produced a back-pressure of 10.5 MPa (1.5 k.p.s.i.). The excitation and emission wavelengths of the detector were 230 and 350 nm, respectively, and the excitation and emission slits were set at 15 and 20 nm, respectively.

*Stock solutions*

Codeine-6-glucuronide was made up as 1.6 mg/ml (based on 80% purity, see Results) in distilled water and was diluted to concentrations ranging from 32 to 1600 ng/ml in drug-free plasma and from 0.32 to 160 μg/ml in drug-free urine. Dihydrocodeine, the internal standard, was diluted in distilled water to a concentration of 4 μg/ml for plasma analysis.

*Sample preparation*

*Plasma.* The extraction of codeine-6-glucuronide from plasma was based on the method of Svensson et al. [13] for morphine-3- and -6-glucuronides. A Sep-Pak $C_{18}$ cartridge (Waters Assoc.) was pretreated with 10 ml of methanol, 5 ml of 25% acetonitrile in 10 m*M* phosphate buffer (pH 2.1) and 10 ml of distilled water. Plasma (0.5 ml), containing 50 μl of 4 μg/ml dihydrocodeine, was mixed with 3 ml of 0.5 *M* ammonium sulphate adjusted to pH 9.3 with ammonia and was slowly passed through the pretreated cartridge. The cartridge was then washed with 20 ml of the 5 m*M* ammonium sulphate buffer and then with 0.5 ml of distilled water. Codeine-6-glucuronide and dihydrocodeine were eluted with 2 ml of 25% acetonitrile in 10 m*M* phosphate buffer (pH 2.1). The eluate was mixed with 3 ml of the 0.5 *M* ammonium sulphate buffer and injected into another cartridge. The cartridge was then washed with 20 ml of the 5 m*M* ammonium sulphate buffer and then with 0.5 ml of distilled water. The two compounds were eluted with 2 ml of methanol. The eluate was

evaporated to dryness in a vortex evaporator (Buchler, Fort Lee, NJ, U.S.A) and redissolved in 200 $\mu$l of 0.05 *M* HCl. An aliquot (10–50 $\mu$l) of the solution was injected on the column via the automatic injector.

*Urine.* Urine was injected onto the HPLC column directly after dilution (1:5) with distilled water.

*Statistical analysis*

Peak heights were measured manually and the peak-height ratio of codeine-6-glucuronide to the internal standard, dihydrocodeine, was calculated. For plasma analysis, standard curves were plotted as peak-height ratio versus concentration and for urine analysis as peak height versus concentration. Standard linear regression analysis was used to determine the slope, intercept, their variability and the strength of the correlation for the above standard curves. Precision was evaluated by adding codeine-6-glucuronide to achieve plasma concentrations of 80 and 1600 ng/ml and urine concentrations of 0.8 and 160 $\mu$g/ml. These samples were analysed nine times within one day and once daily on six separate occasions and the coefficients of variation were calculated. Accuracy was assessed by adding codeine-6-glucuronide to achieve plasma concentrations of 80 and 1600 ng/ml and urine concentrations of 0.8 and 160 $\mu$g/ml. Each sample was assayed nine times and the estimated concentrations were calculated from concurrently processed standard curves.

*Stability*

Stability of codeine-6-glucuronide was assessed by adding codeine-6-glucuronide to achieve plasma concentrations of 80 and 1600 ng/ml and urine concentrations of 0.8 and 160 $\mu$g/ml. These were stored at $-20\,^{\circ}$C and assayed for codeine and codeine-6-glucuronide on several occasions during a twelve-week period.

*Assay application*

A healthy female subject (aged 27 years, weight 48 kg) ingested a 30-mg codeine phosphate tablet. Through an indwelling catheter, kept patent with a stylet (Jelco$^{TM}$, Critikon, Tampa, FL, U.S.A.), placed in a forearm vein, multiple blood samples were collected for 12 h and all urine for 48 h. These samples were assayed for codeine-6-glucuronide concentrations by the above method. Codeine concentrations in these samples were also determined by a recently described HPLC assay using solvent–solvent extraction [14]. This study was approved by the Human Ethics Committee of the Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide.

## RESULTS AND DISCUSSION

To verify the identity of the synthesised codeine-6-glucuronide, a $^{13}$C NMR spectrum was measured on a solution of the product in $^{2}H_2O$ and recorded on

a Varian Gemini 200 RT NMR spectrometer (Fig. 1). Assignments were based on resonances reported for codeine by Carroll et al. [15]. An attached proton test (APT) was also measured (Fig. 1), using a procedure reported previously [16].

The APT pulse sequence causes signals for quaternary carbons and $CH_2$ groups to appear above the baseline of the spectrum whilst the signals for CH and $CH_3$ groups point below. The results of the APT are in agreement with the assignments given for the $^{13}C$ resonances.

The results of the studies to assess the degree of purity of the synthesized codeine-6-glucuronide sample are shown in Table I. Both hydrolytic methods gave essentially the same result and indicated that the purity of the original sample was about 80%. The buffer incubation of codeine-6-glucuronide with 4000 U/ml $\beta$-glucuronidase resulted in incomplete hydrolysis. At concentrations of 3.2, 16 and 160 $\mu$g/ml, hydrolysis was $81 \pm 0$, $79 \pm 0.4$ and $56 \pm 0.7\%$ (mean $\pm$ S.D.), respectively. This result is in agreement with that reported previously [7]. Urine had a marked inhibitory effect on the hydrolytic capacity of $\beta$-glucuronidase. In acetate buffer, 4000 U/ml $\beta$-glucuronidase resulted in

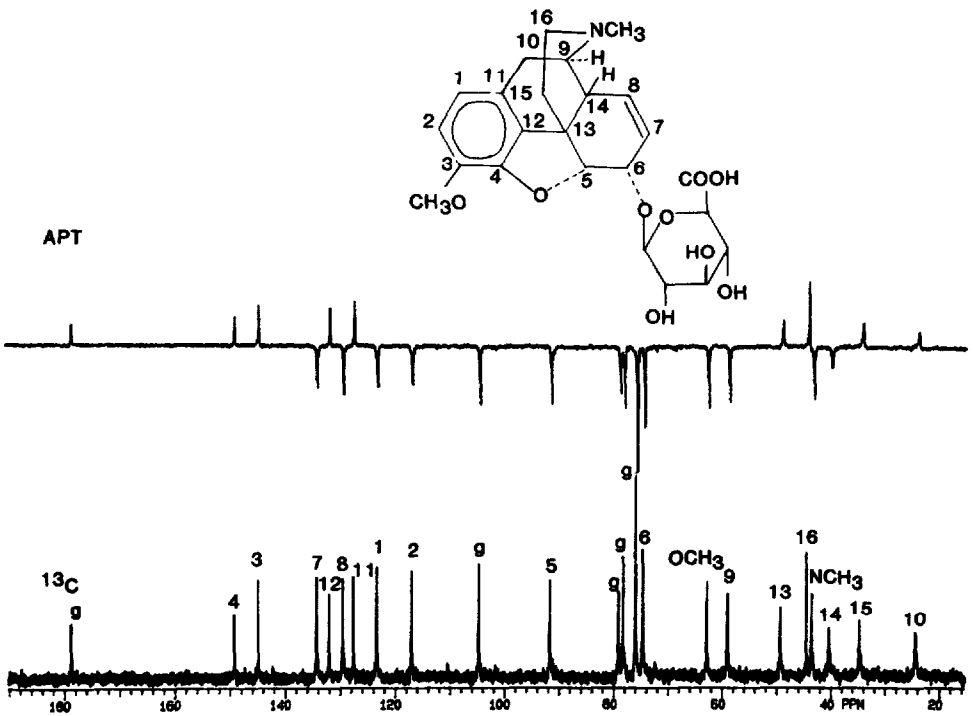


Fig. 1. $^{13}C$ and APT (attached proton test) NMR spectra of codeine-6-glucuronide in $^2H_2O$. Numbers above signals in the $^{13}C$ NMR spectrum refer to structure. Symbol g refers to carbons in sugar residue.

TABLE I

PURITY OF THE SYNTHESIZED SAMPLE OF CODEINE-6-GLUCURONIDE

| Hydrolysis method | Concentration ($\mu$g/ml) | Purity (mean ± S.D., $n$=5) (%) |
|---|---|---|
| $\beta$-Glucuronidase | 3.2 | 80.2 ± 0 |
| | 16.0 | 80.3 ± 0.7 |
| | 160.0 | 81.7 ± 1.6 |
| Hydrochloric acid | 3.2 | 81.3 ± 0 |
| | 16.0 | 80.9 ± 2.3 |
| | 80.0 | 85.0 ± 1.6 |

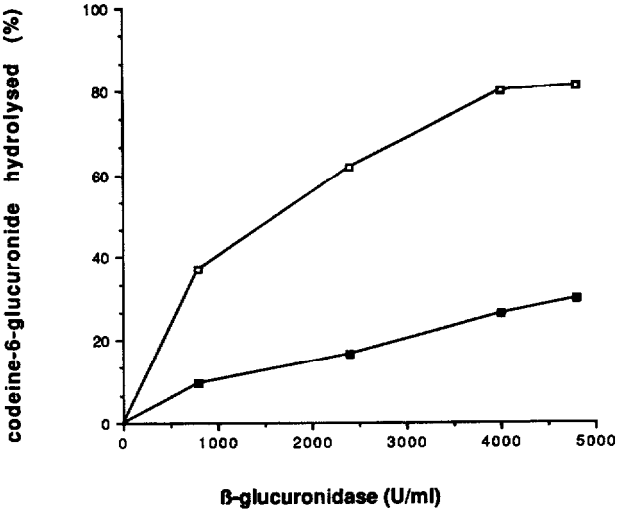


Fig. 2. Influence of $\beta$-glucuronidase concentration on the hydrolysis of codeine-6-glucuronide (16.0 $\mu$g/ml) in 0.2 *M* acetate buffer (□) and in urine (■). Each point is the mean of two measurements.

maximal, but incomplete release of the aglycone at a codeine-6-glucuronide concentration of 16 $\mu$g/ml (Fig. 2). When urine was added to the incubate (urine–acetate buffer, 1:1, v/v), the hydrolysis was inhibited by 69 ± 2% at a $\beta$-glucuronidase concentration of 4000 U/ml (Fig. 2). The inhibition of hydrolysis by urine was related to the concentration of urine in the incubate (Fig. 3). These results suggest that urine contains inhibitors of $\beta$-glucuronidase activity. Saccharo-1,4-lactone and sulphate ions have been shown to inhibit this enzyme [17–19]. Whilst acid hydrolysis successfully resulted in complete release of codeine, the extreme acidic conditions employed could result in the degradation of the aglycone of other glucuronide conjugates [20]. These results strongly support the need to assay glucuronide conjugates of xenobiotics in biological fluids by direct methods.

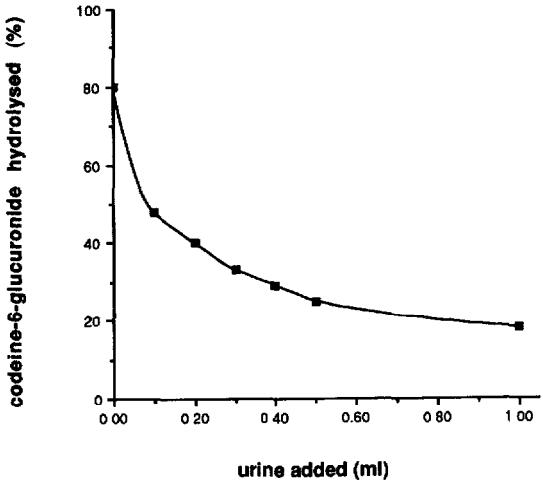


Fig. 3. Influence of urine volume on the $\beta$-glucuronidase (4000 U/ml) hydrolysis of codeine-6-glucuronide at a concentration of 16.0 $\mu$g/ml. Each point is the mean of two measurements.


Fig. 4. Chromatogram of a standard solution of 1.6 $\mu$g/ml codeine-6-glucuronide (1) and 3.3 $\mu$g/ml dihydrocodeine (2).

A representation chromatogram from an injection of a solution containing a mixture of codeine-6-glucuronide and the internal standard dihydrocodeine is shown in Fig. 4. Codeine-6-glucuronide had a retention time of 5.9 min and a capacity factor of 2.9 and dihydrocodeine had a retention time of 11.5 min and a capacity factor of 6.7. We initially tried a cyano column because this had proved successful in assaying codeine, morphine and norcodeine in plasma and urine [14]. With this column, the internal standard peak was interfered with by endogenous substances from plasma. Alterations in the composition and pH of the mobile phase were unsuccessful in obtaining complete separation. The octadecyl-silane column and mobile phase as described above resulted in

good separation of codeine-6-glucuronide and dihydrocodeine from endogenous substances in plasma and urine (Fig. 5).

Interference was studied by preparing and chromatographing aliquots of solutions of pure drugs and by analyzing plasma and urine samples from patients on multiple-drug therapy. None of the drugs tested in Table II interfered with the assay of codeine-6-glucuronide.

In contrast to codeine [14], codeine-6-glucuronide could not be extracted from plasma by conventional solvent–solvent extraction techniques. In addition, protein precipitation and injection of the supernatant onto the HPLC column resulted in interference by endogenous substances and substantial reduction in sensitivity. The Sep-Pak cartridge has been successfully used by Svensson et al. [13] to assay morphine glucuronides in plasma and proved equally successful for codeine-6-glucuronide. The use of a second Sep-Pak cartridge to further purify the sample was necessary. The recovery of codeine-6-glucuronide using this extraction method was $74 \pm 2\%$ (mean $\pm$ S.D., $n = 4$) at

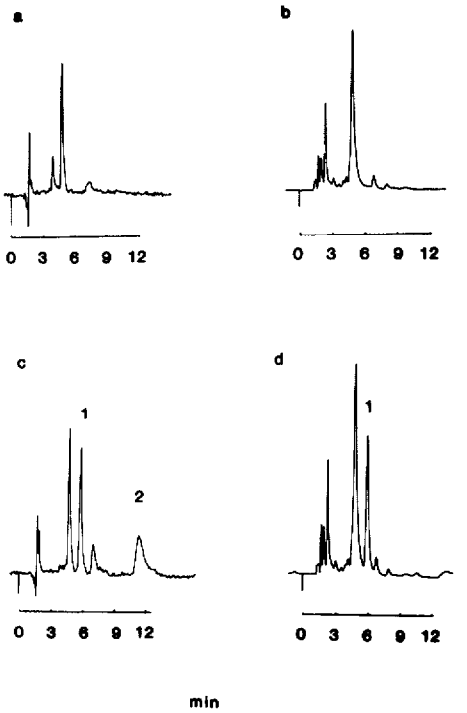


Fig. 5. Chromatograms of (a) blank plasma, (b) blank urine, (c) a plasma sample from the subject 1.25 h after ingestion of 30 mg codeine phosphate (codeine-6-glucuronide concentration = 1426 ng/ml) and (d) a 0–12 h urine sample from the same subject (codeine-6-glucuronide concentration = 92.8 μg/ml; sample diluted 1:5). Peaks: 1 = codeine-6-glucuronide; 2 = dihydrocodeine (internal standard).

TABLE II

DRUGS SHOWN NOT TO INTERFERE WITH THE CODEINE-6-GLUCURONIDE ASSAY

| Drugs in patients' plasma and urine | Pure drug preparations |
|---|---|
| Captopril | Cocaine |
| Digoxin | Dextromethorphan |
| Frusemide | Dextrorphan |
| Glyceryl trinitrate | Dextropropoxyphene |
| Metoclopramide | Ethylmorphine |
| Multivitamins | Naloxone |
| Paracetamol | Oxycodone |
| Polystyrene sulphonate | Pholcodine |
| Pseudoephedrine | Morphine-3-glucuronide |
| Quinine bisulphate | Morphine-6-glucuronide |
| Salbutamol | Morphine-3-sulphate |
| Spironolactone | Morphine |
| Temazepam | Normorphine |
| Theophylline | Codeine |
| Tolbutamide | Norcodeine |

a plasma concentration of 1600 ng/ml and was similar between 32 and 1600 ng/ml; for dihydrocodeine the recovery was $33 \pm 2\%$. Although the use of Sep-Pak cartridges adds substantially to the cost of the assay, it was possible to re-use a cartridge at least thirty times.

The relationship between peak-height ratios and concentrations from 32 to 1600 ng/ml for codeine-6-glucuronide in plasma ($r > 0.99$) and between peak height and concentrations from 0.32 to 160 $\mu$g/ml for codeine-6-glucuronide in urine ($r > 0.99$) was linear. For plasma and urine standard curves, the 95% confidence intervals of the intercepts included the origin, and the standard errors of the slopes were less than 5%. The assay showed good precision at low and high concentrations in plasma and urine. Table III shows the intra- and inter-day assay precision, whose coefficients of variation were less than 10%. The assay showed good accuracy for codeine-6-glucuronide in plasma and urine and the coefficients of variation were less than 10%.

Fig. 6, illustrating the utility of the assay, shows plasma codeine-6-glucuronide and codeine concentrations with time for a subject who had ingested a single dose of 30 mg codeine phosphate. In this subject the area under the plasma concentration–time curve of codeine-6-glucuronide was seventeen times higher (calculated as codeine base) than that of codeine, but the apparent plasma half-lives were similar (2.3 and 2.7 h, respectively). In urine of this subject, 11% of the dose was recovered as codeine and 67% as codeine-6-glucuronide.

In summary, an HPLC assay has been developed for the direct determina-

TABLE III

ASSAY VARIABILITY FOR CODEINE-6-GLUCURONIDE IN PLASMA AND URINE

| | Concentration | Coefficient of variation (%) | *n* |
|---|---|---|---|
| *Plasma (ng/ml)* | | | |
| Intra-day | 80 | 4.2 | 9 |
| | 1600 | 3.4 | 9 |
| Inter-day | 80 | 8.2 | 6 |
| | 1600 | 1.8 | 6 |
| *Urine (μg/ml)* | | | |
| Intra-day | 0.8 | 7.0 | 9 |
| | 160.0 | 2.0 | 9 |
| Inter-day | 0.8 | 6.6 | 6 |
| | 160.0 | 1.8 | 6 |

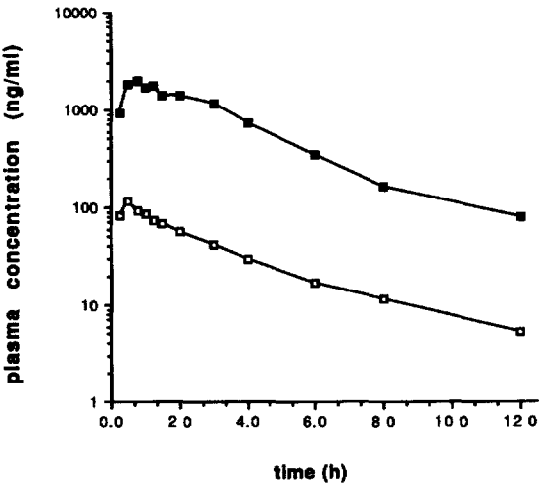


Fig. 6. Semilogarithmic plot of plasma concentration versus time profile for codeine-6-glucuronide (■) and codeine (□) after a single oral dose of 30 mg codeine phosphate in a human subject.

tion of codeine-6-glucuronide in plasma and urine. The method shows good precision, accuracy, selectivity and sensitivity and is currently being used to determine the pharmacokinetics, pharmacodynamics and metabolism of codeine in patients.

ACKNOWLEDGEMENTS

Dr. Chen is supported by the National Health and Medical Research Council of Australia. Dr. Somogyi was supported by the Royal Adelaide Hospital Research Fund.

REFERENCES


1 T.K. Adler, J.M. Fujimoto, E.L. Way and E.M. Baker, J. Pharmocol. Exp. Ther., 114 (1955) 251.
2 N. Nomof, H.W. Elliott and K.D. Parker, Clin. Toxicol., 11 (1977) 517.
3 E.L. Way, T.K. Adler, Bull WHO, 26 (1962) 51.
4 A.L. Misra, in M.L. Ader, L. Manara and R. Samanin (Editors), Factors Affecting the Action of Narcotics, Raven Press, New York, 1978, p. 297.
5 J.W.A. Findlay, R.F. Butz and R.M. Welch, Clin. Pharmacol. Ther., 22 (1977) 439.
6 D.R.P. Guay, W.M. Awni, C.E. Halstenson, J.W.A. Findlay, J.A. Opsahl, P.A. Abraham, E.C. Jones and G.R. Matzke, J. Clin. Pharmacol., 27 (1987) 983.
7 H. Yoshimura, K. Oguri and H. Tsukamoto, Chem. Pharm. Bull, 16 (1968) 2114.
8 J. Axelrod and J. Inscoe, Proc. Soc. Exp. Biol. Med., 103 (1960) 675.
9 H. Yoshimura, S. Ida, K. Oguri and H. Tsukamoto, Biochem. Pharmacol., 22 (1973) 1423.
10 G.W. Pasternak, R.J. Bodner, J.A. Clark and C.E. Inturrisi, Life Sci., 41 (1987) 2845.
11 R. Osborne S. Joel, D. Trew and M. Slevin, Lancet, i (1988) 828.
12 H. Yoshimura, M. Mori, K. Oguri and H. Tsukamoto, Biochem. Pharmacol., 19 (1970) 2353.
13 J.-O. Svensson, A. Rane, J. Säwe and F. Sjöqvist, J. Chromatogr., 230 (1982) 427.
14 Z.R. Chen, F. Bochner and A. Somogyi, J. Chromatogr., 491 (1989) 367.
15 F.I. Carroll, C.G. Moreland, G.A. Brine and J.A. Kepler, J. Org. Chem., 41 (1976) 996.
16 S.L. Patt and J.N. Shoolery, J. Magn. Reson., 46 (1982) 535.
17 J. Combie, J.W. Blake, T.E. Nugent and T. Tobin, Clin. Chem., 28 (1982) 83.
18 G.A. Levvy, Biochemistry, 52 (1952) 464.
19 E. Boyland and K. Williams, Biochem. J., 76 (1960) 388.
20 F. Fish and T.S. Hayes, J. Forensic Sci., 19 (1974) 676.