Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 4/5, pp. 339–343, 1993
Printed in Great Britain

0731–7085/93 \$6.00 + 0.00
© 1993 Pergamon Press Ltd

Influence of hydrolysis procedures on the urinary concentrations of codeine and morphine in relation to doping analysis

F.T. DELBEKE* and M. DEBACKERE

Vakgroep Farmacologie en Toxicologie, Faculteit Diergeneeskunde, Universiteit Gent, Casinoplein 24, B-9000 Gent, Belgium

Abstract: A method is described for the GC–NPD determination of urinary codeine and morphine after derivatization with trifluoroacetic anhydride. The lower limit for accurate quantitative determination was 0.05 μg ml^{-1}. After the oral administration of Bisolvon Griblettes® corresponding to 30 mg codeine phosphate to seven subjects maximum codeine concentrations were obtained after 1–2 h and codeine remained detectable generally 24 h post dosing. The mean maximum level was 5.1 ± 2.8 μg ml^{-1} found after enzymatic hydrolysis with Suc *Helix pomatia* juice (SHP). Based on these and previous results (mean 6.3 ± 3.4 μg ml^{-1}) a threshold level for codeine of 16 μg ml^{-1} is proposed. Significant differences were noticed between urinary codeine concentrations found after enzymatic hydrolysis with SHP, β-glucuronidase from *Patella vulgata* and acid hydrolysis, respectively. Generally, highest values were obtained after SHP, while β-glucuronidase and especially acid hydrolysis resulted in much lower levels. No morphine could be detected after acid hydrolysis. Concerning doping analysis, in particular the uniformity of methods and interpretation of the results, it is recommended that the hydrolysis method should be specified in the rules of those sporting federations allowing codeine and/or morphine.

Keywords: *Codeine; morphine; hydrolysis; doping.*

Introduction

Codeine is a commonly-used analgesic drug available worldwide. It is metabolized mainly by conjugation with glucuronic acid, and minor routes of metabolism involve O-demethylation to morphine and N-demethylation to nor-codeine [1]. Although the abuse liability of codeine is lower than that of morphine, codeine was classified as a banned drug by different organizations, including the International Olympic Committee. The presence of codeine, irrespective of its concentration in urine, automatically resulted in a positive doping test. For the International Amateur Athletic Federation codeine is permitted only for the treatment of a disorder. The Medical Commission of the International Cyclist Union (UCI) allows urinary concentrations of codeine and morphine (as a metabolite of codeine) below 1 μg ml^{-1} [2].

In a previous study [3] we determined the urinary concentration range that might be encountered after the administration of therapeutic amounts of several preparations containing codeine and tried to establish a threshold level. In the present work the influence of different hydrolysis procedures on the concentration of codeine after the therapeutic administration of a preparation containing 30 mg codeine phosphate will be evaluated.

Materials and Methods

Experimental design and subjects

The study was performed on seven healthy volunteers. The nature and purpose of the study was explained to each volunteer before asking their consent to participate. The volunteers were asked not to take any medication for 1 week before the experiment. Two tablets of the antitussive Bisolvon Griblettes® (Boehringer, Ingelheim, Germany) each containing acetylsalicylic acid (300 mg), bromhexine hydrochloride (4 mg) and codeine phosphate (15 mg) were administered.

Total urine was collected in capped bottles before (0 h) and 2, 4, 6, 9, 12, 24 and 30 h after administration of codeine and was either analysed immediately or stored deep-frozen for later analysis. All samples were analysed in duplicate for each hydrolysis procedure. When

* Author to whom correspondence should be addressed.

necessary, dilutions were made with blank urine.

Reagents and apparatus

Codeine base was obtained from Sigma Chemie (Deisenhofen, Germany). Morphine and ethylmorphine were obtained from Bios (Brussels, Belgium).

Ammonia buffer (pH 9.5) was prepared by the addition of ammonia to a saturated ammonium chloride solution. Sodium acetate and acetic acid were used for the preparation of 1 M acetate buffer (pH 5.2) and 0.1 M acetate buffer (pH 3.8). *Helix pomatia* juice (SHP) containing β-glucuronidase 100,000 Fishman units ml^{-1} and sulphatase 1,000,000 Roy units ml^{-1} was obtained from IBF (Villeneuve, France).

The enzyme preparation β-glucuronidase from limpets (*Patella vulgata*) containing 2,400,000 β-glucuronidase units g^{-1} solid and 32,000 sulphatase units g^{-1} solid was from Sigma. Trifluoroacetic anhydride (TFAA) was obtained from Pierce (Oud-Beyerland, The Netherlands).

All chromatograms were generated in the split mode (split ratio 1:10) on a Varian (Walnut Creek, CA, USA) 3400 gas chromatograph fitted with a 25 m × 0.25 mm i.d. fused silica OV1 Permabond column (Machery–Nagel, Düren, Germany) with a film thickness of 0.23 μm. The GC was equipped with an autosampler and a nitrogen specific detector and was interfaced with an IBDH data processor. Injector and detector temperatures were maintained at 280°C. The oven temperature was programmed as follows: initial temperature 160°C (1 min); temperature program rates 5°C min^{-1} to 190°C followed by 10°C min^{-1} to 290°C. Helium was used as the carrier gas at an inlet pressure of 1.1 bar. Detector make-up flow rate was 25 ml min^{-1}.

Hydrolysis procedure

Two enzymatic hydrolysis methods were used:

(i) A 5 ml volume of urine in a screw-capped tube was buffered by adding 1 ml acetate buffer pH 5.2 and hydrolysed with 50 μl SHP (corresponding to 5000 Fishman units and 50,000 Roy units) during 2 h at 56°C.

(ii) A 1 ml volume of acetate buffer (pH 3.8) was added to 5 ml urine and the hydrolysis performed during 2 h at 56°C after the addition of 50 μl β-glucuronidase from *Patella vulgata* (corresponding to 5000 units).

Acid hydrolysis was done by adding 0.5 ml concentrated HCl to 5 ml urine and heating for 30 min at 100°C. After cooling, 0.5 ml 12 M NaOH was added to the hydrolysate.

Determination method

Codeine and morphine were assessed using a modified method [3]. Briefly, a small amount of sodium chloride was added to the hydrolysate followed by 0.5 ml ammonia buffer and 50 μl internal standard solution (ethylmorphine 50 μg ml^{-1} in methanol). After controlling the pH, the hydrolysate was extracted by rolling with 5 ml CH_2Cl_2–MeOH (9:1, v/v) for 15 min. After centrifugation the organic phase was dried over anhydrous Na_2SO_4 and evaporated under nitrogen at 50°C. The residue was redissolved in 100 μl ethyl acetate and 50 μl of TFAA was added. Derivatization was performed at 56°C for 30 min. The excess reagent was then removed by evaporation under nitrogen at 56°C and the residue was dissolved in 200 μl ethyl acetate and transferred to an autosampler microvial (200 μl); 1 μl was injected on the chromatograph.

A standard curve was constructed by analysing spiked urine samples (final concentrations 0.05, 0.1, 0.25, 0.50, 1 and 2 μg ml^{-1}) in quadruplicate for each concentration. The accuracy of the assay was measured for three different codeine and morphine concentrations.

Results are expressed as mean ± standard deviation (SD).

Results and Discussion

Under the chromatographic conditions described TFAA derivatized codeine, morphine and ethylmorphine gave sharp peaks with retention times of 12.63, 12.17 and 13.07 min, respectively. From the chromatograms obtained after the extraction of spiked urine, standard curves were generated. The calibration graphs showed good linearity between peak-area ratios and concentrations of 0–2 μg ml^{-1} with r^2 = 0.995 and 0.994 for codeine and morphine, respectively. The lower limit for accurate quantitative determination (signal-to-noise ratio = 4) was 0.05 μg ml^{-1}. The accuracy of the assay is summarized in Table 1.

The concentrations of codeine after the intake of Bisolvon Griblettes by seven subjects

Table 1
Accuracy of the codeine and morphine assay (n = 3)

Conc. added (μg ml^{-1})	Codeine	RSD* (%)	Morphine	RSD* (%)
0.10	0.100 ± 0.002	2.0	0.124 ± 0.010	8.1
0.50	0.499 ± 0.009	1.8	0.520 ± 0.040	7.7
2.00	2.014 ± 0.049	2.4	1.960 ± 0.130	6.6

*RSD = relative standard deviation.

and after different hydrolysis procedures are summarized in Table 2.

Generally maximum concentrations were obtained 1–2 h after the administration and codeine remained detectable 24 h post dosing in four out of the seven subjects. The intake of this antitussive codeine preparation in therapeutic amounts would result in a UCI positive doping test (>1 μg ml^{-1}) for at least 3 h (excepting Subject 4) while Subjects 1 and 5 should be positive even during 9 and 12 h, respectively (SHP hydrolysis).

The mean maximum codeine level in seven subjects was 5.1 ± 2.8 μg ml^{-1}. In previous work [3] where the codeine excretion after the therapeutic administration of different preparations was compared, the highest codeine concentrations were found after Bisolvon Griblettes respectively 5.1, 13.3, 6.4 and 9.3 μg ml^{-1} in four subjects. Taking into account these additional values, the mean maximum level would be 6.3 ± 3.4 μg ml^{-1} (n = 11). Introducing a permitted level for codeine implies admitting and consenting to the therapeutic use as an antitussive [4]. From the results obtained with Bisolvon Griblettes a codeine threshold level ($P < 0.5\%$; mean + 3 SD) of 16 μg ml^{-1} (SHP hydrolysis) is therefore proposed.

Hydrolysis of the urine is recommended as 52 or 58% of the dose is recovered as codeine glucuronide after an oral [5] or intramuscular administration [6], respectively. However, as illustrated in Table 2 noticeable differences in codeine concentration occurred depending on the hydrolysis procedure. Enzymatic hydrolysis with SHP resulted in the highest codeine values. The differences (Student t-test) between SHP and *P. vulgata* or acid hydrolysis were significant ($P < 0.001$). As it is known that β-glucuronidase cannot completely hydrolyse codeine-6-glucuronide and that substances naturally present in urine interfere with or inhibit the activity of β-glucuronidase [7, 8], acid hydrolysis was generally recommended for the cleavage of codeine conjugates. However, in this study better results were obtained with enzymatic hydrolysis. The destruction of many drugs, including morphine by the extreme conditions of acid hydrolysis [9] could account for the lower codeine values and for the fact that morphine was not detected in five out of seven subjects after acid hydrolysis (Table 3). Although morphine is excreted in free and conjugated form the relative amount after codeine administration is small compared to the amount of total codeine excreted [10]. This is confirmed in this work as morphine was scarcely detected after enzymatic hydrolysis with SHP (Table 3).

Inter-subject variability in the degree of metabolic conversion of codeine to morphine as noticed respectively after intramuscular [6] and oral [5] administration could account for the relatively higher morphine amounts and longer detection period in Subject 1. Previous work [11] in equine doping analysis has shown that *P. vulgata* is the preferred source of β-glucuronidase for cleaving morphine glucuronide. Nevertheless the values obtained here using comparable amounts of enzyme demonstrated that SHP seemed to be superior, confirming the results of Solans *et al.* [12] indicating that the hydrolysis of morphine-3-glucuronide is almost complete after enzymatic hydrolysis with β-glucuronidase from SHP.

Some side-effects of the opioids including drowsiness, respiratory depression, nausea and vomiting are clearly detrimental to athletic performance. Notwithstanding codeine was formerly a banned drug since in endurance sports it could be abused especially for its analgesic effect. Some sport federations however allowed codeine for therapeutic use or introduced permitted levels for codeine and morphine. As indicated previously [3] the proposed threshold value of 1 μg ml^{-1} for codeine was too low. Moreover it is clear that quantitative methods should be uniform preventing an athlete from testing positive or

Table 2
The influence of different hydrolysis procedures on the concentrations (μg ml^{-1}) of codeine after the intake of Bisolvon Griblettes

T	Subj. 1			Subj. 2			Subj. 3			Subj. 4			Subj. 5			Subj. 6			Subj. 7		
(h)	Ac	PV	SHP	Ac	PV	SHP	Ac	PV	SHP	Ac	PV	SHP	Ac	PV	SHP	Ac	PV	SHP	Ac	PV	SHP
2	1.82	2.85	3.28	1.63	1.90	2.37	3.20	4.30	4.42	4.66	4.78	5.06	5.66	5.70	6.09	3.05	1.74	3.10	3.06	3.12	3.57
4	1.40	2.66	2.73	2.27	4.08	5.01	1.06	1.66	1.85	0.54	0.65	0.76	10.19	10.35	11.25	1.43	0.81	1.54	2.90	2.58	3.40
6	1.26	1.50	1.75	2.25	3.18	4.64	1.00	1.04	1.32	0.42	0.38	0.56	2.38	2.48	2.98	0.64	0.49	0.57	1.45	1.46	1.70
9	1.49	1.53	1.68	0.53	0.56	0.64	0.27	0.31	0.40	0.48	0.45	0.60	1.61	1.46	1.74	0.16	0.00	0.18	0.84	0.57	0.69
12	0.52	0.56	0.67	0.08	0.10	0.16	0.09	0.17	0.22	0.24	0.24	0.31	1.68	1.40	1.64	0.10	0.00	0.10	0.19	0.17	0.19
24	0.13	0.13	0.13	0.11	0.08	0.12	0.00	0.00	0.00	0.18	0.00	0.20	0.34	0.20	0.38	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Ac, Acid hydrolysis.
PV, Enzymatic hydrolysis with β-glucuronidase from *P. vulgata*.
SHP, Enzymatic hydrolysis with β-glucuronidase–aryl sulphatase from Suc *Helix pomatia*.

Table 3
The influence of different hydrolysis procedures on the urinary concentrations ($\mu g\ ml^{-1}$) of morphine after the intake of Bisolvon Griblettes*

T	Subj. 1			Subj. 2		Subj. 3		Subj. 4	Subj. 5		Subj. 6	Subj. 7		
(h)	Ac	PV	SHP	PV	SHP	PV	SHP	SHP	PV	SHP	SHP	Ac	PV	SHP
2	0.83	0.76	1.96	0.00	0.40	0.76	0.71	0.35	0.00	0.00	0.58	0.91	0.64	1.46
4	1.40	1.04	1.76	0.95	0.74	0.00	0.00	0.00	0.70	1.65	0.62	0.98	0.71	1.50
6	0.96	0.44	1.45	0.00	0.15	0.00	0.00	0.00	0.00	0.69	0.00	0.75	0.00	0.66
9	0.53	0.54	1.08	0.00	0.28	0.71	0.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Only those subjects and hydrolysis procedures where morphine was found are shown.
Abbreviations as in Table 1.

negative depending on the analytical methodology. As demonstrated in this study noticeable differences were obtained depending on the hydrolysis procedure. In this way sporting federations allowing threshold levels for codeine and morphine should specify the analytical method and in particular the hydrolysis procedure by which these drugs should be quantified.

The highest codeine concentrations found after enzymatic hydrolysis with SHP however do not constitute a definite proof of complete hydrolysis. Therefore direct determination methods of codeine, morphine and the respective glucuronides using HPLC [5, 13–15] would be more reliable and accurate alternatives for the currently employed hydrolysis procedures. Finally, with respect to the interpretation of analytical findings it should be stressed that as only single untimed urine samples are collected from athletes several presently unknown factors including urinary pH and dilution could influence the urinary excretion of codeine and morphine. Moreover, several drugs have been shown to interfere with the metabolism and conjugation of codeine [16] while inter-ethnic differences in codeine excretion and metabolism were also noticed [13].

Therefore, since several non-banned analgesics and different non-narcotic antitussive preparations are available, a complete ban of codeine and morphine seems to be more appropriate and more adequate than the current regulations which provide little hold for the athletes and are open to misinterpretation.

Acknowledgements — The authors wish to thank Ms K. Buyle, Mr K. Roels and Ing. F. Maertens for their technical assistance. Thanks are also due to Mrs G. Demey for typing the manuscript.

References

[1] T.K. Adler, J.M. Fujimoto, E.L. Way and E.M. Baker, *J. Pharm. Exp. Ther.* **114**, 251–262 (1955).
[2] *Le monde cycliste*, April 1990, Report on the UCI Medical Commission meeting of 28 January 1990.
[3] F.T. Delbeke and M. Debackere, *J. Pharm. Biomed. Anal.* **9**, 959–964 (1991).
[4] *The Pharmacological Basis of Therapeutics* (A. Goodman Gilman, T.W. Rall, A.S. Nies and P. Taylor, Eds), p. 497. Pergamon Press, Oxford (1990).
[5] Z.R. Chen, A.A. Somogyi, G. Reynolds and F. Bochner, *Br. J. Clin. Pharmac.* **31**, 381–390 (1991).
[6] E.J. Cone, P. Welch, B.D. Paul and J.M. Mitchell, *J. Anal. Toxicol.* **15**, 161–166 (1991).
[7] C.-H.L. Shackleton, A.L. Charro-Salgado and F.L. Mitchell, *Clin. Chim. Acta* **21**, 105–118 (1968).
[8] E. Bodd, K.M. Beylich, A.S. Christophersen and J. Morland, *Pharmac. Toxicol.* **61**, 297–300 (1987).
[9] F. Fish and T.S. Hayes, *J. Forensic Sci.* **19**, 676–683 (1974).
[10] G.J. Mannering, A.C. Dixon, E.M. Baker III and T. Arami, *J. Pharmacol. Exp. Ther.* **111**, 142–146 (1954).
[11] J. Combie, J.W. Blake, T.E. Nugent and T. Tobin, *Clin. Chem.* **28**, 83–86 (1982).
[12] A. Solans, R. de la Torre and J. Segura, *J. Pharm. Biomed. Anal.* **8**, 905–909 (1990).
[13] Q.Y. Yue, J.-O. Svensson, C. Alm, F. Sjoquist and J. Sawe, *Br. J. Clin. Pharmac.* **28**, 629–637 (1989).
[14] Z.R. Chen, G. Reynolds, F. Bochner and A. Somogyi, *J. Chromatogr.* **493**, 313–324 (1989).
[15] C.P.W.G.M. Verwey-van Wissen, P.M. Koopman-Kimenai and T.B. Vree, *J. Chromatogr.* **570**, 309–320 (1991).
[16] Q.Y. Yue, C. Von Bahr, I. Odar-Cederlöf and J. Säwe, *Pharmac. Toxicol.* **66**, 221–226 (1990).

[*Received for review 4 September 1992; revised manuscript received 14 December 1992*]