Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 1, pp. 23–28, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

The influence of diuretics on the excretion and metabolism of doping agents — V. Dimefline

F.T. DELBEKE* and M. DEBACKERE

Laboratorium voor Farmacologie en Toxicologie der Huisdieren, Faculteit Diergeneeskunde, Rijksuniversiteit Gent, Casinoplein 24, B-9000 Gent, Belgium

Abstract: A sensitive method for the quantitative determination of the respiratory stimulant dimefline in 5 ml urine using capillary gas chromatography with nitrogen specific detection is presented. After the oral administration of a therapeutical amount of 16 mg dimefline to five subjects only 0.26 ± 0.16% of the dose is excreted as the conjugated drug in 24 h. The maximum excretion rate occurred 3 h after dosing, the peak concentration being 154 ± 60 ng ml^{-1}. The influence of diuretics taken 2 h after the administration of dimefline was studied in three subjects. From these results it appeared that the use of acetazolamide and hydrochlorthiazide in order to circumvent a positive dimefline doping case is questionable. Due to the potent diuretic effect of furosemide, the intake of this diuretic could result in a suppression of the dimefline concentration below the detection limit of 10 ng ml^{-1}.

Keywords: *Dimefline; acetazolamide; hydrochlorthiazide; furosemide; diuretics; doping.*

Introduction

The use of respiratory stimulants including dimefline {8[(dimethylamino)methyl]-7-methoxy-3-methyl-2-phenyl-4H-1benzopyran-4-one}, prethcamide, fenspiride and doxapram is not allowed in competitive sports. Therefore these drugs figure on the list of doping agents issued by the Flemish Community Executive in 1987.

Although a number of pharmacological and clinical studies have been carried out [1], little work has been published on the renal elimination and metabolism of dimefline, excepting the study by Scanni *et al.* [2]. These authors assayed the urinary concentration of dimefline and metabolites with a spectrodensitometric procedure after thin layer chromatography, but they considered the metabolites also as dimefline in the quantitative study.

In order to perform doping analysis properly it is obvious that not only a detection method should be elaborated but that the metabolism and the urinary excretion of forbidden substances need to be studied too. Furthermore as improved detection methods have led to a lengthening of the time period during which a drug can be detected, these detection times should be reconsidered. Therefore, a sensitive gas chromatographic method for the assay of dimefline is reported and applied here.

Previous studies [3–6] on the influence of diuretics on the excretion of doping agents revealed that loop diuretics generally exert a diluting effect, whilst the carbonic anhydrase inhibitor acetazolamide suppressed the excretion of weak basic drugs during at least 22 h. From studies in horses [7] it appeared that the diluting effect of furosemide was much more pronounced for drugs excreted in the conjugated form. As the respiratory stimulant dimefline was reported to be excreted as a conjugate [2], it could be interesting to study the influence of three differently acting diuretics on the excretion of this drug.

Experimental

Reagents and apparatus

Dimefline·HCl and fentanyl citrate were gifts of Bournonville-Pharma (Braine-l'Alleud, Belgium) and Janssen Pharmaceutica (Beerse, Belgium), respectively. Dichloromethane, methanol and diethyl ether (analytical grade) were from Merck (Darmstadt, FRG). The ammonia buffer was a saturated ammonium chloride solution adjusted to pH 9.5 with ammonia. *Helix pomatia* juice (SHP) containing β-glucuronidase 100,000 Fishman units ml^{-1} and sulphatase 1,000,000 Roy units ml^{-1} was from IBF (Villeneuve, France). β-Glucur-

* Author to whom correspondence should be addressed.

onidase (*Escherichia coli*) from Sigma (St. Louis, USA).

A Model 3700 chromatograph (Varian, Walnut Creek, CA, USA) equipped with a nitrogen specific detector (NPD) was adapted for capillary gas chromatographic work by installing an inlet splitter kit from Chrompack (Antwerp, Belgium). All chromatograms were generated in the split mode (split rate 1:10) using a 25 m × 0.22 mm i.d. fused silica methylsilicone CP-Sil 5-CB column (Chrompack) with a film thickness of 0.12 μm.

Injector and detector temperatures were monitored at 280 and 300°C, respectively. The oven temperature was programmed as follows: initial temperature 210°C, initial hold 1 min, temperature programme rate 6°C min^{-1}, final temperature 265°C. Helium was used as the carrier gas at an inlet pressure of 18 psi. Detector make-up flow rate was 30 ml min^{-1}. A Shimadzu CR-4A integrator was used for quantitative work.

Determination method

Five millilitres of urine, in a screw capped tube, were buffered with 1 ml 1 N sodium acetate buffer (pH 5.3) and 100 μl SHP was added. The urine was hydrolysed during 2 h at 52°C. After cooling, the hydrolysate was made alkaline by adding 0.5 ml ammonium buffer followed by 50 μl internal standard solution (fentanyl citrate 20 μg ml^{-1}) and extracted by rolling with 5 ml CH_2Cl_2–CH_3OH (9:1, v/v) during 15 min. After centrifugation, the organic phase was transferred to a screw capped tube and evaporated under nitrogen at 50°C. The residue was dissolved in 1 ml 0.1 M HCl and vortexed with 2 ml diethyl ether for 30 s. The organic phase was discarded and the aqueous solution made alkaline by adding 0.5 ml ammonium buffer. Extraction with 5 ml CH_2Cl_2–CH_3OH (9:1, v/v) was performed by rolling for 15 min.

After centrifugation (5 min) the organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated under nitrogen at 50°C. The residue was redissolved in 50 μl of a mixture ethyl acetate and methanol (9:1), and 1 μl injected. A stock solution containing 50 μg ml^{-1} of dimefline, calculated as free base, was prepared in methanol. Different volumes of diluted stock solutions were put into screw capped tubes and evaporated under nitrogen at 50°C. The residues were dissolved in 5 ml blank urine to give final concentrations of 0, 10, 25, 50, 100, 250 and 500 ng ml^{-1}. These spiked urine samples were then subjected to the above extraction procedure in quadruplicate for each concentration. From the chromatograms obtained, a standard curve was generated by plotting the dimefline to fentanyl peak height ratio against the dimefline concentration. The accuracy of the assay was measured by adding different amounts of dimefline to blank urine.

Human investigations

The five male subjects (non-smokers) granted written informed consent prior to participation in this study.

In the first series of experiments two tablets of Remefline^R equivalent to 16 mg dimefline were given orally to five human volunteers at 09.00 a.m. On a weekly basis, in three further experiments three of the five volunteers each received the same dose of dimefline at 09.00 a.m. followed by either 250 mg acetazolamide, 40 mg furosemide or 25 mg hydrochlorthiazide 2 h later. In order to control the diuretic effect of caffeine, volunteers abstained from coffee, tea and other caffeine containing beverages during the day of the experiment. All the urine was collected in capped bottles during fixed periods (0, 1, 2, 3, 4, 6, 9, 12 and 24 h) after the ingestion and either used immediately or stored deep-frozen for later analysis. Urinary volume was measured and an aliquot stored in capped bottles. Excepting the samples collected in the evening, the urinary pH was measured as soon as the urinary temperature reached 25°C. All samples were analysed in duplicate.

Results and Discussion

Urinary extraction under normal conditions

Under the chromatographic conditions described herein dimefline and fentanyl gave sharp peaks with retention times of 9.28 and 9.74 min, respectively. Typical chromatograms obtained by processing control urine and urine after the intake of Remefline^R are shown in Fig. 1.

The statistical data for slope, *y*-intercept and the correlation coefficient of the standard curve were 14.97, 0.82 and 0.989, respectively. Starting with 5 ml urine, the detection limit for quantitative work was 10 ng ml^{-1} (signal to noise ratio = 3).

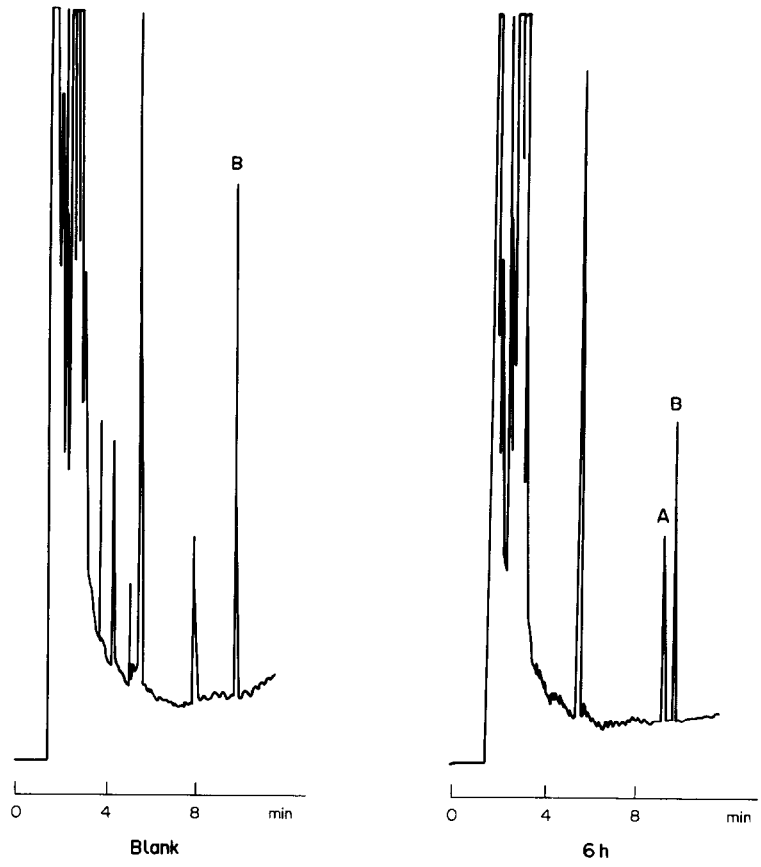

Figure 1
Chromatograms obtained after the extraction of blank urine and urine 6 h after the intake of two tablets of Remefline^R. Peak A, dimefline; peak B, internal standard fentanyl.

Using the described extraction method and adding different amounts of dimefline to blank urine the accuracy of the assay method was evaluated and summarized in Table 1. The recovery is moderate but reproducible.

The time peak for excretion of dimefline, the maximal concentration and the percentage excreted over a 12-h period in five subjects are given in Table 2.

Generally, dimefline excretion peaks at 3 h with a mean maximum concentration value of 154 ± 60 ng ml^{-1}. The proportion of the dose excreted as conjugates after 12 h is very low and varies from 0.15 to 0.52%, with a mean value of 0.26 ± 0.16%. Furthermore, compared with the cumulative excretion after 6 h (0.23 ± 0.11%) there is no significant difference, indicating that the renal clearance of conjugated dimefline virtually ceases after 6 h.

It is well known that the urinary excretion of weak basic drugs such as amphetamines is strongly correlated with the urinary pH [8]. However, for dimefline such an influence was not found either in the excretion pattern or in the cumulative excretion as a function of the mean urinary pH (Table 1).

Generally, when a therapeutic dose of 16 mg is administered orally, dimefline remains de-

Table 1
Recovery and accuracy of the assay method (n = 4 for each concentration)

Amount of dimefline added (ng)	Amount calculated (ng)	Recovery (%)
25	25.3 ± 2.9	69 ± 3
100	94.8 ± 3.0	64 ± 2
250	251.0 ± 19.0	63 ± 3

Table 2. Mean urinary pH, excretion peak time, maximum concentration and cumulative percentage of the dose excreted after the oral intake of 16 mg dimefline (Remefline^R)

Subject	Peak (h)	Max. conc. (ng ml^{-1})	% Cumulative excretion after 2 h	4 h	6 h	12 h	Mean urinary pH*
1	3	106	0.07	0.18	0.20	0.20	5.5
2	3	112	0.02	0.09	0.15	0.15	5.7
3	3	173	0.08	0.26	0.41	0.52	6.4
4	4	251	0	0.19	0.27	0.30	6.1
5	2	127	0.07	0.12	0.13	0.13	5.7

*Calculated as Σ pH$_i$ Δt_i/g.

tectable in urine from 2 h on till 9 h, as illustrated in Fig. 2. This period is considerably shorter, compared with the detection time periods of at least 72 h when therapeutical amounts of amphetamines are administererd [5]. In order to detect dimefline in the urine samples hydrolysis with SHP is required. When unhydrolysed urine was analysed, dimefline was found in the 3–4-h samples in three out of five subjects. However, the amount recovered only represents 0.013–0.025% of the administered dose. When buffered urine samples were heated during 2 h at 52°C and subsequently analysed, dimefline was not detected. On the other hand, hydrolysis with glucuronidase from *E. coli* gave rise to the same results as found with the mixture of glucuronidase and sulphatase in SHP, proving that dimefline is conjugated with glucuronic acid.

Finally, using this gas chromatography method, no metabolites were detected in the urine samples.

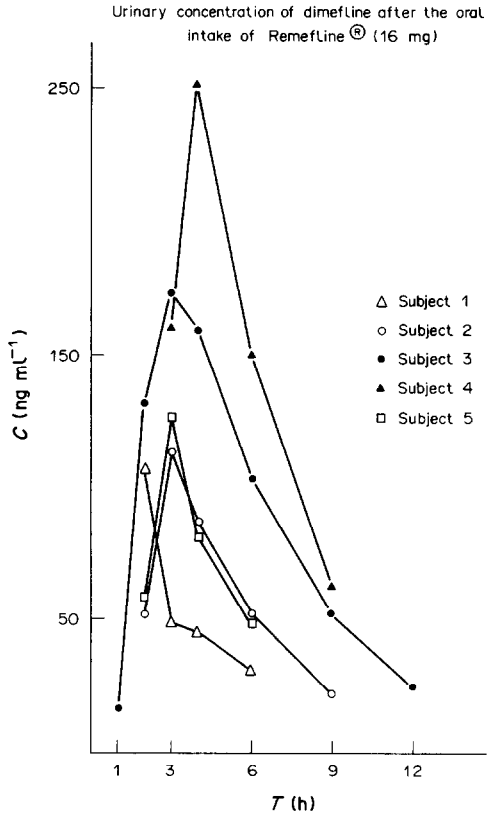

Figure 2
Urinary dimefline concentration–time curve in five subjects.

Effect of diuretics

The effect of three different diuretics including furosemide, acetazolamide and hydrochlorthiazide on the cumulative excretion of dimefline is summarized in Table 3.

When acetazolamide is administered as a diuretic in order to circumvent a positive test result, one could say that its use is rather questionable. From our results it appeared that the influence of acetazolamide on dimefline concentration is strongly connected with the diuretic effect. Indeed, in subjects 1 and 5, dimefline is quantitatively detected (>10 ng ml^{-1}) in urine samples till 6 h (4 h post-diuretic). On the other hand, the high urinary flow of 600 ml h^{-1}, 2 h after acetazolamide in subject 2 prevents the quantitative detection of dimefline. From this point of time onwards, however, a dimefline peak corresponding to a concentration below 10 ng ml^{-1} was observed in the chromatograms until 6 h after the intake. Due to the low amount, this peak could not be accurately quantified. It should be clear that, due to the clean extracts and the resulting low chromatographic background, increasing the urine volume or the detection sensitivity could be helpful in order to detect dimefline in those exceptional cases where the diuretic effect of acetazolamide is potent and short-lived. The fraudulent use of acetazolamide in competitive sports is mainly based on the carbonic anhydrase inhibiting effect of this diuretic, resulting in an alkaline urine and

Table 3
Influence of diuretics on the cumulative excretion of dimefline

	Subject	Normal	Furosemide	Acetazolamide	Hydrochlorthiazide
Cumulative excretion after 9 h (μg)	1	32	16	32	17
	2	24	32	15	24
	5	21	27	25	24
Diuresis time period	1		½–2 h	1–2	1–2
	2		1–2 h	1–2	2–7(?)
	5		½–2 h	1–4	1–7(?)
Mean urinary flow (ml h^{-1}) during diuresis	1		590	295	182
	2		353	425	146
	5		631	330	178

tubular reabsorption of the weak basic drugs [3–5]. From the results obtained here, however, the change in urinary pH has little effect on the excretion of dimefline. This could be due to the fact that dimefline is excreted as a conjugate.

It is well known that the diuretic effect of furosemide is potent and short-lived [3–5]. As illustrated in Table 3 the diuresis after 40 mg Lasix^R lasts from 0.5 to 2 h with high urinary flow rates. In the case of stimulant amines, the urinary concentration dropped by a factor 3–4 after the intake of furosemide, but the drugs remain detectable [3–5]. Due to the relatively low dimefline concentration values under normal conditions the administration of furosemide, however, could result in very low dimefline values. In subject 1 with a high urinary flow rate after furosemide, the dimefline concentration is suppressed below the detection limit of 10 ng ml^{-1} during 1.5–2 h after furosemide.

In subject 2, with a moderate response to the diuretic effect of furosemide, dimefline remains detectable, although its concentration dropped by a factor of 2–3 during 1–2 h after the intake of the diuretic (Fig. 3). Finally, dimefline was detected in the urine of subject 5, half an hour post-diuretic but afterwards was suppressed below the detection limit for quantitative work of 10 ng ml^{-1} due to the high urinary flow rate (Fig. 4). As with acetazolamide in subject 2, the influence of furosemide on dimefline excretion should be mainly attributed to the high urinary flow rate. These results also correspond with earlier findings in horses [7], where furosemide exerts a considerable effect on drugs excreted as conjugates.

The influence of the thiazide diuretic hydrochlorthiazide is not obvious. The time period of

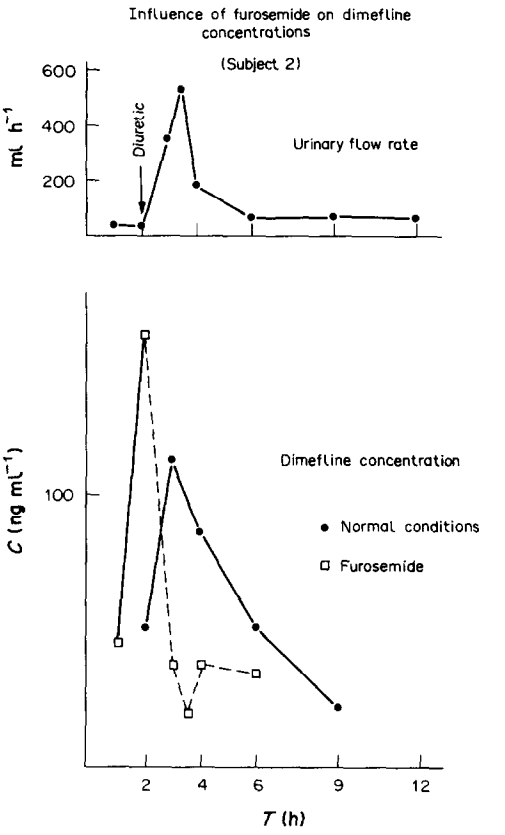

Figure 3
Influence of furosemide on the urinary dimefline concentration in subject 2.

diuresis is rather extended and could not be defined properly in the three subjects. As hydrochlorthiazide gives rise to a moderate diuretic effect, its use in order to circumvent a positive dimefline doping case is rather questionable since the respiratory stimulant remains detectable in the three subjects.

In earlier work it was demonstrated that the fraudulent use of diuretics to suppress the caffeine concentration below the doping value

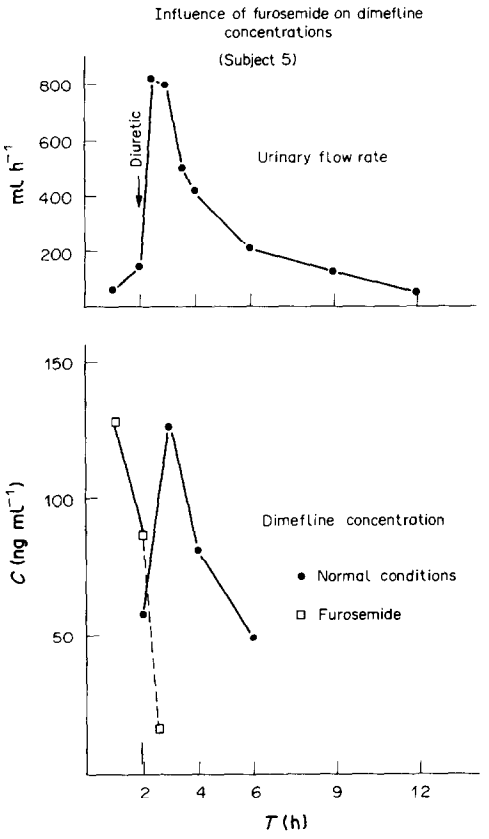

Figure 4
Influence of furosemide on the urinary dimefline concentration in subject 5.

of 12 μg ml^{-1} was senseless as the caffeine excretion depends on the urinary flow rate [6]. The use of diuretics in order to circumvent a positive doping test for stimulant amines including mephentermine [3], phentermine [4] and ethylamphetamine [5] was rather questionable, excepting the administration of the carbonic anhydrase inhibiting drug acetazolamide. From this work, however, the use of furosemide in order to mask the excretion of drugs like dimefline showing up low urinary concentrations under normal circumstances and being excreted as conjugates, could be successful. For these reasons it should be clear that diuretics should be banned in competitive sports.

Acknowledgements — The authors wish to thank de Bournonville and Janssen Pharmaceutica for the gifts of the reference compounds. Ing. B. Van Belleghem, Ing. F. Maertens and Miss M. Geerinck are gratefully acknowledged for their technical assistance. Thanks are also due to Mrs G. Demey for typing the manuscript and to the NFWO for financial support.

References

[1] G. Gunnella and C. Galleri, *Sett. Ospedali* **19**, 1–9 (1977).
[2] A. Scanni, M. Biraghi, M. Tomirotti and A. Cova, *Boll. Chim. Farm.* **116**, 353–357 (1977).
[3] F.T. Delbeke and M. Debackere, *J. Pharm. Biomed. Anal.* **3**, 141–148 (1985).
[4] F.T. Delbeke and M. Debackere, *Arzneim. Forsch. Drug Res.* **36**, 134–137 (1986).
[5] F.T. Delbeke and M. Debackere, *Arzneim. Forsch. Drug Res.* **36**, 1413–1416 (1986).
[6] F.T. Delbeke and M. Debackere, *Biopharm. Drug Disp.* **9**, 137–145 (1988).
[7] T. Tobin, B.L. Roberts and J.R. Miller, *J. Equine Med. Surg.* **1**, 402–409 (1977).
[8] A.H. Beckett and M. Rowland, *J. Pharm. Pharmacol.* **17**, 628–639 (1965).

[Received for review 4 October 1989; revised manuscript received 28 September 1990]