MAYNARD: In British Columbia, they also extracted some degradation products of penicillin which were picked up on the GC mass spectrometer. They are investigating it at present.

TOBIN: Model 2 assumes a single site from which absorption of an intra-muscular injection occurs and there is a discrepancy. We can only account for about half the drug absorbed and we assume a single first order rate of absorption.

DEBACKERE: Is it a question of protein-binding?

TOBIN: I see no reason if it is a simple question of protein binding why the simple model should fit subcutaneous but not fit intramuscular administration, it is an event subsequent to absorption, protein-binding occurring in the central compartment. We have to assume binding in the tissue of some sort, this seems to be practised by the drug right through. The calculated half-life is a little slower than we observed for subcutaneous administration, the same, however, was much more marked for intramuscular and the same applied to procaine penicillin and intra-articular injections. There are also discrepancies between the urinary levels and the plasma levels. Some sort of binding seems to be a constant characteristic of the kinetics of this drug.

DEAN: Following a normal therapeutic dose of procaine penicillin injection, for how long could an analytical chemist detect it in the urine?

TOBIN: It would depend on the analytical chemist! We reckon to find the drug for 2 weeks.

BLAKE: The GC derivative method that Tobin was referring to, detects about 1 to 2 picograms injected on the column, depending upon how large blank is.

LAMBERT: With the routine method used in Ireland, we are finding procaine penicillin after about 40 hours, but we wouldn't go on looking for it for 2 weeks.

JAGGARD: We cannot find procaine HCl after 24 hours by our routine procedure. With procaine penicillin we find it for maybe 48 hours, with azomycin, we can find it for 3 or 4 days but that is our limit.

EXCRETION AND METABOLISM OF NIKETHAMIDE IN THE HORSE

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ABSTRACT

It is well known that nikethamide (N,N-diethylnicotinamide, Coramine^R) is metabolized very rapidly to nicotinamide. Hence, there is difficulty in proving that nikethamide has been used as a doping substance because nicotinamide is a normal physiological metabolite in the organism as well as a vitamin preparation. However, an intermediate metabolite (N-ethylnicotinamide) was found by us in the urine of horses treated with Coramine^R. This was characterized by gas chromatography/mass spectrometry, and synthesized and identified as being N-ethylnicotinamide.

The excretion and metabolism of nikethamide after intramuscular injection in the horse was followed using quantitative gas chromatography of urinary extracts over a period of several hours and the results of these experiments are reported.

Changes in urinary pH had no significant effect upon either the metabolism or rate of excretion of the drug.

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Introduction

N,N-diethylnicotinamide (nikethamide) is a widely used respiratory stimulant for oral and parenteral use which is formulated alone or in multi-component pharmaceutical dosage forms.

After oral administration of nikethamide (CORAMINER[®] Ciba) to human beings, an unknown substance was isolated from the urine by means of extraction and TLC (thin layer chromatography) procedures. This substance was identified as N-ethylnicotinamide, an intermediate metabolite of nikethamide. The metabolism and excretion of nikethamide was followed in the horse after intramuscular injection, by means of qualitative and quantitative GLC measurements of both substances.

Several papers on thin layer chromatographic methods for the detection of nikethamide in biological materials have been published (1, 2, 3, 4). Infra-red and ultra-violet spectrophotometric determination of the drug extracted from saliva following its isolation by TLC have also been described in connection with the control of doping in race-horses (5). Qualitative gas liquid chromatographic determinations with a range of stationary phases have been published for nikethamide alone and in the presence of nicotinic acid (6, 7). Quantitative essays of nikethamide in pharmaceutical dosage forms (8) and recently in injectable preparations (9) have been described using spectrophotometric and gas liquid chromatographic methods respectively.

Metabolism of Nikethamide

In 1974 the central nervous stimulant nikethamide was added to the list of forbidden drugs published by the Medical Commission of the International Cyclists Union. As our laboratory is also involved in the doping control of cyclists a study was undertaken to detect nikethamide in human urine. Using the extraction and TLC procedures already described in an earlier paper (4) and slightly modified as mentioned here we found that after oral administration of the drug a spot with an R_f value different from that of nikethamide occurred on the silica gel plate.

Fig 1. The Metabolism of NIKETHAMIDE

Since it is well known that nikethamide is metabolized finally to nicotinamide which is a normal physiological metabolite as well as a vitamin preparation, it was assumed that the additional spot resulted from the intermediate metabolite N-ethylnicotinamide (Fig. 1) and that the presence of this compound could be used to prove a 'positive' nikethamide case. N-ethylnicotinamide was occasionally obtained during the synthesis of CORAMINER[®] as a result of the presence of monoethylamine impurity in the diethylamine used. Nevertheless the two solvent systems used in the quantitative TLC determination (8) failed to separate this compound from nikethamide. After the administration of nikethamide to guinea-pigs, Chambon (10) using a spectrophotometric method detected only the water soluble N-oxide derivative of N,N-diethylnicotinamide. No nikethamide was found in the urine. Amine oxides of nicotinamide were also found in biological materials of rats (11) and mice (12, 13, 14, 15). After the injection of nikethamide in horses the substance could be detected by a TLC method equally well in urine, blood and saliva but only up to 3 hours after its administration (4).

Extraction Procedure and GLC of Nikethamide and N-Ethylnicotinamide

Synthesis of N-Ethylnicotinamide

Nicotinic acid (12.3 g) and freshly distilled thionylchloride (18.0 g) were heated with occasional shaking for 1 hour or until the evolution of hydrogen chloride ceased.

The unchanged thionylchloride was distilled off and the contents of the flask washed with benzene, filtered and dried. The residue was then treated with a monoethylamine solution (70% in water), made alkaline with 50% sodium hydroxide solution and extracted with chloroform.

After evaporating the extract *in vacuo*, the resulting oil was redissolved in ether and purified by bubbling hydrochloric acid in the solution giving rise to the crystalline N-ethylnicotinamide hydrochloride.

This compound was dissolved in water, the solution made alkaline and extracted with chloroform. The chloroform extract was distilled under reduced pressure (160°C/1mm Hg) to obtain the crystalline N-ethylnicotinamide (m.p. 59°C).

The structure of this derivative was confirmed by chemical ionization mass spectrometry. Field ionization mass spectrometry yielded the molecular ion peak at m/e 170.

Since the metabolite nicotinamide is a normal physiological substance and moreover very slightly soluble in

chloroform (Recovery: 0%), the metabolism of nikethamide was studied by following the excretion of nikethamide and N-ethylnicotinamide.

Urine (25 ml) was pipetted into a glass-stoppered tube. After adjusting the pH to 11-12 with a few drops of a 50% sodium hydroxide solution, the urine was extracted three times with chloroform (7 ml) using a mechanical shaker (20 minutes), centrifuged, and the organic phase separated. The combined chloroform extracts were then dried over anhydrous sodium sulphate, treated with 1 ml of an ethereal solution of hydrochloric acid and evaporated to dryness *in vacuo* at 40°C. For qualitative work the extract was transferred to a small tube using 1% diethylamine (1 ml) in ether and evaporated at ambient temperature. The extract was redissolved in chloroform, methanol or diethylether (20 μ l) and gas chromatographed (1-2 μ l).

Fig. 2. GC of an urinary extract of NIKETHAMIDE and N-ETHYLNICOTINAMIDE

For quantitative purposes 0.5 ml (or 0.25 ml) of an internal standard solution (0.5 μ g/ μ l benzphetamine in diethylamine:chloroform, 1:100) was added and 1 μ l injected into the gas chromatograph. Gas chromatography was performed using a VERSAMID 900 column. The conditions are described in Fig. 2.

Recovery using the Extraction Procedure

The standard curves were obtained using different concentrations of nikethamide and N-ethylnicotinamide dissolved in chloroform containing 0.5 μ g/ μ l benzpheta-

Fig. 3. STANDARD CURVE N,N-DIETHYLNICOTINAMIDE

mine. The regression equations are given in Figures 3 and 4. After addition of nikethamide and N-ethylnicotinamide to blank urine in order to obtain final concentrations of respectively 2.5-5-10 μ g/ml and 5-12.5-25-50 μ g/ml four urine samples for each concentration were extracted and gas chromatographed as described above. The peaks were integrated using a VARIAN CDS 101 Integrator. The recoveries obtained were 80.4% ($\sigma = 3.016$) and 35.8% ($\sigma = 1.875$) for nikethamide and N-ethylnicotinamide respectively.

Experimental Animals and Sampling Techniques

Six mares weighing from 375 to 450 kg were fasted for 24 hours up to the time of the intramuscular injection of the drug. Doses varied from 2.5 to 4.0 gm. Half an hour before the experiment a quantity of water equal to 1% of the body weight was administered by stomach tube to obtain a good diuresis. For two of the six horses used (Nos. 5 and 6) an additional experiment after treatment with ammonium chloride was carried out. For this treatment the following procedure was used: 24 Hrs. before the experiment 10 g ammonium chloride dissolved in 2 l water was administered by stomach tube and oats treated with 5 g ammonium chloride given to the horse, followed by 5 g ammonium chloride mixed in the oats 18 Hrs. before the injection. Immediately before the administration of the drug another dose (10 g + 5 g ammonium chloride) was given by the same procedure as that used 24 hr before.

In all but one experiment (Horse No. 1) urine was collected every hour from 0 hr. to 6 hr. and every three hours from 6 hr. to 12 hr. after the injection using a bladder catheter.

A rubber endotracheal tube (40 cm in length and 1 cm in diameter) provided with a thin-walled balloon served as a bladder catheter. After introducing the

Fig. 4. STANDARD CURVE N-ETHYLNICOTINAMIDE

catheter into the bladder and blowing up the balloon with air, the catheter was connected to a plastic tube which conveyed the urine continuously into a plastic receptacle.

After measuring the volume and the pH, 25 ml aliquots of the urine samples were immediately extracted in quadruplicate and gas chromatographed as described above.

Results and Discussion

The results of the experiments are summarized in Table I and II and a typical pattern in the excretion and metabolism is given in Figs. 5 and 6.

1. Excretion of Unchanged Nikethamide

The total amount of unchanged nikethamide excreted during the 9 hour-period after the injection showed very great individual variations between the six horses without ammonium chloride pretreatment. This was probably due partly to the difference in volume of urine excreted but also to the excretion rate of the product itself as can be seen from the average concentrations expressed in $\mu\text{g}/\text{ml}$. This varied between 1.8 and 6.79 $\mu\text{g}/\text{ml}$ or a 3-4 fold variation. For the horses No. 2 and No. 4 the total amount of unchanged nikethamide excreted in the urine was extremely high by comparison with the amounts for the other horses. For the last horse mentioned this can be attributed to the great volume of urine excreted, mainly during the first two hours, but for the first horse this was not the case and here the high concentration of nikethamide must be responsible. It seemed possible that this was due to the lower pH values (7.5 – 7.7) in the beginning of the experiment (0-2 hr)

Fig. 5. Urinary pH and Volume

with respect to the normal pH values (8.2 – 8.8). However, by relating the pH value to the concentration for each urine sample it was seen that the fluctuation of the pH during all the experiments had no significant influence on the concentration of nikethamide in the urine.

For all the horses injected the total amount of unchanged drug excreted in the urine was only a very small percentage (0.07 – 0.37) of the dose administered. The maximum excretion of nikethamide generally occurred two hours after its administration, except for horse No. 4 which received 4 l water two hours before the injection of the drug, resulting in a great volume of urine

Fig. 6. Urinary pH and Volume

Excretion (mg / Hr)
Concentration (ppm)
HORSE 8 - NH_4Cl
WEIGHT : 425 Kg
INJECTED : 3.750 g CORAMINE®
○ Nikethamide
△ N-EthylNicotinamide

at the beginning of the experiment. This was also the case for the total excretion (mg/hr) as well as for the concentration ($\mu\text{g}/\text{ml}$). After 5-6 hours the concentration of nikethamide reached a minimum value below the detection limit of $0.4 \mu\text{g}/\text{ml}$ urine. Nevertheless, using the qualitative procedure described above a detection limit of $0.2 \mu\text{g}/\text{ml}$ in urine was obtained resulting in a possible detection of nikethamide even after 6 hr.

The low percentage of unchanged drug excreted, together with the very quick disappearance of nikethamide from the urine suggest that nikethamide is metabolized very rapidly in the horse, in which case this should be

confirmed by the excretion pattern of its metabolite N-ethylNicotinamide.

2. Excretion of N-ethylNicotinamide

For all the horses studied there was already a marked excretion of this metabolite 1 hr after the injection of nikethamide. Furthermore the rate of excretion of the metabolite as well as its concentration increased progressively to a maximum level at 3-4 hours after the injection. Thereafter the elimination and concentration curves showed a very slow decline so that even 12 hours after the injection pronounced quantities of the metabolite could still be detected (detection limit $1 \mu\text{g}/\text{ml}$). This means that despite the rapid metabolism of nikethamide the elimination of one of its metabolites takes at least twice as long as for the original drug. Hence, in the control of doping in racehorses, a "positive" case for doping with nikethamide could easily be proved by means of the extraction and GLC procedures described, even 12 hours after the administration of the drug. This conclusion is only valid for urine samples and not for saliva. Indeed saliva samples have been examined with the same qualitative procedure up to 3 hours after the injection, but, despite nikethamide being detectable in urine up to 6 hours and N-ethylNicotinamide up to 12 hours after the injection, none of these substances could be detected in the saliva samples examined.

Nevertheless, except for two cases (horses Nos. 1 and 5) the maximum concentration of N-ethylNicotinamide was never higher than that for nikethamide. For the quantity of metabolite excreted per hour this was only the case in 2 of the 6 experiments (Nos. 2 and 4). The total amount of the metabolite eliminated within 12 hours after the injection was at least 1.5-2 times higher than for the original drug except for the horse No. 4 and this was due to a loss of urine during this experiment between 6 and 9 hrs. As for the unchanged drug itself the percentage of the original dose excreted as N-ethylNicotinamide was very low with a mean value of 0.34% ($\sigma = 0.116$), although it must be emphasized that this percentage was at least twice as high as that for the original drug. This means that for a given dose of nikethamide the quantity of its metabolite N-ethylNicotinamide excreted is at least double that of the parent drug. It seems therefore that the total amount of nikethamide together with its metabolite N-ethylNicotinamide, detectable in the urine of horses up to 12 hours after the injection together do not exceed 1% of the original dose. This may be because nikethamide is very rapidly metabolized, and because N-ethylNicotinamide is only an intermediate in the metabolism of nikethamide to nicotinamide, which was not determined because this compound is not extracted by the procedure described. However it is possible that alternative metabolic pathways also exist in the horse such as have been demonstrated for guinea-pigs (10), rats (11) and mice (12, 13, 14, 15). This needs further investigation.

3. Excretion of both Nikethamide and N-EthylNicotinamide after Acidification of the Urine.

As already mentioned, the normal pH fluctuations in the range of 6.0-8.8 during the different experiments had no significant influence on the concentration of either nikethamide or N-ethylNicotinamide in the urine.

In order to evaluate the influence of the urinary pH on the excretion of both substances an attempt was made to acidify drastically the urinary pH below 6 by means of ammonium chloride as described above. Therefore horses Nos. 5 and 6 were submitted to an additional experiment (Nos. 5A & 6A) after treatment with ammonium chloride. As can be seen from the results the urinary pH dropped between 5.0 and 5.3 in contrast to the normal values of 6.0-8.6. By comparing the results for the horse 5 with 5A and 6 with 6A respectively it is not possible to conclude that there were distinct changes in the excretion pattern of the original drug and its metabolite, except perhaps that the total amount of nikethamide and N-ethylNicotinamide excreted over the whole experimental period was much higher for horse No. 6 after ammonium chloride treatment than before.

However, this difference did not show up in horse No. 5. In the second experiment No. 6A on horse No. 6, the maximum level of nikethamide was reached at 1 hr, instead of the 2 hr observed previously, without the ammonium chloride pretreatment. Again there was no difference in the other horse, No. 5, whether he had had the ammonium chloride pretreatment or not. The one and only similarity for both pairs of experiments was the higher average concentration for the two substances under acidic conditions. However these concentrations were still in the range of the values for the other experiments without acidified urine.

central stimulant amines, the excretion of nikethamide and its metabolite N-ethylNicotinamide in the urine of horses is scarcely influenced by changes in the urinary pH, even when this occurs over a range of more than .3 pH units.

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TABLE I
NIKETHAMIDE (mg/hr) and Volume of Urine (ml/hr)
Excreted by the Horse

Horse No.	1	2	3	4	5	6	Repeat with ammonium chloride pre-treatment	
							5A	6A
Weight (Kg)	375	425	575	425	450	425	450	425
CORAMINE injected (g)	2.5	3.75	4.0	3.75	3.75	3.75	3.75	3.75
Urinary pH	7.4-8.8	7.4-8.3	7.5-8.6	7.4-8.7	7.0-8.4	6.0-8.6	5.0-5.3	4.9-5.3
1 Hr	0.952 (195)	1.543 (370)	1.153 (108)	6.390 (1795)	0.615 (315)	0.237 (237)	0.133 (100)	1.763 (127)
2 Hr	1.000 (170)	3.719 (330)	0.608 (32)	5.196 (1280)	1.576 (325)	1.066 (185)	1.254 (202)	1.424 (130)
3 Hr	0.497 (130)	2.565 (380)	2.048 (154)	0.423 (149)	0.977 (740)	0.424 (120)	0.445 (152)	1.096 (455)
4 Hr		1.514 (320)	0.449 (60)	0.531 (196)	0.445 (500)	0.457 (225)	0.170 (129)	0.360 (580)
5 Hr		0.711 (280)	0.552 (180)	0.936 (600)	0.159 (261)	0.237 (275)	0.131 (180)	— (440)
6 Hr	0.130 (245)	0.360 (310)	0.192 (198)	0.259 (267)	— (115)	0.057 (335)	— (27)	0.101 (202)
9 Hr	— (405)	0.070 (160)	— (175)	— (100)	— (230)	— (288)	— (232)	— (513)
TOTAL EXCRETED(mg)	2.839	10.623	4.972	13.735	3.772	2.478	2.133	4.744
% EXCRETED	0.11	0.28	0.12	0.37	0.10	0.07	0.06	0.13
AVERAGE CONCENTR. µm/ml	2.31	4.30	6.79	3.23	1.89	1.80	2.80	3.18

The figures between brackets refer to the urinary volume (ml/hr).

TABLE II
N-ETHYLNICOTINAMIDE (mg/hr) and Volume of Urine (ml/hr)
Excreted by the Horse

Horse	1	2	3	4	5	6	Repeat with ammonium chloride pre-treatment	
							5	6
Weight (Kg)	375	425	575	425	450	425	450	425
CORAMINE injected (g)	2.5	3.75	4.0	3.75	3.75	3.75	3.75	3.75
Urinary pH	7.4-8.8	7.4-8.3	7.5-8.6	7.4-8.7	7.0-8.4	6.0-8.6	5.0-5.3	4.9-5.3
1 Hr	0.337 (195)	0.371 (370)	0.191 (108)	0.164 (1795)	0.187 (315)	0.037 (237)	— (100)	0.752 (127)
2 Hr	0.935 (170)	1.983 (330)	0.263 (32)	3.261 (1240)	1.703 (325)	0.703 (185)	1.398 (202)	1.411 (130)
3 Hr	1.028 (130)	2.398 (380)	1.897 (154)	0.526 (149)	2.109 (740)	0.623 (120)	1.167 (152)	3.772 (455)
4 Hr	—	2.243 (320)	0.755 (60)	0.731 (196)	1.475 (500)	1.206 (225)	0.689 (129)	3.468 (580)
5 Hr	—	1.963 (280)	2.241 (180)	2.088 (600)	0.814 (261)	1.040 (275)	1.071 (180)	2.200 (440)
6 Hr	1.068 (245)	1.922 (310)	1.887 (198)	0.625 (267)	0.277 (115)	1.135 (335)	0.253 (27)	1.004 (202)
9 Hr	0.799 (405)	0.835 (160)	1.356 (175)	a	0.463 (230)	0.367 (288)	0.948 (232)	1.114 (513)
12 Hr	0.295 (310)	0.750 (217)	0.683 (97)	0.154 (117)	0.330 (277)	0.132 (110)	0.209 (143)	0.302 (128)
TOTAL EXCRETED (mg)	8.785	15.635	13.352	7.857	8.945	6.243	8.047	16.853
% EXCRETED	0.38	0.45	0.36	0.23	0.26	0.18	0.23	0.49
AVERAGE CONCENTR. $\mu\text{g/ml}$	2.61	5.35	8.91	2.70	2.47	3.31	4.44	4.37

a Not measured

The figure between brackets refer to the urinary volume (ml/hr).

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DISCUSSION

JOHNSTON (Canada): The last nikethamide positive we had several years ago was almost missed because there appeared to be a larger amount of the metabolite than the pure drug and initial detection was through UV screening. On checking the thin-layer chromatogram, we noticed that the spot was almost co-incidental with nicotine. However there was a smaller spot above, which through GC we were able to prove to be nikethamide. At that time we did not have our mass spectrometer and were not able to identify the metabolite.

TOBIN: Did you note any behavioural effects on these horses after this very substantial dose of nikethamide.

DEBACKERE: No, we gave these doses to horses which are known not to be easily excited.