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DETERMINATION OF NIKETHAMIDE AND N-ETHYLNICOTINAMIDE IN THE BLOOD AND URINE OF GREYHOUNDS

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SUMMARY

Following the intramuscular administration of nikethamide to a series of greyhounds, both plasma and urine excretion levels were obtained.

A qualitative urine screening procedure for both nikethamide and its major metabolite has been devised. The method involves solvent extraction, thin-layer and a two-system gas chromatographic system.

INTRODUCTION

The illicit use of the medullary stimulant drug, nikethamide, as a doping agent in greyhounds, has prompted a study of its metabolism, excretion and subsequent detection in both urine and in plasma.

Nikethamide (N,N-diethylnicotinamide) has been reported as a doping agent in racehorses[1–3]. Its pharmacokinetics have been studied in humans and horses[2], yet there appears to have been little published information as to its metabolic fate in racing greyhounds.

Delbeke and Debackere[2] reported that the major urinary metabolite of nikethamide in man and in the horse was N-ethylnicotinamide.

Preliminary experiments conducted in laboratory greyhounds revealed, in addition to unchanged nikethamide, an unknown compound in the urine following a single administration of nikethamide. Subsequent analysis of this compound by mass spectrometry (MS) indicated the chemical structure to be N-ethylnicotinamide.

A method is presented for the detection and identification of nikethamide and its major urinary metabolite in greyhounds. The method is suitable as part of a routine drug screening system. The system employs solvent extraction followed by thin-layer chromatography (TLC) and a two-system gas–liquid chromatographic (GLC) confirmation. Kováts's retention data, as used by Kazyak and Permisohn[4], are presented for both GLC systems.

EXPERIMENTAL

Reagents and equipment

All reagents were analytical-reagent grade and were used without further purification.

TLC plates were prepared by mixing a slurry of 20 g of silica gel GF_{254} (Merck) with 40 ml of distilled water, and spreading a layer 0.25 mm thick. The plates (20 × 20 cm) were air dried for 5 min and then baked at 100° for 30 min. The TLC solvent was ethyl acetate–acetone–chloroform–ammonia (100:10:5:5).

The phosphate buffer was prepared by dissolving 10 g of potassium dihydrogen orthophosphate (KH_2PO_4) in 100 ml of distilled water. Dilute sodium hydroxide solution was added to produce a solution of pH 8.

The potassium triiodide spray was 10 g of potassium iodide and 10 g of iodine dissolved in 250 ml of ethanol. 60 ml of concentrated hydrochloric acid were added and the solution was made up to 500 ml with distilled water.

The ultraviolet (UV) lamp was Mineralight (Ultraviolet Products), and the extraction tubes 15 ml Quickfit (for plasma) and 50 ml polypropylene with PTFE-faced screw caps (Bel Art Products, for urine). A Clements rotating extractor (30 rpm) was used.

The internal standard was phenacetin in chloroform (5 μg per 8 ml). Nikethamide Injection (25%, w/v) was purchased from Abbott Labs. (Sydney, Australia) and N-ethylnicotinamide (1 mg/ml in chloroform) was a generous donation by Professor M. Debackere (Rijksuniversiteit, Gent, Belgium).

Procedure

Nikethamide (10 mg/kg) was administered by intramuscular injection to six male greyhounds (weight range 25–34 kg). Prior to administration, control blood and urine samples were collected; thereafter hourly specimens were obtained up to 6 h. The greyhounds were allowed water *ad libitum* during the experiments.

Blood samples (10 ml) were taken from the femoral vein, centrifuged and the plasma frozen until analysed.

Urine samples were obtained by walking the dogs and collecting specimens at the appropriate moment. The samples were frozen until analysed.

Extraction

Plasma. To 1 ml of plasma contained in a 15-ml glass-stoppered centrifuge tube was added 0.3 ml of pH 8 buffer, followed by 8.0 ml of chloroform containing 5 μg of phenacetin. The plasma was extracted on a rotary extractor for 20 min, centrifuged and the aqueous phase discarded. The chloroform was dried with anhydrous sodium sulphate and was then gently evaporated to dryness at 40° under a stream of nitrogen.

Urine. 20 ml of urine was adjusted to pH 5–6 and extracted for 20 min with 2 × 20 ml chloroform. The combined organic phases were washed with 5 ml of saturated sodium bicarbonate solution, dried through filter paper (Whatman No. 1) into an evaporating beaker and gently evaporated to dryness on a water bath at 40° under a stream of nitrogen.

Analysis

Thin-layer chromatography of urine samples. The urine extracts were dissolved in 100 μl of chloroform, and 5 μl spotted on to a TLC plate. Standard nikethamide and N-ethylnicotinamide in chloroform were also spotted. The plate was developed in ethyl acetate–acetone–chloroform–ammonia (100:10:5:5) to a distance of 15 cm. The developed plate was air dried and the spots located under UV light (254 nm).

followed by spraying with potassium triiodide reagent. Spots due to the presence of nikethamide and N-ethylnicotinamide appeared dark brown.

Gas–liquid chromatography. Following visualisation of the spots on TLC, confirmation was achieved using GLC. Two columns were used: (a) 3% OV-1 on Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.); (b) 5% Free Fatty Acid Phase (FFAP) on Chromosorb W (Varian, N. Springvale, Australia).

Conditions were as follows. Instrument: Bendix 2500 Series gas chromatograph fitted with flame ionisation detectors; carrier gas: nitrogen, flow-rate 40 ml/min; air flow-rate: 300 ml/min; hydrogen flow-rate: 40 ml/min; temperatures: injector, 245°; detector, 245°; oven, 155° (column a) or 225° (column b); recorder Perkin-Elmer Model 56, chart speed 5 mm/min.

Plasma extracts were dissolved in 100 μl of chloroform and 1 μl injected into column a. Quantitation was achieved by calibration using a standard curve and phenacetin as internal marker.

Urine extracts were dissolved in 0.5 ml of chloroform and suitable aliquots injected. Dilutions were made when necessary. Quantitation was achieved by comparison of peak heights to those of standards.

Column b was used as an additional confirmation system. Good separation was achieved between both compounds and naturally occurring constituents.

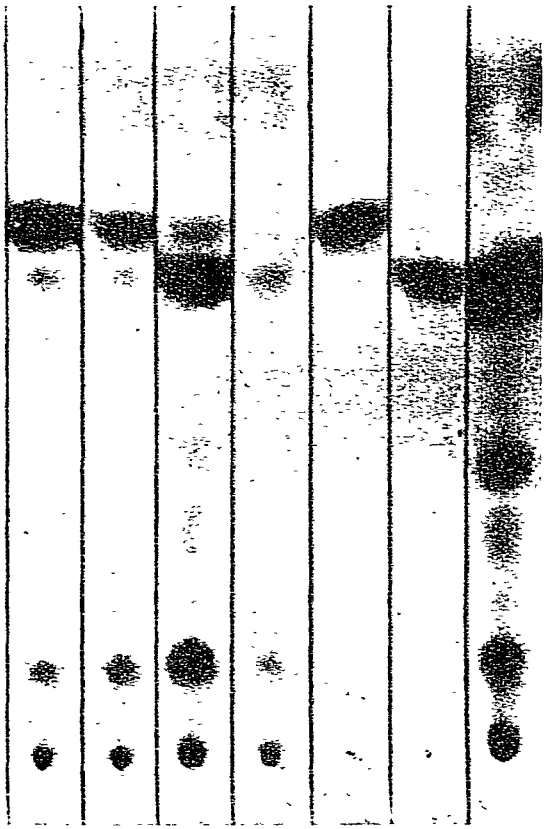


Fig. 1. TLC of urine extracts following nikethamide administration. Left to right: 2 h, 3 h, 4 h, 5 h, nikethamide, N-ethylnicotinamide, 6 h.

GLC–MS. Urine extracts found to contain nikethamide and N-ethylnicotinamide were subjected to chemical ionisation GLC–MS, and their spectra compared with the authentic reference drugs (Finnigan 3200 gas chromatograph–mass spectrometer fitted with Finnigan 6110 data system).

Entire urine extracts were spotted on to TLC plates and developed as before. The appropriate bands were located by UV light, scrapped off and eluted into methanol.

Recovery. Recovery experiments were conducted using plasma and urine to which authentic drugs had been added. For nikethamide, the recovery was 83–95%

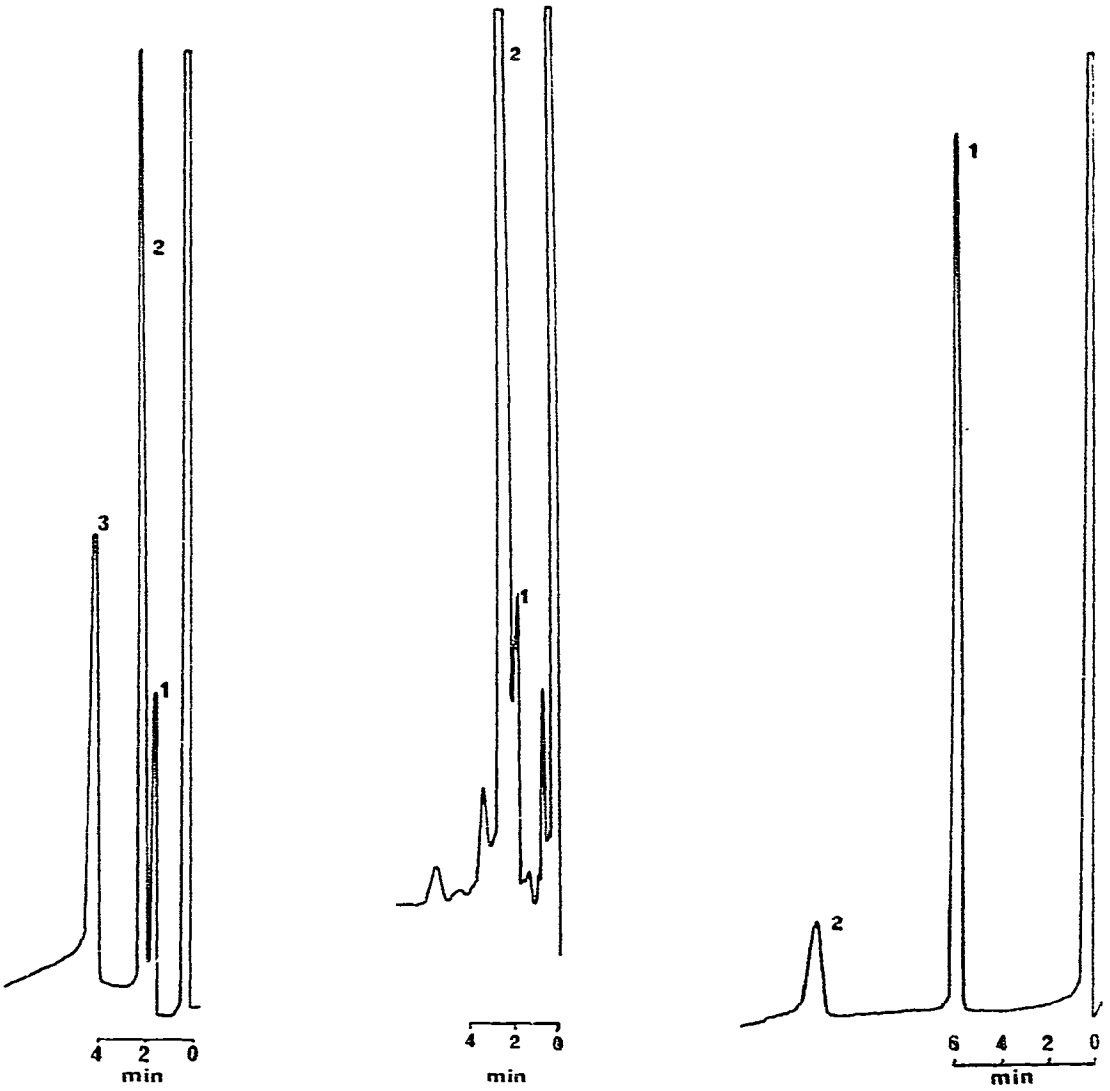


Fig. 2. GLC on OV-1 of plasma extract at 155°. 1 = N-Ethylnicotinamide; 2 = nikethamide, 3 = phenacetin.

Fig. 3. GLC on OV-1 of urine extract at 155°. 1 = N-Ethylnicotinamide; 2 = nikethamide.

Fig. 4. GLC on FFAP at 225°. 1 = Nikethamide; 2 = N-ethylnicotinamide.

(blood) and 55–65% (urine); for N-ethylnicotinamide, 88–95% (blood) and 60–70% (urine).

RESULTS

TLC of urine samples

Good separation of both drugs from urinary pigments was achieved (Fig. 1). R_F values: Nikethamide, 0.70; N-ethylnicotinamide 0.57.

GLC

On column a good separation of the two drugs was attained in plasma (Fig. 2) and in urine (Fig. 3). Excellent resolution was obtained on column b (Fig. 4). The gas chromatographic data are shown in Table I.

TABLE I

GLC RETENTION DATA

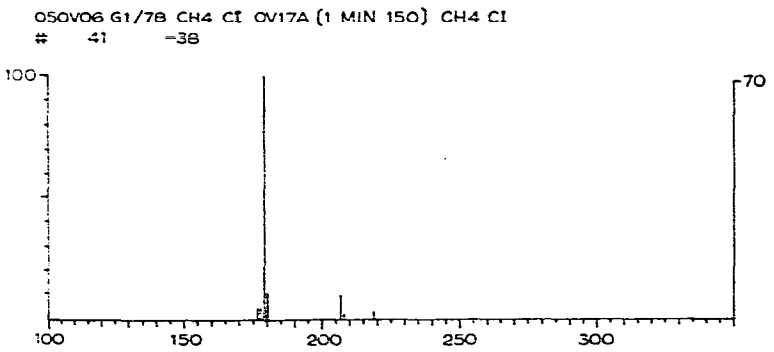
	Nikethamide		*N-Ethylnicotinamide*	
	Column a	*Column b*	*Column a*	*Column b*
Temperature	155	225	155	225
Relative retention time (phenacetin = 1.0)	0.50	—	0.39	—
Kováts retention index	1492	2569	1443	2767

GLC–MS

Chemical ionisation MS gave identical spectra of samples and reference compounds (Figs. 5 and 6).

The results of the plasma and urine concentrations of both unchanged nikethamide and N-ethylnicotinamide are shown in Figs. 7 and 8. Individual concentrations have been plotted for each time interval measured and the curve drawn through the median.

Following the intramuscular administration of nikethamide, peak plasma levels (17 μg/mg) were obtained 1 h after dosing. The levels dropped rapidly and nikethamide was undetected in the plasma 4 h after dosing. N-Ethylnicotinamide



g. 5. Chemical ionisation mass spectrum of nikethamide.

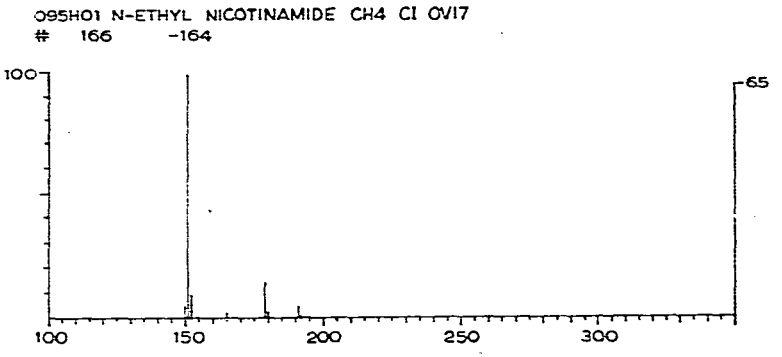


Fig. 6. Chemical ionisation mass spectrum of N-ethylnicotinamide.

could be detected in the plasma 6 h after dosing, peak levels (8 μg/ml) occurring at 2 h. Unchanged nikethamide was detected in the urine up to 4 h after administration, the maximum excretion occurring at 2 h (17 μg/ml). N-Ethylnicotinamide levels reached a peak 3 h after administration (20 μg/ml), and continued to be excreted at 6 h.

DISCUSSION

One of the problems of drug detection in racing greyhounds is the question of the estimated time of administration of the drug. The significance of the time factor is twofold. Firstly, under the rules of racing[5], no drugs or medications are permitted within 72 h of the dog racing. Hence it is necessary to ensure that drugs which may

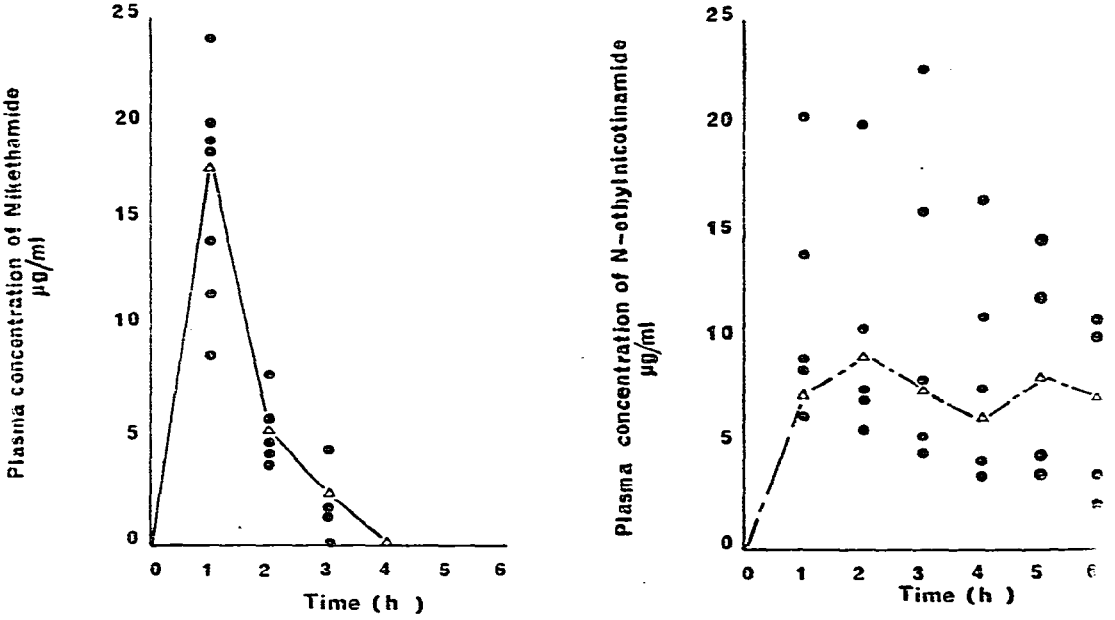


Fig. 7. Plasma concentration of nikethamide and N-ethylnicotinamide *versus* time following the intramuscular administration of nikethamide (10 mg/kg) to a series of six greyhounds.

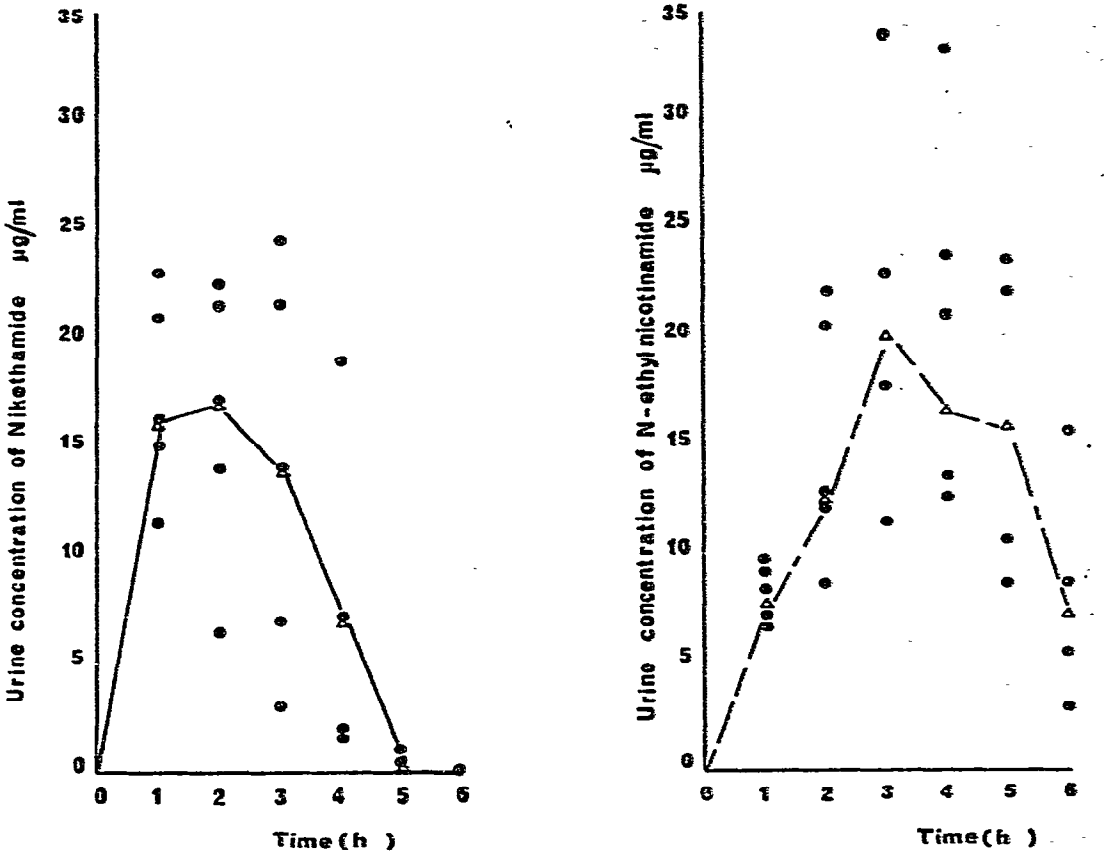


Fig. 8. Urine concentration of nikethamide and N-ethylnicotinamide *versus* time following the intramuscular administration of nikethamide (10 mg/kg) to a series of six greyhounds.

be used for medication, are not detected in the urine, provided that treatment has ceased within that time interval. Secondly, under local regulations, racing greyhounds are kennelled 2 h prior to the first race. During this time period, strict security is maintained over the dogs. In these experiments, nikethamide was found to be rapidly metabolised, and hence after 1 h, both drugs were detected in the urine. If, in a urine specimen taken from a greyhound, only nikethamide had been detected, one could predict that it was highly likely that the drug had been administered within 30 min of sampling, and hence during the kennelling period. Conversely, the presence of only the metabolite, N-ethylnicotinamide, would suggest that at least 5–6 h had elapsed since administration.

A comparison of the excretion patterns of nikethamide in the greyhound and horse shows a distinct similarity. Allowing for variation between individual animals, in both species the urinary excretion reached a maximum 2 h after administration and the parent compound was not detected 5–6 h later. Similarly, N-ethylnicotinamide was strongly excreted after 1 h, reached a maximum at 3–4 h and was easily detected at 6 h.

Although metabolic studies were not carried past 6 h owing to collection

difficulties, it could be reasonably predicted that N-ethylnicotinamide could be detected more than 6 h after administration.

Hence, urine screening for the illicit use of nikethamide could be effected by the presented method. The use of two dissimilar GLC columns, OV-1 and FFAP, in conjunction with TLC provided confirmation of the presence of the two compounds. Unequivocal proof could be obtained by GLC–MS after a simple clean-up procedure

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