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Short Communication

Simultaneous determination of N,N-dimethylformamide, N-monomethylformamide and N-hydroxymethyl-N-methylformamide in rat plasma by capillary gas chromatography

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ABSTRACT

A capillary gas chromatographic method with nitrogen–phosphorus detection for the simultaneous quantitative determination of N,N-dimethylformamide, and its two metabolites, N-monomethylformamide and N-hydroxymethyl-N-methylformamide, in rat plasma has been developed. The method involves a single extraction step with ethyl acetate–acetone (4:1, v/v). The extract is injected into a fused-silica capillary column coated with Carbowax 20M. A temperature gradient (65–110°C) is applied, and the three products can be separated within 10 min. The quantitation limits, using 25 μl of rat plasma, for N,N-dimethylformamide, N-monomethylformamide and N-hydroxymethyl-N-methylformamide are 0.4, 0.4 and 2 μg/ml, respectively. This method is suitable for toxicokinetic studies in rats.

INTRODUCTION

N,N-Dimethylformamide (DMF) (Fig. 1), a widely used industrial organic solvent, has been shown to be hepatotoxic, and its toxicity is probably mediated through its metabolites [1].

In rodents and humans, DMF is metabolized *in vivo* to N-hydroxymethyl-N-methylformamide (HMMF) and, to a lesser degree, to N-monomethylformamide (NMF) and formamide (F) (Fig. 1) [2,3]. DMF and metabolites have been determined in urine with gas chromatography

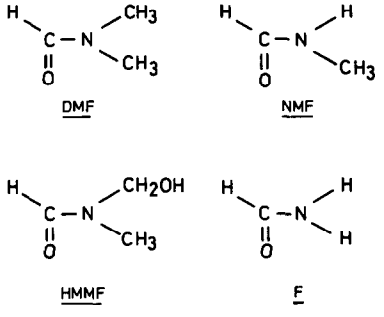

Fig. 1. Structures of N,N-dimethylformamide (DMF) and its metabolites, N-methylformamide (NMF), N-hydroxymethyl-N-methylformamide (HMMF) and formamide (F).

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(GC), but until now distinction between NMF and HMMF using GC has not been possible [4–6], owing to the thermal decomposition of HMMF to NMF at the injection port or on the GC column. NMF, with the possible presence of HMMF as a urinary metabolite, was determined after derivatization of HMMF with heptafluorobutyric anhydride using packed-column GC and a nitrogen detector [7].

No data are available on the plasma concentrations of DMF and its metabolites, HMMF and NMF. We therefore developed a capillary GC method with nitrogen–phosphorus detection and on-column injection for the simultaneous determination of these three substances in plasma.

EXPERIMENTAL

Materials

DMF and N,N-dimethylacetamide (DMA), the internal standard, were purchased from Aldrich (Milwaukee, WI, USA). NMF was obtained from Sigma (St. Louis, MO, USA). HMMF was synthesized according to the patent BASF 2327574. Methanol, ethyl acetate and acetone (analytical grade) were obtained from Lab Scan (Dublin, Ireland), Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germany), respectively.

Standards

Stock solutions of each compound (1 mg/ml) were prepared in methanol and were stored at −20°C. Appropriate dilutions were made every week and stored at 4°C. Known aliquots from these dilutions were added to plasma.

Instrumentation

A Hewlett Packard (Avondale, PA, USA) Model 5890 gas chromatograph, equipped with an HP nitrogen–phosphorus detector and an HP on-column injector was used. The detector was operated with hydrogen at 3.5 ml/min and with air at 75 ml/min. The detector temperature was 300°C. Samples were injected with a Hamilton 10-μl syringe and a fused-silica needle (10 cm × 0.18 mm O.D.). An HP 20M (Carbowax 20M) fused-silica capillary column (5 m × 0.32 mm I.D., 0.30 μm film thickness) was used. Helium served as the carrier gas at a flow-rate of 6.0 ml/min. Helium at a flow-rate of 25 ml/min was used as make-up gas for the detector. Two minutes after the injection, the oven temperature was programmed from 65°C to 110°C at 20°C/min, and then maintained at 110°C for 7 min.

Synthesis, purity and identification of HMMF

For the synthesis of HMMF, NMF was mixed with formaldehyde in the presence of potassium carbonate as described in the patent. Purity was checked by capillary GC by several 0.2-μl injections ($n = 11$) of a solution of 20 μg of HMMF in 125 μl of ethyl acetate–acetone (4:1, v/v) under the conditions described in *Instrumentation*.

HMMF was identified by GC–mass spectrometry and proton NMR spectroscopy. A ^1H NMR spectrum from synthesized HMMF in ^2H_2O as solvent was obtained using a Brucker AM-500 instrument at 500 MHz. [$^2H_4$]-3-(Trimethylsilyl)-propionic acid sodium salt was used as calibration standard. A mass spectrum was obtained on a Varian Saturn mass spectrometer (Walnut Creek, CA, USA) equipped with a ion-trap detector and coupled to a Varian 3400 gas chromatograph. An HP cross-linked 5% phenylmethylsilicone fused-silica capillary column (25 m × 0.32 mm I.D., 0.32 μm film thickness) was programmed from 60 to 100°C at 4°C/min. The carrier gas was helium.

Extraction procedure

The internal standard (400 ng in 4 μl of methanol) was added to 25 μl of rat plasma in a glass-stoppered conical tube. The mixture was extracted with 125 μl of ethyl acetate–acetone (4:1, v/v) by shaking for 10 min. After centrifugation (10 min, 2200 *g*, 4°C) the organic phase was transferred with a Pasteur pipette into a 6-ml glass-stoppered conical tube. These samples were stored at −20°C until analysis. A 0.2-μl aliquot was used for capillary GC.

Calibration curves

Calibration curves from 0.4 to 200 μg/ml (DMF), from 0.4 to 50 μg/ml (NMF) and from

2.0 to 150 μg/ml (HMMF) were prepared. The least-squares regression lines of the calibration curves were obtained by plotting peak-area ratios of product to internal standard against the plasma concentration of the product.

Extraction efficiency and limit of quantitation

The extraction recoveries of DMF and its metabolites were calculated by comparing the signals obtained after extraction of plasma spiked with DMF and its metabolites (with addition of the internal standard after extraction) with the signal obtained following the direct injection of the same amounts of DMA, DMF and its metabolites in ethyl acetate–acetone (4:1, v/v). The limit of quantitation for the three products was evaluated by replicate analysis of five spiked control samples at the lowest concentration of the standard curves.

Precision and accuracy

Within-day precision and accuracy were evaluated for DMF, NMF and HMMF at three different plasma concentrations by replicate analyses of five spiked control plasma samples on the same day. Between-day precision and accuracy were evaluated by analysis of spiked control plasma samples stored at −20°C and analysed with each run over five days.

Application

DMF, HMMF and NMF were measured in plasma of male Wistar rats after intraperitoneal administration of 100 mg/kg DMF. Blood samples were obtained from the tail vein as a function of time. Plasma samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Fig. 2 shows examples of chromatograms of extracts of control rat plasma, spiked rat plasma and rat plasma obtained after intraperitoneal administration of 100 mg/kg DMF. Under the conditions described, DMF is fully separated from its metabolites NMF and HMMF and from the internal standard (DMA). The retention times for DMF, DMA, NMF and HMMF are 0.65, 1.03, 3.16 and 8.21 min, respectively. There is no interference by endogenous plasma components, as is apparent from the chromatogram of rat control plasma.

Synthesized HMMF contains 2.70 ± 0.07% (S.D.) (n = 11) NMF, as determined by capillary GC, and its mass spectrum shows peaks at m/z 90 ($[M + H]^+$), 89 (M^+), 72 ($[M + H - H_2O]^+$) and 60 ($[M + H - CH_2O]^+$). They are identical with those reported by Scailteur *et al.* [5]. The ^1H NMR signals for the methyl protons of synthesized HMMF have chemical shifts identical with those reported for the methyl protons for HMMF and an integral ratio of *ca.* 5:1 [3].

Because HMMF is known to be converted into NMF on GC, attention was paid to the optimal column temperature for HMMF analysis to prevent degradation of HMMF to NMF. Fig. 3 shows the percentage of maximal HMMF/DMF ratio response (peak-area ratio) as a function of column temperature. Between 80 and 110°C this response is constant, and degradation of HMMF to NMF starts at a column temperature above 110°C. Kawai *et al.* [6] selected an injection port temperature of 250°C for a 100% conversion of HMMF into NMF.

The regression curves were linear for the plasma concentration range evaluated from 0.4 to 200 μg/ml for DMF, from 0.4 to 50 μg/ml for NMF and from 2 to 150 μg/ml for HMMF. Typical linear regressions are $y = -0.1019 + 0.0896x$ with $r = 0.9995$ for DMF, $y = 0.0189 + 0.0634x$ with $r = 0.9996$ for NMF, and $y = 0.193 + 0.0231x$ with $r = 0.9976$ for HMMF.

After a single-step extraction, the mean (± S.D.) recovery yields were: for DMF in the 0.4–150 μg/ml concentration range, 87.5 ± 5.8% (n = 6); for NMF in the 0.4–50 μg/ml range, 84.5 ± 6.7% (n = 6); and for HMMF in the 2–100 μg/ml range, 74.7 ± 5.2% (n = 6).

For a small volume of rat plasma (25 μl), the limits of quantitation are 0.4 μg/ml for DMF, 0.4 μg/ml for NMF and 2 μg/ml for HMMF. At these concentrations the mean calculated value deviates 15% for DMF, 17% for NMF and 20% for HMMF (n = 5). The coefficients of variation

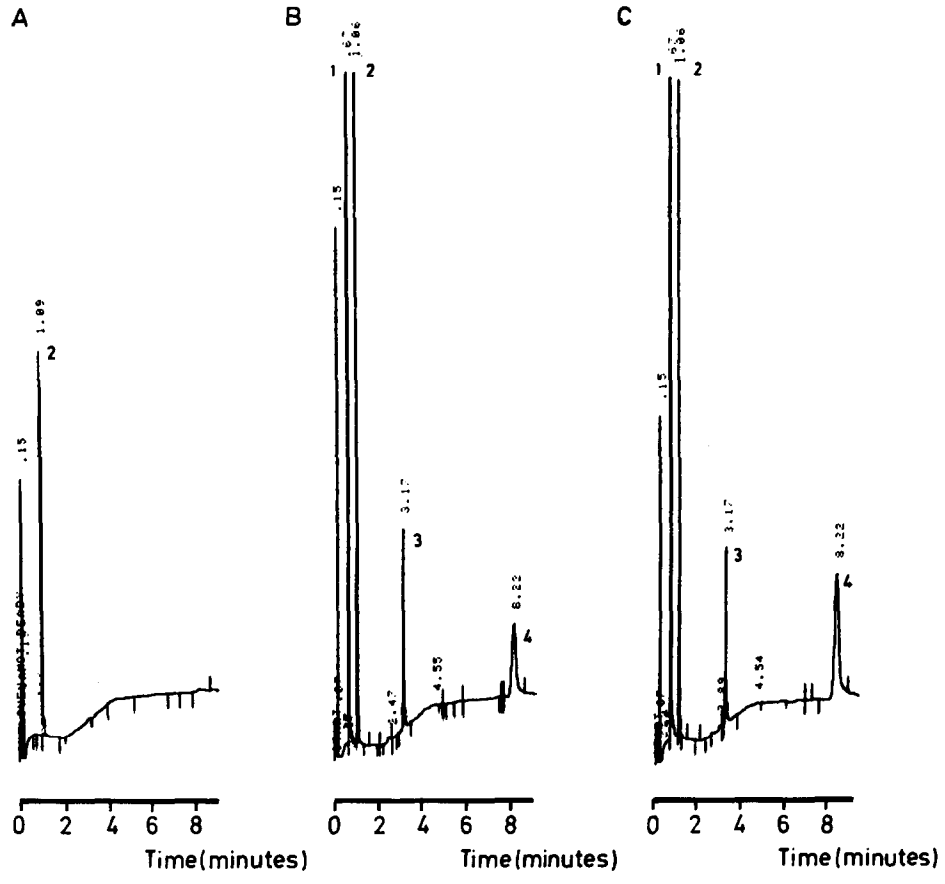

Fig. 2. Chromatograms of extracts. (A) Control rat plasma spiked with 16 μg/ml DMA, the internal standard; (B) control rat plasma spiked with 20 μg/ml DMF, 3.2 μg/ml NMF, 20 μg/ml HMMF and 16 μg/ml DMA, the internal standard; (C) plasma of the same rat 300 min after intraperitoneal administration of DMF (100 mg/kg) and spiked with 16 μg/ml DMA, the internal standard. Peaks: 1 = DMF; 2 = DMA; 3 = NMF; 4 = HMMF.

(C.V.) are less than 20% for all these compounds.

The within- and between-day precision and accuracy results for DMF, NMF and HMMF are shown in Table I. For the three products the between-day C.V. is less than 15% and there is no more than 10% deviation from the nominal value. The within-day accuracy and C.V. are acceptable.

Fig. 4 shows a representative example of the plasma concentration–time curves of DMF, NMF and HMMF after administration of 100 mg/kg DMF to a rat. DMF is rapidly absorbed after intraperitoneal administration. Plasma concentrations of HMMF and NMF could be measured. The AUC for HMMF is much higher than that of NMF. The maximum concentration occurs for HMMF at 12 h and for NMF at 14 h after DMF administration. These data present evidence for the metabolism of DMF to NMF.

CONCLUSION

Using a short fused-silica capillary HP Carbowax 20M column and nitrogen–phosphorus detection with a injection temperature below 100°C and a maximum column temperature of 110°C, a specific, sensitive and reproducible analysis of DMF, NMF and HMMF in plasma is possible.

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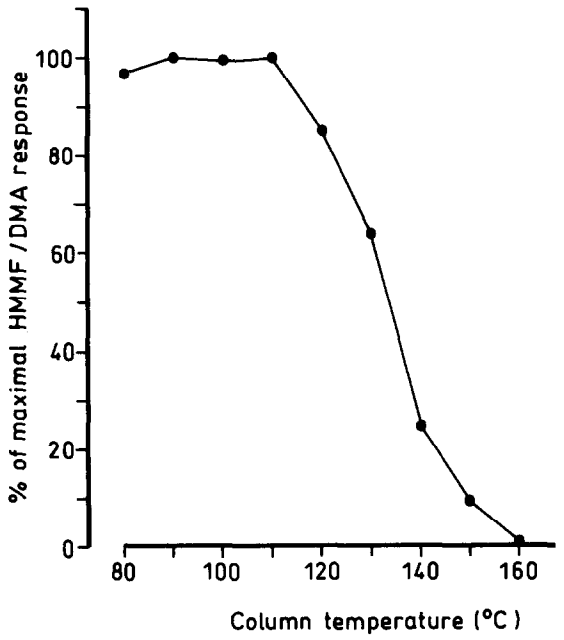

Fig. 3. Percentage of maximal HMMF/DMA response (peak-area ratio) as a function of column temperature. A 0.2-μl aliquot of a solution containing 40 μg of HMMF and 3 μg of DMA per ml ethyl acetate–acetone (4:1, v/v) was injected at different column temperatures.

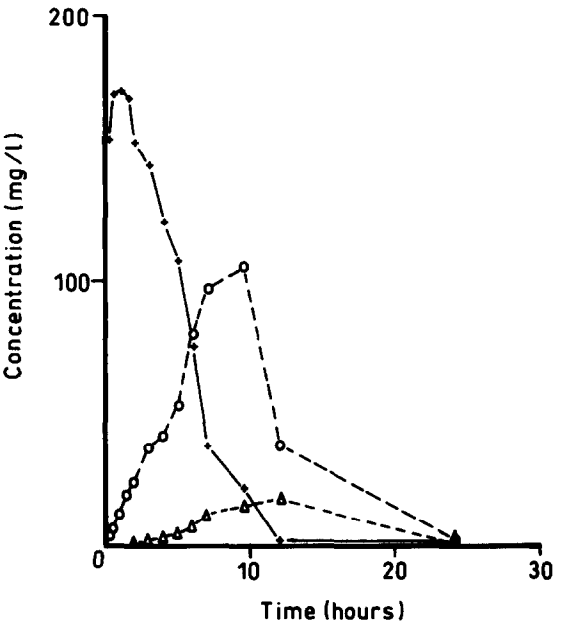

Fig. 4. Plasma concentration–time curves for DMF (+) and its metabolites NMF (△) and HMMF (○) after intraperitoneal administration of 100 mg/kg DMF to a rat.

TABLE I

WITHIN-RUN AND BETWEEN-RUN ACCURACY AND PRECISION (n = 5)

Compound		Concentration (μg/ml)	Accuracy (%)	C.V. (%)
DMF	Within-run	5	98.2	10.2
		120	101.1	4.7
	Between-run	5	99.6	15.1
		40	92.5	7.7
		120	97.0	5.6
NMF	Within-run	1.6	102.1	9.8
		20.0	99.3	6.7
	Between-run	1.6	105.0	15.1
		3.2	105.6	14.2
		20.0	101.3	8.9
HMMF	Within-run	5.0	95.6	9.2
		80.0	101.2	5.7
	Between-run	5.0	90.2	13.5
		50.0	102.2	5.9
		80.0	98.7	7.5

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