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Rapid and simple method for the determination of nimustine hydrochloride in human blood and brain by high-performance liquid chromatography

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SUMMARY

A simple method for the determination of nimustine hydrochloride in blood and brain by high-performance liquid chromatography was developed. A pH 4.52 buffer was used in the extraction from blood and a pH 5.0 buffer was used for brain. A pre-packed Extrelut column was used to make the extraction procedure uncomplicated. At room temperature light-resistant test-tubes were unnecessary. The lower limit of detection was 50 ng/ml for blood and 100 ng/g for brain. This method may be useful for the determination of nimustine hydrochloride in blood and brain samples from patients.

INTRODUCTION

Nimustine, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloro-ethyl)-3-nitrosourea hydrochloride (NSC-245382), is an antineoplastic nitro-sourea with the molecular structure shown in Fig. 1. It is water-soluble and also lipid-soluble at physiological pH, and has been used in the treatment of primary brain tumours because it crosses the blood–brain barrier [1,2].

Nishigaki et al. [3] and Nakamura et al. [4] reported that nimustine was labile at physiological pH.
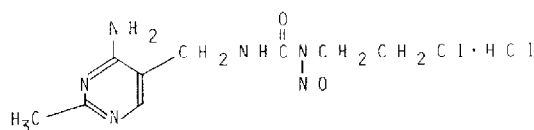
Fig. 1 Structure of nimustine.

A few methods for the determination of nimustine have been reported. Yamada et al. [5] measured the radioactivity of [ethylene-^{14}C]nimustine in rat blood and brain. However, their method may not be appropriate for the monitoring of nimustine levels in human blood and brain. Nakamura et al. [1] reported the quantitive determination of nimustine by high-performance liquid chromatography (HPLC) based on an extraction with 1,2-dichloroethane using a reversed-phase column and an ion-pair technique. The specimens were frozen instantaneously after sampling to prevent the decomposition of nimustine. In addition, all procedures were carried out under cold conditions using light-resistant glass vessels.

This paper describes a simpler HPLC method for the determination of nimustine.

EXPERIMENTAL

Materials

Nimustine was kindly provided by Sankyo (Tokyo, Japan). Methanol was of HPLC grade. Extrelut was obtained from E. Merck (Darmstadt, F.R.G.). Other reagents were of reagent grade.

The buffer solutions were prepared by mixing 0.2 M Na_2HPO_4 and 0.1 M citric acid (McIlvaine buffer) over the pH range 2.52–8.03.

Extraction of nimustine from blood and brain

To 0.5 ml of blood in a glass test-tube, 0.5 ml of buffer (pH 4.5) was added, and the mixture was vortex-mixed for 1 min and applied to a pre-packed column containing Extrelut. After 10 min, it was eluted with 6 ml of chloroform. The eluate was evaporated to dryness under a nitrogen stream. The residue was dissolved in 0.5 ml of methanol containing p-aminobenzoic acid as internal standard. A 10-μl aliquot was injected into the HPLC system.

To 0.2 g of brain in a centrifuge tube, 4.0 ml of McIlvaine buffer (pH 5.0) were added. The sample was homogenized by an Ultra-Turrax (Janke Kunkel, F.R.G.) for 5 min and then centrifuged at 1200 g for 5 min. The aqueous layer was pipetted into a glass test-tube. Then 3 ml of the aqueous layer were applied to a pre-packed column of Extrelut. After 10 min, the column was eluted with 15 ml of chloroform. The subsequent procedure was the same as that for blood.

HPLC conditions

The HPLC apparatus consisted of a Waters M-6000 pump, an M-441 UV detector set at 254 nm, a WISP 710B automatic injector and an M-730 data module (Waters Assoc., Milford, U.S.A.). A 30 cm × 3.9 mm I.D. stainless-steel μBondapak C_{18} column (10 μm particle size, Waters Assoc.) was used for the separation. The mobile phase was 30% (v/v) methanol in 0.1% (w/v) $NH_4H_2PO_4$, adjusted to pH 4.50 with orthophosphoric acid. The mobile phase was filtered through a membrane filter (Sartorius, 0.2 μm) and degassed by sonication before use. The flow-rate was 1.0 ml/min.

Stability tests of nimustine

To investigate the effect of pH on the decomposition of nimustine, the tubes containing the sample McIlvaine solutions (50 μg/ml) were capped and allowed to stand in a water-bath, which was regulated by a thermostat at 20°C.

To study the effect of light on the stability of nimustine, 10-ml aliquots of sample solution were transferred (a) into amber-coloured test-tubes and (b) into transparent test-tubes. These test-tubes were kept in room light at ambient temperature (20–25°C).

Aliquots of 0.1 ml were sampled at various times and immediately mixed with 0.1 ml of methanol containing internal standard. Aliquots of 10 μl of the mixture were injected into the HPLC system.

Calibration curves of nimustine

Standard solutions were prepared as follows. Blood spiked with nimustine was prepared by mixing 3 ml of drug-free blood with 0.2 ml of an aqueous solution of nimustine (0.8–480 μg/ml). For brain, 0.2 ml of an aqueous solution of nimustine (0.1–50 μg/ml) was added to 0.2 g of rat brain.

A calibration curve was obtained by plotting peak-height ratios of nimustine to *p*-aminobenzoic acid against the concentration of nimustine in each spiked sample over the range 0.05–30 μg/ml for blood and 0.1–50 μg/g for brain.

Recovery and precision

The assay recovery of nimustine was assessed for six replicate samples. For the spiked blood samples (0.2 and 5 μg/ml), one was immediately frozen and mixed with buffer after thawing and the other was mixed with the buffer before freezing. The assay was initiated by thawing. For the brain samples, nimustine was spiked at levels of 0.2 and 5 μg/g and homogenized.

The peak-height ratios of nimustine to internal standard obtained from these spiked samples were compared with those of standard solutions.

The precision was examined by assaying six preparations. The intra-assay precision of the method was determined by replicate analysis of the drug-free blood samples spiked with 5.0 μg/ml and the drug-free brain samples spiked

with 5.0 μg/g nimustine. The inter-assay precision was measured by analysis of the same spiked blood and brain samples.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Typical chromatograms of spiked nimustine in blood and brain are shown in Fig. 2. The peaks of nimustine and p-aminobenzoic acid were well separated. The drug-free sample chromatograms showed that no interference occurred from endogenous substance in the blood and brain.

Each calibration curve showed good linearity over the range of concentrations examined. The linear regression equations for calibration were $y = 0.2793x + 0.0173$ for blood and $y = 0.1947x - 0.0018$ for brain, where x is the concentration of spiked nimustine and y is the peak-height ratio of nimustine to p-aminobenzoic acid. Correlation coefficients obtained were 0.999 and 0.999, respectively.

The lower limit of detection was 50 ng/ml for blood and 100 ng/g for brain at signal-to-noise ratio of 4.

Stability of nimustine

Because Nakamura et al. [1] reported that nimustine is rapidly decomposed in blood, its stability in buffer was investigated.
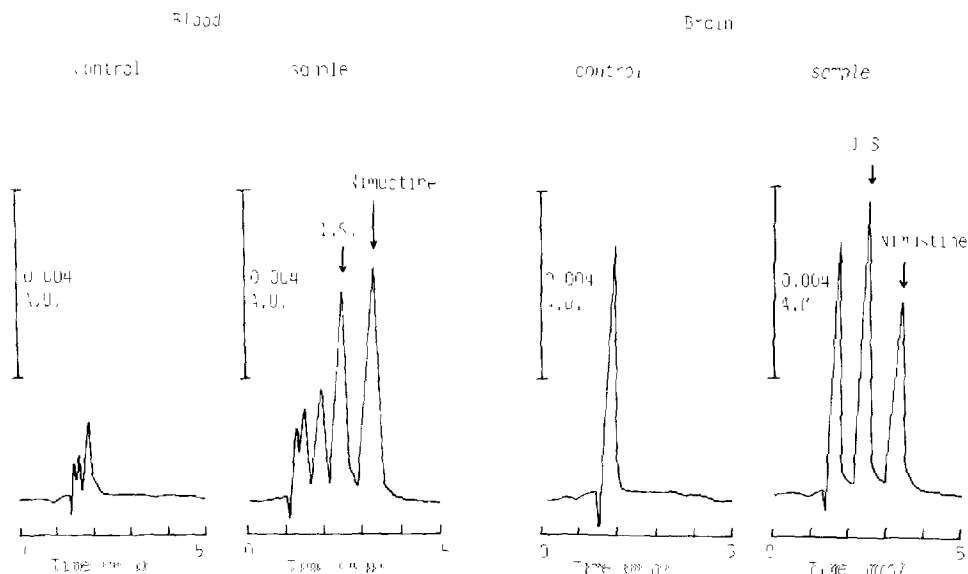

Fig. 2 Chromatograms of nimustine in human blood and rat brain samples Column, μBondapak C_{18} (30 cm $\times$ 3.9 mm I.D.); mobile phase, methanol–0.1% $NH_4H_2PO_4$ (30 70, v/v, pH 4.50), flow-rate, 1.0 ml/min; detection, UV detector (254 nm).

Time courses for the decomposition of nimustine in buffer solutions of various pH are shown in Fig. 3. Nimustine in the buffer solution was stable at pH 2.5–4.5, although it rapidly decomposed at pH 6, 7 and 8.
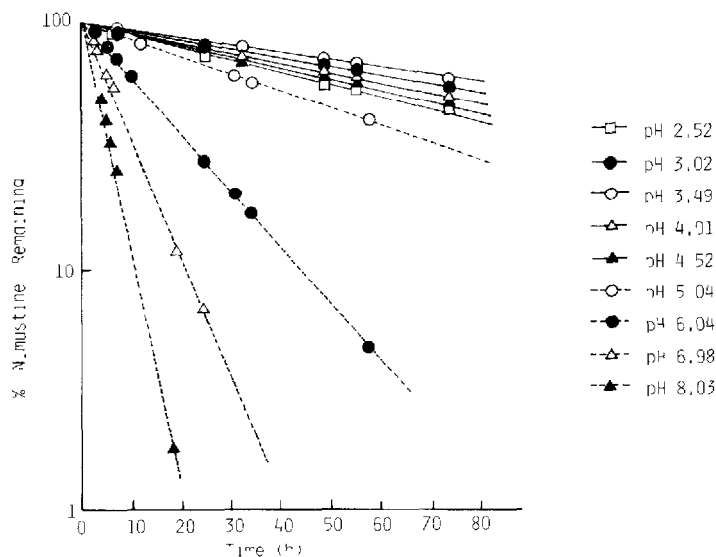

Fig. 3 Time course of the concentration of nimustine in McIlvaine buffer solutions of various pH at 20°C. The experiment was performed in the light.
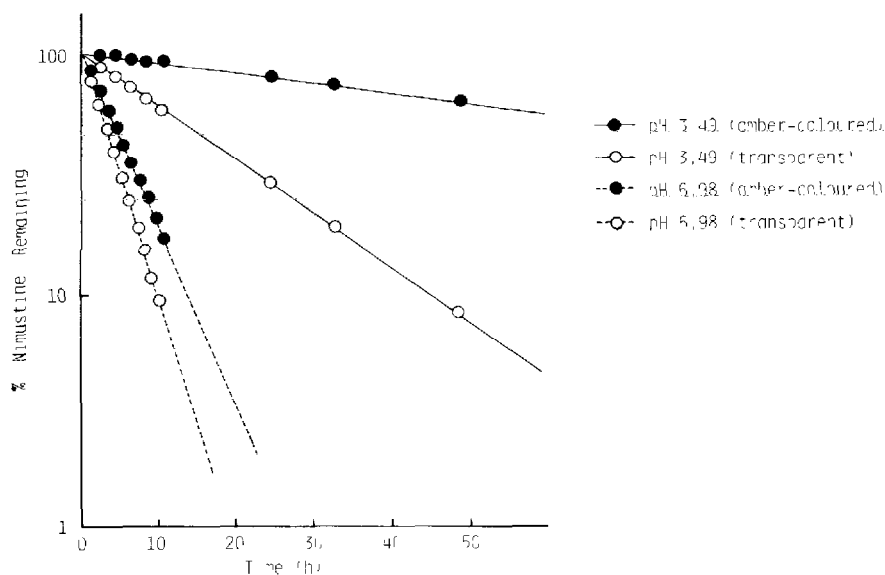

Fig. 4 Effect of light on the stability of nimustine solutions in test-tubes at room temperature (20–25°C).

As shown in Fig. 4, in the amber-coloured test-tubes nimustine was slowly decomposed at pH 3.49.

Nakamura et al. [1] reported that all procedures for the determination of nimustine should be carried out under cold conditions using amber-coloured glass vessels. As shown in Fig. 4, the decomposition of nimustine in pH 3.49 buffer was only slightly affected by light. The degradation rate constants are 0.008 h^{-1} (pH 3.49, amber-coloured), 0.040 h^{-1} (pH 3.49, transparent), 0.135 h^{-1} (pH 6.98, amber-coloured) and 0.161 h^{-1} (pH 6.98, transparent), respectively. Nimustine in buffer was less decomposed at pH 3.49 in transparent test-tubes than at pH 6.98 in amber-coloured test-tubes.

It took 20 min to extract nimustine from blood and brain before applying it to the pre-packed columns of Extrelut. The stability of nimustine in buffer after 1 h was ca. 95.0% at low pH. It is suggested that the decomposition of nimustine in this procedure is minimal. With a buffer of low pH, the present method could be carried out at room temperature without the use of a light-resistant vessel.

Recovery and precision of nimustine from human blood and rat brain

Known amounts of nimustine was added to drug-free human blood. One was immediately frozen, and the buffer solutions (pH 3.52–7.42) were mixed after the frozen blood was thawed. The other was mixed to the same volume with pH 4.52 and 7.42 buffer before freezing. As shown in Tables I and II, in the case of mixing with pH 4.52 and 7.42 buffers before freezing, the recovery of nimustine was better than after thawing.

In the case of brain samples, higher pH values gave lower recoveries of nimustine. As shown in Table III, the recovery of nimustine was fairly constant

TABLE I

EFFECT OF BUFFER pH ON RECOVERY OF NIMUSTINE WHEN BLOOD WAS MIXED WITH BUFFER AFTER THAWING

The concentration of nimustine was 5 μg/ml.

Buffer pH	Recovery (mean ± S.D., $n=6$) (%)
3.52	71 7 ± 2 6
4 52	78.4 ± 0.7
4.75	74.9 ± 0.6
5.00	71.0 ± 0.7
5 52	73.8 ± 1.0
7.42	73 6 ± 2.5

TABLE II

EFFECT OF BUFFER pH ON RECOVERY OF NIMUSTINE WHEN BLOOD WAS MIXED
WITH BUFFER BEFORE FREEZING

Buffer pH	Concentration of spiked nimustine (μg/ml)	Recovery (mean $\pm$ S.D., $n=6$) (%)
4.52	0.2	93.5 $\pm$ 3.5
	5.0	95.4 $\pm$ 2.1
7.42	0.2	86 0 + 7.0
	5.0	87.0 $\pm$ 5 2

TABLE III

EFFECT OF BUFFER pH ON RECOVERY OF NIMUSTINE FROM RAT BRAIN

Buffer pH	Concentration of spiked nimustine (μg/g)	Recovery (mean $\pm$ S.D , $n=6$) (%)
3.52	5.0	29 1 $\pm$ 0.8
4.01	5.0	57.6 $\pm$ 0.5
4.52	0.2	78 4 $\pm$ 5.9
	5.0	83.9 $\pm$ 1.6
4.76	0 2	83.4 $\pm$ 2.6
	5.0	87.4 $\pm$ 0.8
5 02	0.2	89.6 $\pm$ 5.4
	5.0	89.5 $\pm$ 2.3
5.50	0.2	86.5 $\pm$ 3.5
	5.0	87.1 $\pm$ 1.8
6.00	0.2	78.9 $\pm$ 3.3
	5.0	85 6 $\pm$ 1.8

in the pH range 4.76–6.00. The recovery of nimustine was best when the brain was homogenized with pH 5.02 buffer.

Nakamura et al. [1] utilized pH 7.4 buffer during the extraction from brain. However, Nishigaki et al. [3] reported that nimustine rapidly decomposed in pH 7.4 buffer at 20°C and suggested that pH 7.4 buffer was not appropriate for the extraction procedure. On the other hand, our recovery values for nimustine at pH 3.5 were poor, as shown in Table III. The pK_a of nimustine is 5.95 [1]. The non-ionized moiety of nimustine is increased by an increase in pH. The non-ionized nimustine is easily transferred to the organic solvent. Therefore, it seems that the recovery of nimustine from blood and brain is better at pH 4.5–5.5 than at pH 3.5. The transfer of nimustine to the organic phase is increased by an increase in pH, although nimustine is unstable. As
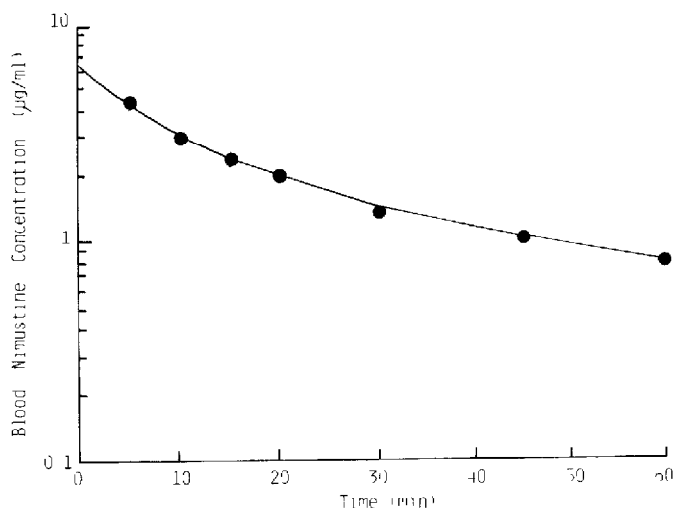
Fig. 5. Time course of the concentration of nimustine in the blood of a patient with a brain tumour after intravenous bolus administration (72 mg).

shown in Tables II and III, the optimum pH of buffer for extraction of nimustine is 4.5 for blood and 5.0 for brain.

The precision was estimated in six preparations. The intra-assay values obtained were $4.85 \pm 0.04 \, \mu g/ml$ [coefficient of variation (C.V.) 0.82%] for blood samples spiked with 5.0 $\mu g/ml$ nimustine and $4.47 \pm 0.14 \, \mu g/g$ (C.V. 3.13%) for brain samples spiked with 5.0 $\mu g/g$. The inter-assay values obtained were $4.82 \pm 0.09 \, \mu g/ml$ (C.V. 1.87%) for blood and $4.41 \pm 0.14 \, \mu g/ml$ (C.V. 3.17%) for brain.

Determination of nimustine in human blood

The present method was applied to the determination of nimustine concentrations in human blood. After intravenous bolus administration of 72 mg of nimustine, the blood levels of the drug declined with time in a biexponential pattern as shown in Fig. 5. The preliminary phase gave a half-life of 5.8 min and the terminal phase gave a half-life of 37.4 min.

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