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Note

High-performance liquid chromatographic method for the determination of 2-nitropropane in rat plasma

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2-Nitropropane (2-NP) is an industrial solvent used in coatings, printing inks and adhesives. In addition, it is used in food processing, as an intermediate in organic synthesis and as a rocket fuel. During the last decade there has been considerable interest in this compound because of its carcinogenic potential in rats after inhalation[1]. In humans 2-NP is known to cause liver damage[2]. Until very recently[3], little was known about the risks from oral exposure and therefore our Institute decided to start a chronic toxicity and carcinogenicity study with rats, which were to be given 2-NP by gavage. In the context of this programme it was decided to develop a high-performance liquid chromatographic (HPLC) method that could be used to monitor plasma levels after oral administration of 2-NP to rats.

In this paper, a convenient and rapid method for the determination of 2-NP in rat plasma is described.

EXPERIMENTAL

Materials

All reagents were of analytical-reagent grade; water was distilled twice in Pyrex glass. 2-Nitropropane (purity 95%) was obtained from Merck (Darmstadt, F.R.G.) and acetonitrile (for HPLC) from Rathburn Chemicals (Walkerburn, U.K.). The liquid chromatograph consisted of two LKB (Bromma, Sweden) Model 2150 pumps, an LKB Model 2152 controller, a Gilson (Villiers le Bel, France) Model 231-401 automatic sampler and a Pye Unicam (Cambridge, U.K.) Model PU 4020 variable-wavelength UV detector. HPLC columns (100 × 4.6 mm I.D.) were packed with Shandon (Runcorn, U.K.) Hypersil ODS 3 μm by means of a Shandon slurry packer according to the manufacturer's instructions.

Methods

Plasma samples (0.3 ml) were obtained from rats using a semipermanent canula inserted into the right jugular vein. The samples were collected in 1-ml screw-capped vials that contained an appropriate amount of heparin. To avoid losses of 2-NP by evaporation, the vials were pre-cooled in ice and closed immediately after collection of the samples, then the samples were centrifuged (1000 *g*) at 4°C as quickly as

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possible. After transferring 100 μl from the supernatant to a new 1-ml vial, each sample was deproteinized by slowly adding 120 μl of acetonitrile. The latter was delivered by means of a metering pump at a flow-rate of 0.2 ml/min. The exit tubing of the pump was fitted with a fused-silica (0.1 mm I.D.) capillary to transfer the liquid and during the addition the sample was agitated by means of a mechanical shaker. The sample vials were then closed and agitated for a further 1 min. Each vial was centrifuged at 1000 *g* for 3 min and 100 μl of the supernatant was transferred to a new vial. Finally, 150 μl of tris(hydroxymethyl)aminomethane (Tris) buffer (12.5 mmol/l, pH 6.0) was added and the contents of the vial were mixed. Calibration standards were prepared by adding 20 μl of a freshly prepared aqueous 2-NP solution to 100 μl of blank plasma and subjecting the mixture to the same treatment as the samples.

Chromatography

The solvent mixture used for separation consisted of acetonitrile and a 12.5 mmol/l solution of Tris in water adjusted to pH 6.0 (20:80, v/v) and was pumped at a flow-rate of 1.0 ml/min. Under these conditions the column back-pressure was approximately 160 bar. The UV spectrometer was operated at 224 nm and chromatograms were recorded at 0.005 a.u.f.s. After 100 μl of sample or calibration standard had been injected the eluent was fed to the column for 11 min and then a 50:50 (v/v) mixture of this eluent and pure acetonitrile was pumped through for 2 min to elute all strongly retained material. Finally, the column was re-equilibrated with the former eluent for 15 min. By operating the chromatography in this way, large numbers of samples could be analysed consecutively without interference or column deterioration problems.

RESULTS AND DISCUSSION

Apart from its volatility and poor solubility in water, which had been expected to create substantial problems during analytical manipulation, initial experiments showed that 2-NP also was unstable in aqueous solutions containing more than approximately 0.02 mol/l of inorganic salts. Degradation was found to be maximal at

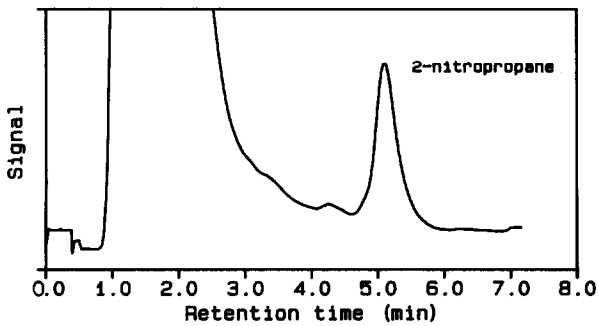

Fig. 1. Chromatogram of a plasma sample spiked with 2-NP to give a concentration of 15 mg/l. For chromatographic conditions, see text.

pH 5–7 and the products that could be identified (nitronic acid and acetone) indicated a reaction mechanism similar to that of the Nef reaction[4,5]. For these reasons it was considered to be of prime importance to keep the analytical manipulations to the absolute minimum and to pay extra attention to the stability of the samples.

Although gas chromatography had been used for the analysis of 2-NP[1], HPLC was considered to be more appropriate in connection with the requirements mentioned above. Early attempts to develop an HPLC system combined with a post-column reactor using the chromogenic reaction of the *aci*-tautomer of 2-NP (nitronic acid) with acidic iron(III) chloride solution failed because the detector noise due to the reagent pump was too great. Therefore, detection by UV absorption at low wavelengths was considered and 224 nm appeared to offer sufficient sensitivity while background absorption due to the eluent could still be compensated for (Fig. 1). The solvent system developed allowed some manipulation of the retention times of plasma interferences relative to that of 2-NP itself by varying the pH. In a later stage this property was used to regain separation when spurious peaks interfered with the 2-NP peak. The absolute detection limit under these conditions was 1 ng injected on the column. Linearity was checked over the range 0–250 ng and, separately, 0–30 ng. Linear regression analysis between peak heights obtained and amounts injected showed good linearity in both instances (correlation coefficients 0.9984 and 0.9918, respectively) and no significant difference between the regression coefficients.

Deproteinization of plasma samples with acetonitrile was found to be a rapid and convenient method because it required minimal manipulation and the composition of the resulting fluid could easily be adapted to match that of the HPLC eluent. Slow, even addition of acetonitrile appeared to be essential for maximal recovery of 2-NP (see Table I) and because of the small sample size (100 μl) the use of a metering pump was necessary. With regard to sample stability an experiment was carried out in which two blank plasma pools were spiked with 0.6 and 3 mg of 2-NP per litre and analysed immediately and after storage for 24 and 96 h at −20°C. The 2-NP concentrations of both plasma pools were found to decrease rapidly resulting, after 96 h, in levels that were only 21 and 50% of the initial concentrations, respectively. From these results it was concluded that analysis should begin immediately after collection of the samples.

To validate the method further, the reproducibility and lowest detectable con-

TABLE I

2-NITROPROPANE RECOVERIES AFTER SLOW OR FAST DEPROTEINIZATION OF PLASMA SAMPLES

Concentration (mg/l)*	*Recovery (%)*		*Significance of difference***
	Slow	*Fast*	
3	91.9 (n = 4)	84.2 (n = 6)	$p < 0.0025$
15	92.5 (n = 4)	83.7 (n = 6)	$p < 0.0005$

* Two blank plasma pools spiked with 2-NP to give the concentrations indicated were subjected to both slow and fast deproteinization.

** Student's t-test.

TABLE II

RESULTS OF REPRODUCIBILITY EXPERIMENTS

Sample No.	*Concentration found (mg/l)*			*ANOVA*	
	Day 1	*Day 2*	*Day 3*	*F*	*Significance*
1	0.39	0.82	0.45		
2	0.51	0.54	0.87		
3	0.76	0.77	0.44		
4	0.45	0.82	0.56		
5	–	0.82	0.50		
6	–	0.65	0.44	3.40	NS*
7	3.42	3.35	3.03		
8	3.36	3.35	3.03		
9	3.33	3.63	3.18		
10	3.61	3.27	3.12		
11	3.36	3.31	2.91		
12	3.36	3.29	3.00	20.21	$p < 0.01$
13	15.51	15.33	14.69		
14	14.76	14.90	14.69		
15	14.73	15.04	14.83		
16	15.04	15.33	15.07		
17	14.79	14.62	14.63		
18	14.79	14.93	14.69	1.61	NS*

* NS = Not significant.

centration of 2-NP in plasma were determined. Three blank plasma pools were spiked with 2-NP to give final concentrations of 0.6, 3 and 15 mg/l. From each pool eight samples were taken and subsequently analysed. Two of these samples were dealt with as if they were standards and used as such to construct a calibration line to calculate the concentrations in the six remaining samples. While the latter procedure obviously was of no use for real calibration, it allowed the inclusion in the final results of a

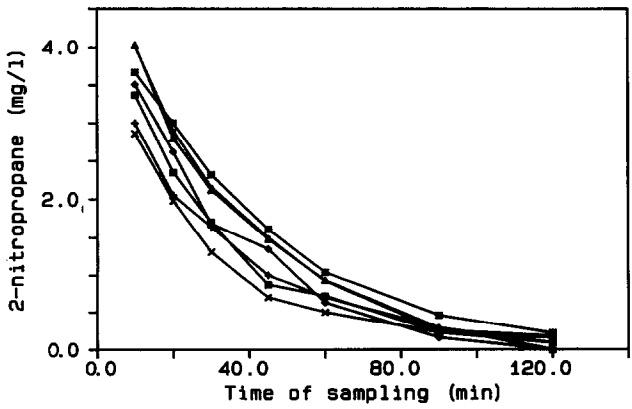

Fig. 2. Concentrations of 2-NP in rat plasma obtained at various times after intravenous administration to seven rats.

variance component due to calibration runs. The whole procedure was carried out on three different occasions using freshly prepared plasma pools each time. Table II shows the individual results and statistical parameters obtained by analysis of variance. The variation coefficients within days varied from 15.8–30, 3.1–3.9 and 1.1–2.0% for the lowest, middle and highest concentrations, respectively. Unexpectedly, a significant between-day variance component was found for the 3 mg/l pool only; *t*-tests performed pairwise on the results of the 3 mg/l pool on different days showed that only the results obtained on the third day differed significantly ($p < 0.001$) from those on days 1 and 2. Therefore, it seems unlikely that a between-day variation exists in general. The lowest plasma concentration of 2-NP that could be determined was 60 μg/l (signal-to-noise ratio = 2).

The method described was successfully applied in toxicokinetic experiments in which 2-NP was administered intravenously to rats. Fig. 2 shows the plasma concentrations at various time intervals after administration to seven different animals. The dose given was 10 mg/kg and extrapolation from Fig. 2 in connection with the detection limit mentioned above suggests that experiments with doses as low as 2 mg/kg should be feasible using this method.

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