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Determination of *p*-chloronitrobenzene in plasma by reversed-phase high-performance liquid chromatography

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ABSTRACT

A simple, accurate and precise isocratic reversed-phase high-performance liquid chromatographic method was developed and validated for the determination of *p*-chloronitrobenzene (p-CNB) in rat plasma. A plasma sample was deproteinized with methanol containing the internal standard (*p*-bromonitrobenzene). The resulting methanol eluate obtained after centrifugation was filtered and injected into a high-performance liquid chromatograph (50 μl each). A column packed with 5 μm octadecylsilane (ODS) spherical particles was used with isocratic elution of methanol–water (45:55, v/v) at a flow-rate of 1.0 ml/min. The compounds were detected by ultraviolet absorbance at 280 nm. The retention times of p-CNB and the internal standard were 12.5 and 15.5 min, respectively, at a column oven temperature of 30°C. The results were linear from 0.05 to 100 μg/ml ($r = 0.999$), and the detection limit was 0.01 μg/ml. The relative error and the coefficient of variation on replicate assays were less than 7 and 10%, respectively, for all concentrations studied. The overall recoveries of p-CNB were between 97 and 105%. Plasma samples could be stored for up to one month at −20°C.

INTRODUCTION

p-Chloronitrobenzene (p-CNB) is widely used as a raw material for dyes, pesticides, medicines and other products, but causes methemoglobinemia in humans and animals [1–7]. Thus, it is very important to be able to measure the amounts of p-CNB absorbed by patients poisoned by p-CNB or by workers exposed to it.

However, although analytical methods have been developed to study the pharmacokinetics of drugs administered to patients, little has been done concerning methods for analysing industrial chemicals in biological samples.

We focused on the widely used p-CNB, and this paper describes a rapid, precise and sensitive method for measuring p-CNB levels in plasma by isocratic reversed-phase high-performance liquid chromatography (HPLC) using a small amount of blood. The sample preparation procedure is simple, requiring only precipitation of proteins with methanol containing an internal standard.

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EXPERIMENTAL

Chemicals

p-CNB [purity: 99.7%; the remaining 0.3% is *m*- (0.1%) and *o*-chloronitrobenzene (0.2%)] was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The internal standard, *p*-bromonitrobenzene (p-BNB), and heparin sodium salt were obtained from Wako (Osaka, Japan). Other chemicals used were all purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan) or Ishizu Pharmaceutical (Osaka, Japan). Methanol was of liquid chromatographic reagent grade. All other chemicals were of reagent grade and used without further purification. Water used during the experiments had been deionized and distilled.

Equipment

The absorbance intensities of p-CNB were measured using a Hitachi Model 200-20 spectrophotometer (Hitachi, Tokyo, Japan) to determine the detection wavelength on HPLC. A supersonic bath was used to degas the mobile phase (Elma Transsonic T570, Singen, Germany). A Tomy Model MC-15A centrifuge was used (Tomy Seiko, Tokyo, Japan). The vortex mixer was purchased from Iuchi (Osaka, Japan). The liquid chromatograph was assembled from components primarily from Hitachi (Tokyo, Japan), which included a Model L-6200 intelligent pump, a Model 655A-52 column oven, a Model L-4000 UV detector and a Model D-2500 chromato-integrator. The injector was a Rheodyne Model 7125 chromatographic injector, with a 50-μl injection loop (Cotati, CA, USA).

Chromatographic conditions

The water for the mobile phase was filtered through an Advantec cellulose nitrate membrane filter (0.45 μm pore size, Toyo Roshi Kaisha, Tokyo, Japan). The mobile phase, methanol–water (45:55, v/v), was prepared freshly on the day of analysis and was subjected to vacuum and supersonic degassing. p-CNB was separated from plasma using an analytical column, LiChrospher 100 RP-18(e) (125 mm × 4 mm I.D., 5 μm particle diameter, Merck, Darmstadt, Germany), protected by the same RP-18 guard column (4 mm × 4 mm I.D.). The mobile phase was pumped at 1.0 ml/min. The column temperature was maintained at 30°C. The UV detector was set at 280 nm for absorption measurements. A 50-μl plasma sample was injected into the chromatographic system with a Rheodyne injection valve.

p-Chloronitrobenzene standard solution

A stock solution of p-CNB was prepared by dissolving 50.0 mg of the compound in methanol in a 50-ml volumetric flask. This stock solution, equivalent to 1.0 mg/ml, was stored under shielded light at 4°C for up to one week. The stock solution was diluted with methanol freshly on each day of analysis to prepare concentrations of 0.050, 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 μg/ml.

Internal standard solution

A stock solution of the internal standard, p-BNB, was prepared by dissolving 10.0 mg of the compound in 10 ml of methanol. The stock solution was stored under shielded light at 4°C and was stable for up to one week. A working solution for the plasma assay was prepared freshly on each day of analysis by adding 0.5 ml of the stock internal standard solution to 49.5 ml of methanol, producing a final internal standard concentration of 10 μg/ml.

Animals

Male Sprague–Dawley rats, purchased from Clea (Osaka, Japan), were used when they were nine weeks old in the weight range 310–335 g. Food (Laboratory Chow MF, Oriental Yeast, Japan) and water were available to all animals *ad libitum*. A single dose of 100 mg/kg body weight p-CNB diluted in olive oil was injected intraperitoneally to each of the rats. Blood was withdrawn from the jugular vein of the rats fixed non-anesthetally by the Kanatake method [8].

Sample preparation

Each blood sample (*ca.* 0.15 ml) was collected in a heparinized polypropylene 1.5-ml snap-cap conical-bottom centrifuge tube (Bio Plastics, Osaka, Japan), and immediately centrifuged for 15 min at 2000 *g* in order to separate the plasma. A 50-μl volume of the supernatant was decanted into a fresh centrifuge tube and diluted with 50 μl of internal standard solution and 50 μl of methanol. The mixture was vortexed for 30 s and then centrifuged for 15 min at 3000 *g*, and the supernatant was passed through an Advantec PTFE-type disposable syringe filter DISMIC-13jp (0.50 μm pore diameter, Toyo Roshi Kaisha) to remove proteins and other particulate matter.

Calibration

Each p-CNB standard solution (0.050–100.0 μg/ml) was combined with an equal volume of internal standard solution and water, and 50-μl aliquots of the mixed solutions were analysed by the present HPLC method to construct the calibration curve.

p-CNB concentrations in the plasma samples were calculated by interpolation from the linear least-squares regression line of the multi-level standard curve plot of peak-area ratio (area p-CNB/area internal standard) *versus* p-CNB concentration in the calibration standard solutions.

Recovery

Plasma samples containing various concentrations (0.075–35.24 μg/ml) of p-CNB were prepared by collection of blood at suitable times after the dosage of p-CNB in the rats. The recovery of p-CNB was calculated by adding known amounts (0.18–90.54 μg/ml) of p-CNB to these plasma samples.

Stability of p-CNB in plasma

Each 3 ml of blood from two rats dosed with p-CNB was collected 1 h after the dosage and was centrifuged to separate the plasma. The plasma samples obtained were combined and vortexed. The plasma solution was transferred in 0.1-ml portions into 25 brown screw-capped vials. p-CNB from one screw-capped vial was analysed immediately after preparation. The concentration determined in this vial was considered to be the p-CNB concentration at time zero. The remaining vials were randomly separated into three groups of eight. Each vial was stored at −20°C, 4°C or room temperature until analysis, and the contents of each were analysed periodically.

RESULTS AND DISCUSSION

Sample preparation

Several methods of sample preparation were investigated on the basis of the recovery of p-CNB from plasma. To analyse plasma samples on a reversed-phase HPLC column, the proteins must first be removed. Therefore, we first tried deproteinization with trichloroacetic acid (TCA). A one-fifth volume of 10% TCA (w/v) per volume of plasma was added to the plasma to remove more than 99% of the plasma proteins [9]. However, this method was not satisfactory, because p-CNB was significantly degraded by TCA, and the recoveries of p-CNB from plasma were *ca.* 25% for 5–100 μg/ml concentrations of p-CNB. Next, we examined deproteinization by addition of an organic as precipitant, *i.e.* methanol, acetonitrile or ethanol. Double the volume of each precipitant was added to the plasma in order to sufficiently remove proteins from the plasma [9]. The use of these organic solvents gave highly reproducible recoveries of p-CNB (*ca.* 100%). Consequently, deproteinization with methanol, which was employed as a component of the mobile phase for the chromatographic method, was chosen to prepare the plasma.

The internal standard had to be a compound resembling p-CNB in chemical properties. p-BNB was found to give a peak that was separated from those of p-CNB and other endogenous constituents of the plasma under the chromatographic conditions. Furthermore, recovery of p-BNB from plasma was more than 99% at the concentration of 10 μg/ml and was reproducible quantitatively. Therefore, p-BNB was selected as the internal standard for the present study.

Chromatography

Optimal HPLC conditions for the analysis of p-CNB in rat plasma samples were determined. UV absorption scans showed an absorption maximum for p-CNB at *ca.* 280 nm in 45% methanol solution. The absorbance of deproteinized control plasma decreased greatly at wavelengths of more than 240 nm. Therefore, the determination of p-CNB in the plasma was performed at 280 nm.

Methanol–water was used as the mobile phase. The effect of the pH of the

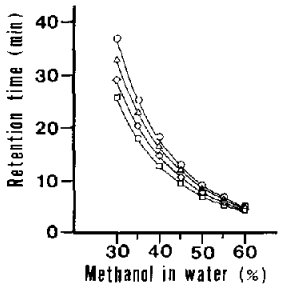

Fig. 1. Effect of analytical temperature and mobile phase composition on retention time of *p*-chloronitrobenzene (p-CNB): (○) 30°C; (△) 35°C; (◇) 40°C; (□) 45°C.

mobile phase on the retention time and the peak area of p-CNB was examined. The pH was changed by the addition 50 m*M* phosphate buffer (pH 2.1–7.0), with the methanol/water ratio held constant (45:55, v/v), and the p-CNB in the plasma sample was determined using each mobile phase. Both the retention time and the peak area of p-CNB were approximately constant for every determination. Therefore, the mixture of methanol and deionized, distilled water without phosphate salt was used as the mobile phase because it was the simplest one.

The variation of the retention time of p-CNB with mobile phase composition (methanol/water ratio) and column oven temperature was determined (Fig. 1).

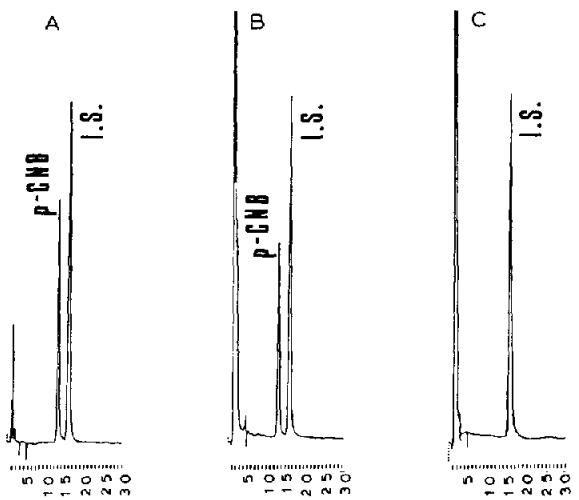

Fig. 2. Typical chromatograms of standard solution of *p*-chloronitrobenzene (p-CNB, 5 μg/ml) (A) and plasma samples of a rat 24 h after the intraperitoneal administration of 100 mg/kg p-CNB (concentration found, 4.2 μg/ml) (B) and of a control rat (C). I.S. = internal standard (*p*-bromonitrobenzene, 10 μg/ml). HPLC conditions: mobile phase, 45% methanol; analytical column, LiChrospher 100 RP-18(e) (125 mm × 4 mm I.D., 5 μm particle diameter) protected by the same RP-18 guard column (4 mm × 4 mm I.D.); flow-rate, 1.0 ml/min; column oven temperature, 30°C; injection volume, 50 μl; detection wavelength, 280 nm.

The retention time gradually shortened as the proportion of methanol and the analytical temperature were increased. Similarly, the chromatographic patterns of the endogenous constituents of the control plasma were examined under each analytical condition. Most of the constituents in the plasma were eluted relatively rapidly at every analytical condition. p-CNB should be eluted as rapidly as possible and must be separated from the other plasma constituents. In this study, p-CNB in the plasma sample was analysed using 45% methanol in the mobile phase at a column oven temperature of 30°C.

HPLC separation of an authentic sample of p-CNB and the internal standard, p-BNB, under the present HPLC conditions is shown in Fig. 2A. The retention times of p-CNB and p-BNB were 12.5 and 15.5 min, respectively. Typical chromatograms of a sample prepared from rat plasma following administration of p-CNB, and of a control plasma sample, are shown in Fig. 2B and C. No significant peaks were observed after the retention time of the internal standard. The section of the chromatogram where p-CNB was eluted was free of interfering peaks. Therefore, the present method appears to be satisfactory for the determination of p-CNB in rat plasma.

Calibration

Statistical calculations at the 99% confidence interval showed that the slopes of the calibration curves that were obtained after addition of p-CNB standards in control plasma or methanol were identical. Therefore, the p-CNB standard dissolved in methanol could be used as the standard solution. Solutions for calibration were prepared by adding internal standard solution and water to the standard solutions. The calibration standards were linear over the concentration range 0.05–100 μg/ml. The normal linear regression line was $y = 0.120x -$

TABLE I

ACCURACY AND PRECISION OF OUR METHOD OF *p*-CHLORONITROBENZENE ANALYSIS

Spiked concentration (μg/ml)	Assayed concentration (mean ± S.D., $n = 6$) (μg/ml)	Coefficient of variation (%)	Relative error (%)
0.050	0.053 ± 0.005	9.4	6.3
0.10	0.102 ± 0.009	9.0	2.1
0.20	0.205 ± 0.009	4.2	2.7
0.50	0.493 ± 0.011	2.1	1.4
1.00	1.016 ± 0.021	2.0	1.6
2.00	1.997 ± 0.039	2.0	0.17
5.00	5.053 ± 0.135	2.7	1.1
10.00	9.986 ± 0.105	1.0	0.14
20.00	19.62 ± 0.276	1.4	1.9
50.00	49.07 ± 1.164	2.4	1.9
100.00	100.23 + 1.004	1.0	0.23

0.0023 ($r = 0.9999$, $n = 11$), where y is the peak-area ratio (*i.e.* p-CNB/internal standard) and x is the p-CNB concentration.

Accuracy and precision

The accuracy and precision of this method were demonstrated by repeated analysis of plasma spiked with p-CNB. Eleven aliquots of pooled control plasma spiked with p-CNB at different concentrations are listed in Table I. The accuracy of the method, indicated by the error of assayed samples relative to their spiked concentrations, was excellent, all errors being less than 7%, with many points showing less than 2% error.

The precision of this method is indicated by the low coefficients of variation in the studies with replicate assays ($n = 6$). They were less than 10%, with concentrations greater than 0.2 μg/ml showing less than 5%, which indicates good reproducibility.

Recovery and detection limit

The variability in the recovery of p-CNB from rat plasma was investigated by spiking plasma samples containing various concentrations (0.075–35.24 μg/ml) of p-CNB with known amounts (0.18–90.54 μg/ml) of p-CNB. As shown in Table II, each experiment done in triplicate gave recoveries between 97 and 105%, indicating adequate recovery.

The detection limit of p-CNB in plasma using this method was *ca.* 0.01 μg/ml at a signal-to-noise ratio 2:1.

TABLE II

RECOVERY OF *p*-CHLORONITROBENZENE FROM PLASMA

p-Chloronitrobenzene concentration (μg/ml) ($n = 3$)			Recovery (%)
Concentration spiked to plasma	Detected from plasma before spiking	Detected from plasma after spiking	
0.18	0.075	0.25	97.0
0.36	0.29	0.64	98.3
0.73	0.25	0.97	98.5
1.53	0.67	2.27	104.6
3.30	2.23	5.65	103.6
5.04	2.16	7.35	103.0
6.88	14.39	21.10	97.5
13.86	17.28	30.93	98.5
20.20	7.77	27.55	97.9
27.18	24.72	52.36	101.7
49.98	28.01	77.49	99.0
90.54	35.24	125.84	100.1

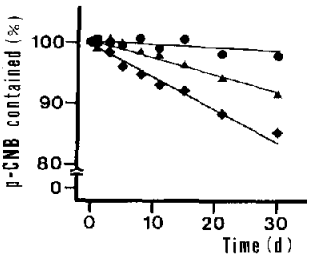

Fig. 3. Stability of *p*-chloronitrobenzene (p-CNB) in plasma at (●) −20°C, (▲) 4°C and (◆) room temperature, at *ca.* 30 μg/ml. p-CNB concentration just before storage (time zero) was set at 100%.

Storage stability

The stability of p-CNB in rat plasma was evaluated at −20°C, 4°C, and room temperature (Fig. 3). No significant p-CNB degradation in frozen (−20°C) samples was observed over one month. On the other hand, though p-CNB in the plasma samples was also relatively stable at both 4°C and room temperature, the loss of p-CNB from plasma samples at 4°C and room temperature was *ca.* 10 and 15%, respectively, over one month. This shows that the plasma sample may be stored by freezing at −20°C until analysis.

The present analytical method is excellent for *in vivo* toxicokinetic studies of p-CNB in rats. Blood can be collected continuously from one rat, because only a small amount (*ca.* 150 μl) is needed for one determination of p-CNB in plasma. The simplicity and high sensitivity of this method make it possible to conduct toxicokinetic studies for concentrations as low as 0.05 μg/ml.

This procedure can be used to determine p-CNB levels in plasma samples from patients suffering from p-CNB poisoning.

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