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# Determination of *p*-chloronitrobenzene and its metabolites in urine 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 (HPLC) using ultraviolet detection was developed for the determination of *p*-chloronitrobenzene (*p*-CNB) and seven of its metaboalites in rat urine. Analysis was performed before and after hydrolysis of the urine samples with acid to determine both free and conjugate forms of the metabolites. An equal volume of methanol was added to the urine sample and after centrifugation the mixed solution was injected into a high-performance liquid chromatograph. A column packed with 5-$\mu$m octadecylsilane (ODS) spherical particles was used at 30°C. The metabolites were divided into three groups, and each group was subjected to three different mobile phase and detection wavelength conditions as follows: water–methanol (60:40, v/v) and 250 nm for *p*-CNB and 2,4-dichloroaniline; 0.005 *M* phosphate buffer (pH 3.6)–methanol (76:24, v/v) containing 1.2 m*M* sodium 1-octanesulphonate and 240 nm for *p*-chloroaniline, 2-chloro-5-nitrophenol, 2-amino-5-chlorophenol, *p*-chloroacetanilide and 4-chloro-2-hydroxyacetanilide; and 0.005 *M* phosphate buffer (pH 6.0)–methanol (80:20, v/v) and 340 nm for N-acetyl-S-(4-nitrophenyl)-L-cysteine. The response was linear at concentrations less than 200.0 $\mu$g/ml ($r$ = 0.9998) for all metabolites, and the detection limits of each metabolite were between 0.05 and 0.2 $\mu$g/ml in non-hydrolysed urine. Analysis of the spiked samples demonstrated good accuracy and precision of the method in both intra- and inter-day assays. Storage stabilities of *p*-CNB and its metabolites at −20°C, 4°C and room temperature were examined for both neutral and acidic urine samples. This method was also shown to be applicable to toxicokinetic study of *p*-CNB following administration to rats.

## INTRODUCTION

*p*-Chloronitrobenzene (*p*-CNB) is widely used as an intermediate in the synthesis of azo and sulphur dyes and as a raw material for pesticides and medicines. However, *p*-CNB causes methaemoglobinaemia in humans and animals [1,2]. Further, *p*-CNB is weakly mutagenic in the Ames test [3] and shows some carcinogenic activity in mice [4]. Therefore, it is very important to be able to measure the amounts of *p*-CNB absorbed by subjects poisoned by it or by workers exposed to it. The amounts of hazardous substances absorbed are commonly monitored by measuring the urinary metabolite levels. However, there are only a few reports concerning the metabolism of *p*-CNB in rabbit [5], yeast [6] and isolated hepatocytes [7] and on the determination of the metabolites. Corbett and Corbett [6] described a method for the determination of *p*-CNB metabolites by high-performance liquid chromatography (HPLC) in a culture medium of incubated yeast with *p*-CNB. In addition, Rickert and Held [7] determined the metabolites in a solution of isolated rat hepatocytes incubated with radiolabelled *p*-CNB by HPLC using radioactivity detection. In both methods, the samples for analysis were relatively clean and the metabolites were eluted with a gradient solvent system. Therefore, these methods do not seem to be suitable for the deter-

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mination of *p*-CNB metabolites in urine samples, which contain many endogeneous components, from workers exposed to *p*-CNB and subjects poisoned by it.

We previously identified urinary metabolites in rats treated with *p*-CNB [8] and in subjects with acute *p*-CNB poisoning [9] using gas chromatography–mass spectrometry. Eight metabolites, 2,4-dichloroaniline, *p*-chloroaniline, 2-chloro-5-nitrophenol, 2-amino-5-chlorophenol, *p*-chloroacetanilide, 4-chloro-2-hydroxyacetanilide, N-acetyl-S-(4-nitrophenyl)-L-cysteine and *p*-chloroformanilide, were detected in the urine of both the rats and the subjects. Traces of unchanged *p*-CNB were also found in the rat urine. The *p*-chloroformanilide detected in both urine samples was considered to have resulted from pyrolysis of a substance originating from *p*-chloroaniline, a *p*-CNB metabolite, in the injection port of the gas chromatograph [10]. Further, the metabolic pathway of *p*-CNB in humans as shown in Fig. 1 was deduced from the above reports concerning the metabolism of *p*-CNB [9]. In rats, *p*-CNB seems to be excreted in urine through the same metabolic pathway as that in humans because the same metabolites were found in both urine samples.

This paper describes a simple, accurate and precise method for measuring *p*-CNB and its seven metabolites, except *p*-chloroformanilide, in urine by isocratic reversed-phase HPLC.

## EXPERIMENTAL

### *Chemicals*

*p*-CNB and *p*-chloroaniline were obtained from Tokyo Kasei Kogyo (Tokyo, Japan), *p*-chloroacetanilide from Aldrich (Milwaukee, WI, USA) and N-acetyl-S-(4-nitrophenyl)-L-cysteine from Toshin Gousei Kogyo (Tokyo, Japan). 2-

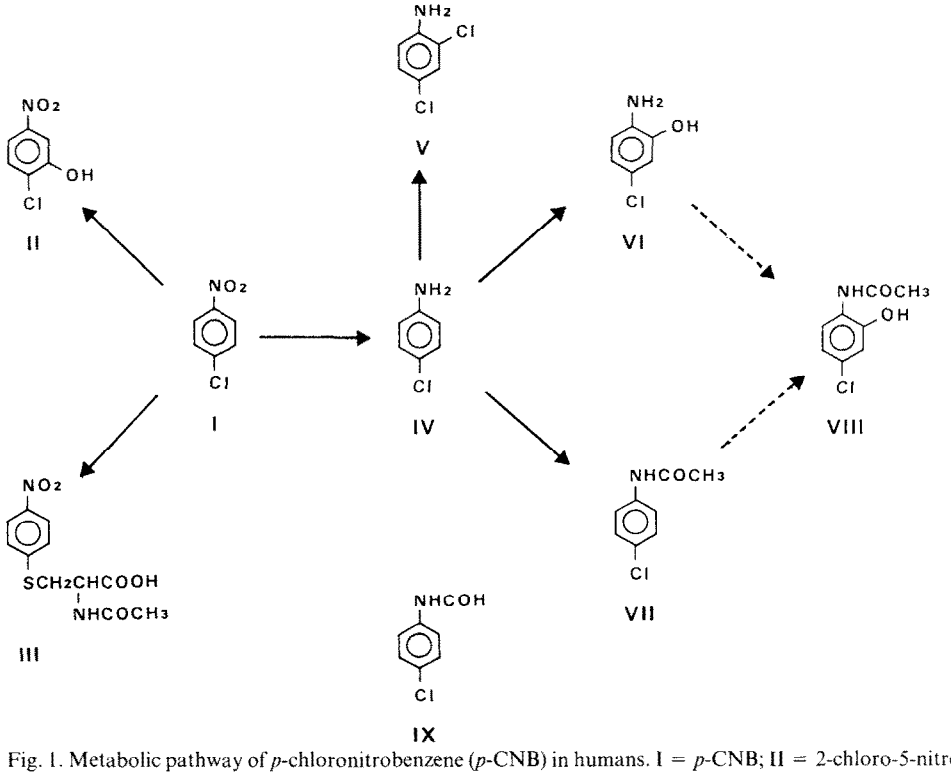


Fig. 1. Metabolic pathway of *p*-chloronitrobenzene (*p*-CNB) in humans. I = *p*-CNB; II = 2-chloro-5-nitrophenol; III = N-acetyl-S-(4-nitrophenyl)-L-cysteine; IV = *p*-chloroaniline; V = 2,4-dichloroaniline; VI = 2-amino-5-chlorophenol; VII = *p*-chloroacetanilide; VIII = 4-chloro-2-hydroxyacetanilide; IX = *p*-chloroformanilide. IX resulted from pyrolysis of a precursor originating from IV at the injection port of the gas chromatograph. Dashed arrows: it was not clear whether VIII was formed by N-acetylation of VI or hydroxylation of VII. This figure is taken from ref. 9.

Chloro-5-nitrophenol, 2-amino-5-chlorophenol and 4-chloro-2-hydroxyacetanilide were synthesized as described previously [11]. Other chemicals were purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan) or Wako (Osaka, Japan). Methanol was of liquid chromatographic reagent grade. All other chemicals were of analytical-reagent grade and were used as received. Water for the experiments had been deionized and distilled.

*Equipment*

The liquid chromatograph was assembled from components primarily from Hitachi (Tokyo, Japan), and 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 (Cotati, CA, USA) Model 7125 chromatographic injector with a 20-$\mu$l injection loop. A supersonic bath was used to degas the mobile phase (Transsonic T570, Elma, Singen, Germany). A Tomy Seiko (Tokyo, Japan) Model MC-15A centrifuge was used. The vortex mixer was purchased from Iuchi (Osaka, Japan).

*Chromatographic conditions*

*p*-CNB and its metabolites were separated from urine using an analytical column, LiChrospher RP-Select B (125 mm × 4 mm I.D., 5 $\mu$m particle diameter) (Merck, Darmstadt, Germany), protected by an RP-Select B guard column (4 mm × 4 mm I.D.). The guard column was replaced with a new one after the analysis of about 200 samples. The water for the mobile phase was filtered through an Advantec cellulose nitrate membrane filter (0.45 $\mu$m pore size) (Toyo Roshi Kaisha, Tokyo, Japan). The urine samples were subjected to three different mobile phase and detection wavelength conditions for absorption measurement, as follows: system I for *p*-CNB and 2,4-dichloroaniline, water–methanol (60:40, v/v) and 250 nm; system II for *p*-chloroaniline, 2-chloro-5-nitrophenol, 2-amino-5-chlorophenol, *p*-chloroacetanilide and 4-chloro-2-hydroxyacetanilide, 0.005 *M* phosphate buffer (pH 3.6)–methanol (76:24, v/v) containing 1.2 m*M* sodium 1-octanesulphonate as paired ion and 240 nm; and system III for N-acetyl-S-(4-nitrophenyl)-L-cysteine, 0.005 *M* phosphate buffer (pH 6.0)–methanol (80:20, v/v) and 340 nm. All mobile phases were prepared freshly on the day of analysis and subjected to vacuum and supersonic degassing. Each isocratic mobile phase was pumped at 1.0 ml/min. The column temperature was maintained at 30°C in all analytical systems. A 20-$\mu$l urine sample was injected into the chromatographic system with a Rheodyne injection valve.

*Standard solutions of p-chloronitrobenzene and its metabolites*

Each stock standard solution of *p*-CNB and its metabolites was prepared separately by dissolving 100.0 mg of the compounds in methanol in 100-ml volumetric flasks. These stock solutions, except *p*-chloroaniline and 2-amino-5-chlorophenol solutions, could be stored under shielded light at 4°C for up to two weeks. The stock solutions of *p*-chloroaniline and 2-amino-5-chlorophenol were prepared freshly on each day of analysis. Equal volumes of the stock standard solutions of *p*-CNB and 2,4-dichloroaniline were combined, and working standard solutions of the two compounds were prepared by diluting the mixed solution, equivalent to 500.0 $\mu$g/ml of each compound, with methanol to yield concentrations of 0.20, 0.50, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 $\mu$g/ml (standard solutions I). Working standard solutions of a mixture of *p*-chloroaniline, 2-chloro-5-nitrophenol, 2-amino-5-chlorophenol, *p*-chloroacetanilide and 4-chloro-2-hydroxyacetanilide (standard solutions II) and of N-acetyl-S-(4-nitrophenyl)-L-cysteine (standard solutions III) were prepared in the same manner. Each standard solution was prepared freshly on each day of analysis.

*Animals and sample collection*

Male Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan). Urine samples from rats treated and untreated with *p*-CNB were collected using Clea (Osaka, Japan) Model CL-0304 metabolic cages. Food (Laboratory Chow MF, Oriental Yeast, Japan) and water

were available to all animals *ad libitum*. Before the subsequent experiments, the urine samples obtained were centrifuged at 2000 *g* for 15 min.

*Sample preparation*

Each urine sample was subjected to analysis before and after hydrolysis to determine both free and conjugate forms of the metabolites.

*Free p-CNB and its metabolites.* A urine sample (*ca.* pH 6–7) of 0.5 ml was decanted into a 1.5-ml polypropylene snap-cap conical-bottomed centrifuge tube (Bio Plastics, Osaka, Japan) and diluted with 0.5 ml of methanol. The mixture was vortex-mixed 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.

*Total (free plus conjugated) p-CNB and its metabolites.* A 1-ml urine sample was hydrolysed with 0.1 ml of concentrated hydrochloric acid for 1 h at 100°C in a screw-capped tube for measurement of total (free plus conjugated) amounts of urinary *p*-CNB and its metabolites, 2,4-dichloroaniline, *p*-chloroaniline, 2-chloro-5-nitrophenol and 2-amino-5-chlorophenol, and cooled to room temperature. The hydrolysate was neutralized by adding 0.24 ml of 5 *M* sodium hydroxide solution and was centrifuged for 15 min at 3000 *g*. A 0.5-ml volume of the supernatant was decanted into the centrifuge tube and subsequent treatment was done in the same manner as above for free *p*-CNB and metabolites.

*Calibration*

Each standard solution (0.20–200.0 μg/ml) of *p*-CNB and its metabolites (standard solutions I, II and III) was combined with an equal volume of water, and 20-μl aliquots of the mixed solutions were analysed by the present HPLC method to construct calibration graphs for the determination of each metabolite in the urine samples.

*p*-CNB and its metabolite concentrations in the urine samples were calculated by interpolation from the linear least-squares regression line of the multi-level calibration plot of peak areas of *p*-CNB and metabolites *versus* these concentrations in the calibration standard solutions.

*Recovery*

The recoveries of the free forms of *p*-CNB and its metabolites (*p*-chloroaniline, 2,4-dichloroaniline, 2-chloro-5-nitrophenol and 2-amino-5-chlorophenol) from urine samples were calculated by heating known amounts (1.0, 5.0, 20.0 and 100.0 μg/ml) of these compounds added to urine free of *p*-CNB and its metabolites (control urine) in order to examine the degradation or disappearance of each hydrolysed compound (free form) by heating.

*Stability of p-CNB and its metabolites in neutral and acidic urine samples*

*Stability in neutral urine sample.* About 1.0 mg of each authentic standard of *p*-CNB and the metabolites was added separately to 50 ml of control urine (*ca.* pH 6–7). Each urine solution was transferred in 1-ml portions into 25 brown screw-capped vials. The contents of each screw-capped vial were analysed immediately after the preparation of each metabolite. The concentrations determined were considered to be those of *p*-CNB and its metabolites 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 its contents were analysed periodically.

*Stability in acidic urine sample.* About 1.0 mg of each authentic standard was added separately to 50 ml of control urine, and each urine solution was made acidic with 5 ml of 1 *M* hydrochloric acid. The urine solutions were treated in the same manner as the above neutral urine samples. The contents of each vial were analysed periodically after neutralization with 1 *M* sodium hydroxide solution.

*Application*

Six rats were used when ten weeks old and weighing 337 ± 30 g (mean ± S.D., $n = 6$). After each rat had been placed in a metabolic cage for one week for acclimatization, a single dose of 100 mg/kg body mass of *p*-CNB diluted

in olive oil was injected intraperitoneally. The urine from the rats was collected after 0–6, 6–27.5, 27.5–50, 50–71, 71–95 and 95–120 h, and *p*-CNB and its metabolites in the urine samples were determined by the proposed method.

## RESULTS AND DISCUSSION

### *Chromatography*

Typical chromatograms of a sample prepared from rat urine spiked with *p*-CNB and 2,4-dichloroaniline and of a control urine sample under the system I conditions, are shown in Fig. 2A and B, respectively. The retention times of *p*-CNB and 2,4-dichloroaniline were 12.5 and 18.6 min, respectively. Similarly, chromatograms of *p*-chloroaniline, 2-chloro-5-nitrophenol, 2-amino-5-chlorophenol, *p*-chloroacetanilide and 4-chloro-2-hydroxyacetanilide under the system II conditions and of N-acetyl-S-(4-nitrophenyl)-L-cysteine under the system III conditions are shown in Figs. 3 and 4, respectively. The retention times

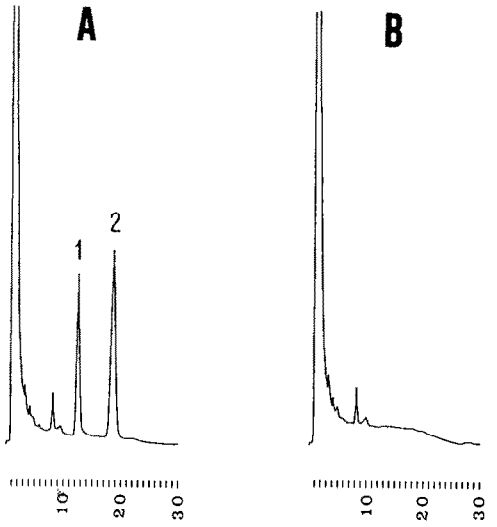


Fig. 2. Typical chromatograms of (A) a sample prepared from rat urine spiked with (1) *p*-CNB and (2) 2,4-dichloroaniline (20 μg/ml each) and (B) a control urine sample. HPLC conditions: mobile phase, water–methanol (60:40, v/v); analytical column, LiChrospher RP-Select B (125 mm × 4 mm I.D., 5 μm particle diameter) protected by an RP-Select B guard column (4 mm × 4 mm I.D.); flow-rate, 1.0 ml/min; column oven temperature, 30°C; injection volume, 20 μl; detection wavelength, 250 nm; detector attenuation, 5 mV full-scale.

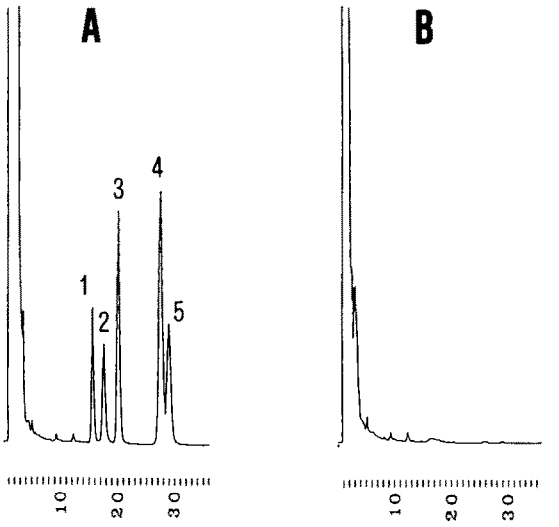


Fig. 3. Typical chromatograms of (A) a sample prepared from rat urine spiked with (1) 4-chloro-2-hydroxyacetanilide, (2) 2-amino-5-chlorophenol, (3) *p*-chloroaniline, (4) *p*-chloroacetanilide and (5) 2-chloro-5-nitrophenol (20 μg/ml each) and (B) a control urine sample. HPLC conditions: mobile phase, 0.005 *M* phosphate buffer (pH 3.6)–methanol (76:24, v/v) containing 1.2 m*M* sodium 1-octanesulphonate; detection wavelength, 240 nm; other conditions as in Fig. 2.

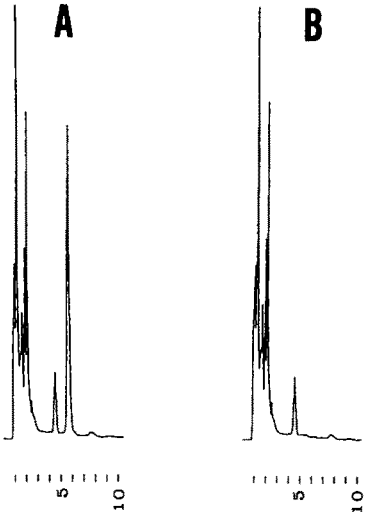


Fig. 4. Typical chromatograms of (A) a sample prepared from rat urine spiked with N-acetyl-S-(4-nitrophenyl)-L-cysteine (20 μg/ml) and (B) a control urine sample. HPLC conditions: mobile phase, 0.005 *M* phosphate buffer (pH 6.0)–methanol (80:20, v/v); detection wavelength, 340 nm; other conditions as in Fig. 2.

of 4-chloro-2-hydroxyacetanilide, 2-amino-5-chlorophenol, *p*-chloroaniline, *p*-chloroacetanilide and 2-chloro-5-nitrophenol were 15.6, 17.4, 19.9, 27.4 and 28.9 min, respectively, and that of N-acetyl-S-(4-nitrophenyl)-L-cysteine was 5.8 min. The sections of the chromatograms where *p*-CNB and its metabolites were eluted were free from interfering peaks under all analytical conditions (system I, II and III conditions). Therefore, the present chromatographic method appears to be satisfactory for the determination of *p*-CNB and its metabolites in rat urine.

*Calibration*

Statistical calculations at the 99% confidence interval showed that the slopes of the calibration graphs that were obtained after combining the standard solutions of *p*-CNB or each metabolite with an equal volume of control urine or water were identical for all the compounds. Therefore, mixed solutions of the standard solutions and water could be used as standard calibration solutions. For each calibration standard, the correlation coefficient of the normal linear regression lines was better than 0.9998 ($n = 10$).

*Precision and accuracy*

The precision and accuracy of the method were demonstrated by repeated analysis of urine spiked with each authentic standard. Six, seven or eight aliquots of pooled control urine spiked with each standard substance at different concentrations are shown in Figs. 5 and 6. Fig. 5 shows the assays performed on the same day and Fig. 6 shows the reproducibility of the assays from day to day. The precision of this method is indicated by the coefficients of variation (C.V.) in the studies with replicate assays ($n = 6$), and the accuracy of the method is indicated by the error of assayed samples relative to their spiked concentrations. The C.V. values of many compounds tended to increase at low concentrations of the compounds, but were less than 10%, which indicates good reproducibility, for all concentrations of each compound in both intra-day and inter-day assays. The accuracy of the intra-day assay was good, all errors being less than ± 10%, with many points being less than ± 5% for most of the compounds. Similar results were obtained for the inter-day assays. The accuracy and precision for the N-acetyl-S-(4-nitrophenyl)-L-cysteine analysis were particularly good in both intra- and inter-day assays. These results showed that the present method can offer excellent accuracy and precision, with satisfactorily reliable data.

*Recovery and detection limit*

The urine samples were hydrolysed for the determination of conjugated *p*-CNB and the metabolites (2,4-dichloroaniline, *p*-chloroaniline, 2-chloro-5-nitrophenol and 2-amino-5-chlorophenol). These conjugated metabolites were considered to be hydrolysed completely by heating the urine samples for 1 h at 100°C with one tenth the volume of concentrated hydrochloric acid because the amounts of these compounds (free forms) in urine, containing high concentrations of them, from rat dosed with *p*-CNB did not increase under more severe conditions for hydrolysis of the conjugates. In order to measure the amounts of these compounds (free forms) that disappeared from the urine and of the compounds degraded by heating, the variabilities in the recoveries of these compounds from urine were investigated by spiking control urine samples with known amounts of the compounds followed by heating the samples for 1 h at 100°C in an oil-bath. As shown in Fig. 7, each experiment, with six replicates, gave recoveries between 94 and 106%, indicating adequate recoveries, and the standard deviations were less than 9% for all concentrations studied for all metabolites. Therefore, the metabolites (free forms) were considered to be stable on heating under the hydrolysis conditions.

The recoveries of *p*-CNB and its seven metabolites from non-hydrolysed urine samples were approximately complete according to the above precision and accuracy experiments.

The detection limits of *p*-CNB and its metabolites in non-hydrolysed urine using this method at a signal-to-noise ratio of 2:1 were 0.05 μg/ml for 2,4-dichloroaniline, *p*-chloroacetanilide and N-acetyl-S-(4-nitrophenyl)-L-cysteine, 0.1 μg/ml for

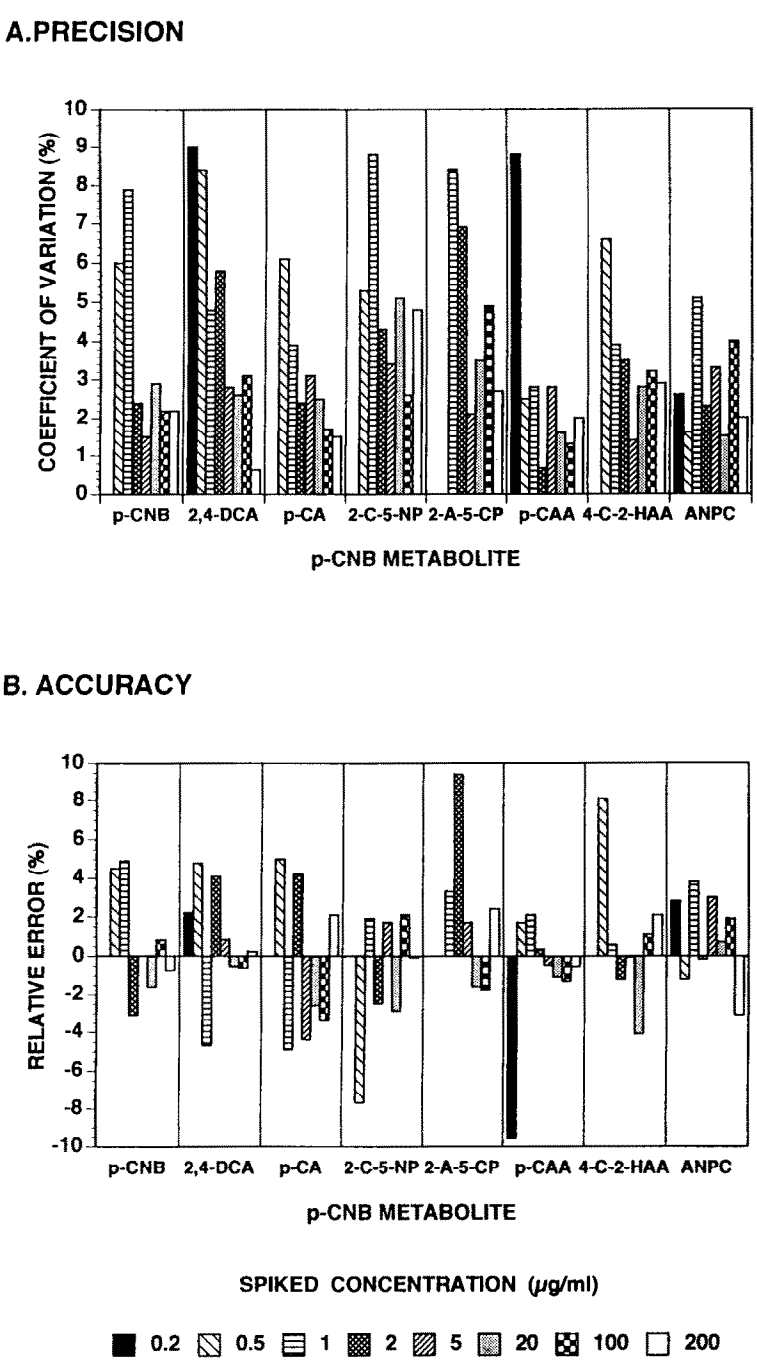


Fig. 5. Intra-day (A) precision and (B) accuracy of the method ($n$ = 6). $p$-CNB = $p$-chloronitrobenzene; 2,4-DCA = 2,4-dichloroaniline; $p$-CA = $p$-chloroaniline; 2-C-5-NP = 2-chloro-5-nitrophenol; 2-A-5-CP = 2-amino-5-chlorophenol; $p$-CAA = $p$-chloroacetanilide; 4-C-2-HAA = 4-chloro-2-hydroxyacetanilide; ANPC = N-acetyl-S-(4-nitrophenyl)-L-cysteine.

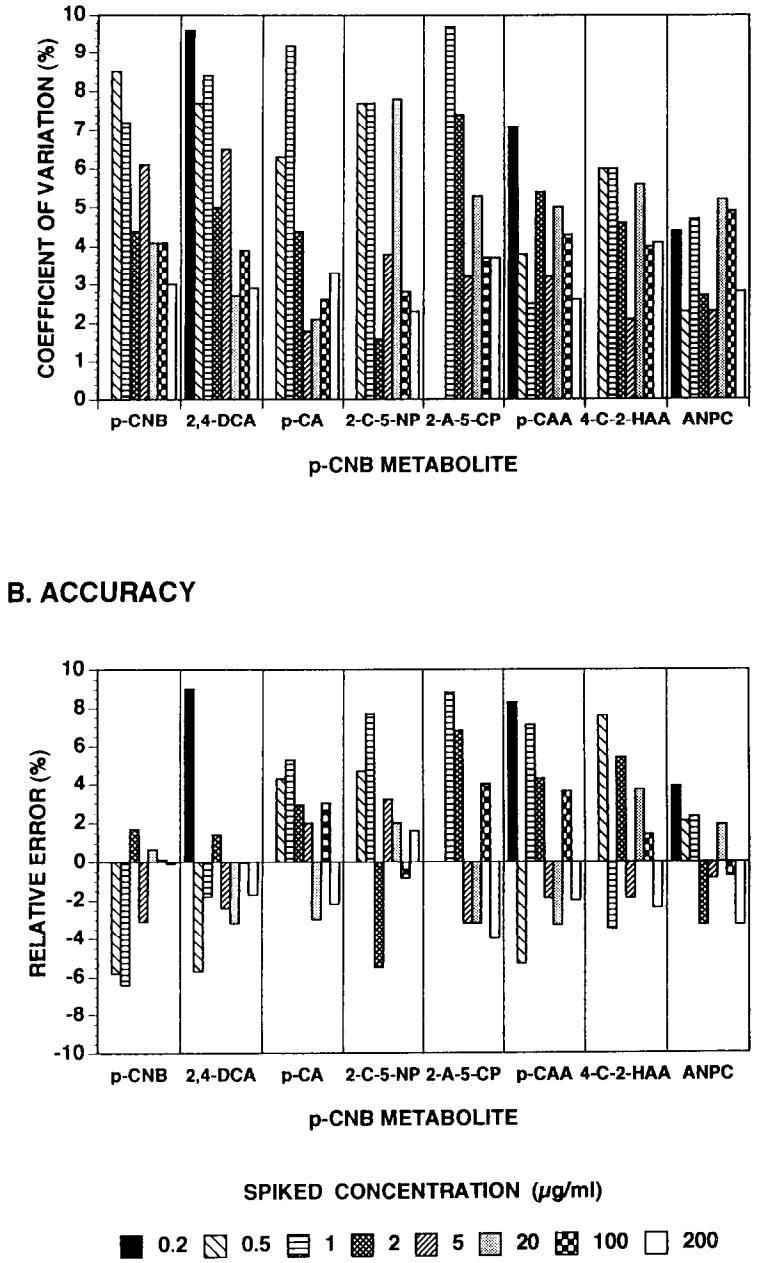


Fig. 6. Inter-day (A) precision and (B) accuracy of the method ($n$ = 6). $p$-CNB = $p$-chloronitrobenzene; 2,4-DCA = 2,4-dichloroaniline; $p$-CA = $p$-chloroaniline; 2-C-5-NP = 2-chloro-5-nitrophenol; 2-A-5-CP = 2-amino-5-chlorophenol; $p$-CAA = $p$-chloroacetanilide; 4-C-2-HAA = 4-chloro-2-hydroxyacetanilide; ANPC = N-acetyl-S-(4-nitrophenyl)-L-cysteine.

$p$-CNB, $p$-chloroaniline, 2-chloro-5-nitrophenol and 4-chloro-2-hydroxyacetanilide and 0.2 μg/ml for 2-amino-5-chlorophenol.

*Storage stability*

The stabilities of $p$-CNB and its metabolites at −20°C, 4°C and room temperature were evaluat-

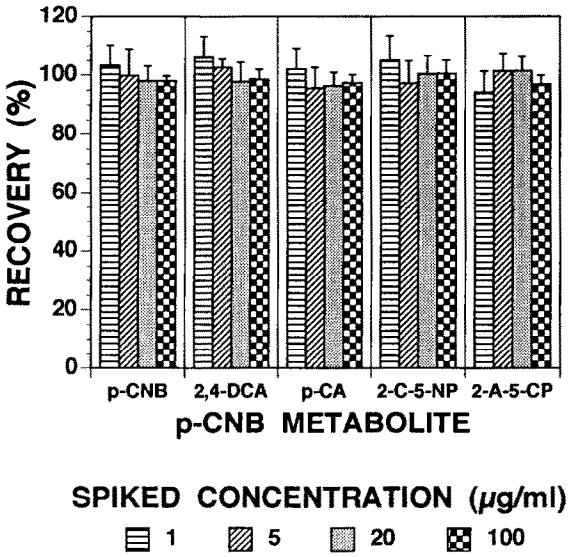


Fig. 7. Recoveries of *p*-CNB and its metabolites from urine heated for hydrolysis. Values are means + S.D. ($n$ = 6). *p*-CNB = *p*-chloronitrobenzene; 2,4-DCA = 2,4-dichloroaniline; *p*-CA = *p*-chloroaniline; 2-C-5-NP = 2-chloro-5-nitrophenol; 2-A-5-CP = 2-amino-5-chlorophenol.

ed for both neutral and acidic urine samples from rats, and the results are shown in Fig. 8. Most of the metabolites were demonstrated to be more stable at low temperature. *p*-CNB, 2,4-dichloroaniline and *p*-chloroaniline were most stable in frozen (−20°C) acidic urine samples, but the losses of *p*-CNB from the urine were *ca.* 10 and 45% over one week and one month, respectively, under these storage conditions. 2-Amino-5-chlorophenol decreased rapidly with time at all storage temperatures in neutral urine samples, and disappeared completely in two weeks at room temperature. On the other hand, in acidic urine, it was relatively stable. 2-Chloro-5-nitrophenol was fairly stable under all storage conditions for one month. Both *p*-chloroacetanilide and 4-chloro-2-hydroxyacetanilide were more stable in neutral than acidic urine samples. No significant degradation in frozen neutral urine samples were observed during one month. In frozen acidic urine, *p*-chloroacetanilide and 4-chloro-2-hydroxyacetanilide were relatively stable for one week, but 20 and 30%, respectively, were lost in one month. These results show that urine samples can be stored in an acidic state by freezing at −20°C until analysis. However, the analysis should be done as soon as possible after sampling.

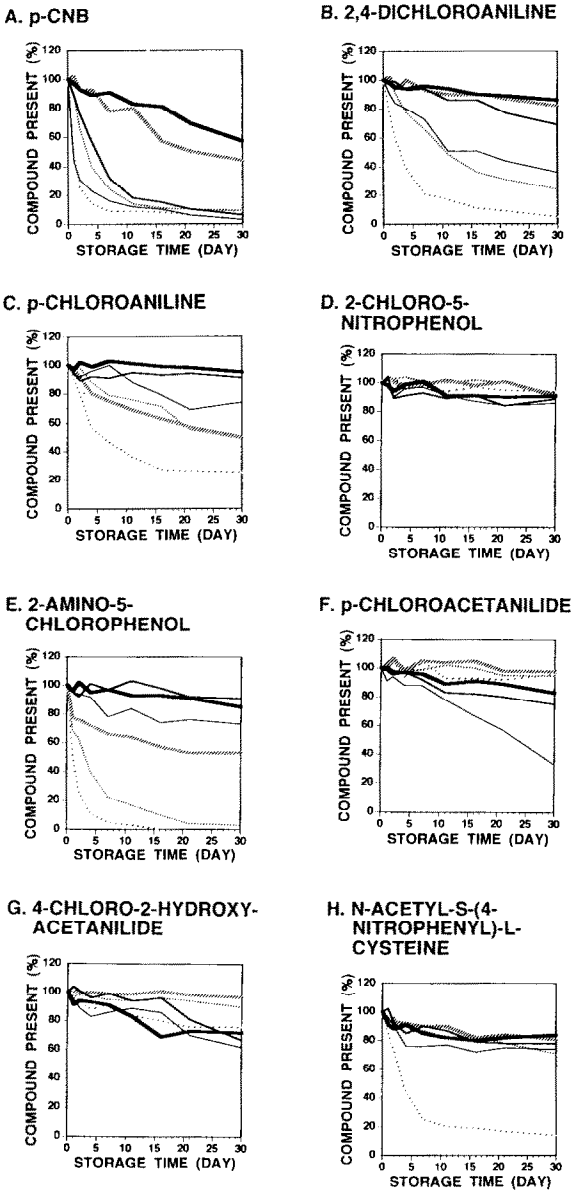


Fig. 8. Stabilities of *p*-CNB and its metabolites at −20°C, 4°C and room temperature (RT) in acidic and neutral urine samples. The concentration of each compound just before storage (time zero) was set at 100% (each compound, *ca.* 20 μg/ml).

TABLE I

TOXICOKINETIC PARAMETER ESTIMATES FOR RATS TREATEDS INTRAPERITONEALLY WITH *p*-CNB (100 mg/kg)

| Metabolite | $X^{\infty}$ (μg)[a] | Percentage of dose[b] | MRT (h)[c] |
|---|---|---|---|
| *Non-hydrolysed urine (free)* | | | |
| 4-Chloro-2-hydroxyacetanilide | 604–1217 | 2.23 ± 0.76 | 41.4 ± 3.85 |
| N-Acetyl-S-(4-nitrophenyl)-L-cysteine | 15 246–22 025 | 29.55 ± 2.42 | 23.1 ± 2.73 |
| *Hydrolysed urine (free + conjugated)* | | | |
| 2,4-Dichloroaniline | 337–691 | 1.40 ± 0.29 | 25.4 ± 3.72 |
| *p*-Chloroaniline | 1632–2095 | 6.68 ± 0.71 | 34.5 ± 3.60 |
| 2-Chloro-5-nitrophenol | 2762–5502 | 9.77 ± 1.80 | 27.5 ± 2.80 |
| 2-Amino-5-chlorophenol | 3928–4554 | 14.12 ± 1.13 | 28.0 ± 2.73 |

[a] Cumulative urinary excretion amount at infinite time of each metabolite. Values are maxima and minima for six rats.
[b] Each compound was converted into *p*-CNB equivalents to calculate the percentage of dose excreted. Mean ± S.D. ($n$ = 6).
[c] Mean residence time of each metabolite in the body. Mean ± S.D. ($n$ = 6).

*Application*

The urine from six rats treated with *p*-CNB was collected over a period and *p*-CNB and its metabolites in the samples were determined by the proposed method. Traces of unchanged *p*-CNB and *p*-chloroacetanilide were detected only for the first period. The changes of the six other metabolites with time were examined. Toxicokinetic parameters of 4-chloro-2-hydroxyacetanilide and N-acetyl-S-(4-nitrophenyl)-L-cysteine in the non-hydrolysed urine samples and of 2,4-dichloroaniline, *p*-chloroaniline, 2-chloro-5-nitrophenol and 2-amino-5-chlorophenol in the hydrolysed urine samples were estimated by moment analysis of the time course data [12], and the amount of each metabolite excreted, the percentage of the dose and the mean residence time of each metabolite are given in Table I.

In conclusion, this reversed-phase HPLC procedure can be used for the precise and accurate determination of *p*-CNB and its metabolites in urine samples from workers exposed to *p*-CNB and subjects poisoned by it.

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