Published in final edited form as:

Clin Toxicol (Phila). 2009 February ; 47(2): 132–136. doi:10.1080/15563650801971390.

TOXICOLOGY LABORATORY ANALYSIS AND HUMAN EXPOSURE TO *p*-CHLOROANILINE

AF Pizon, AR Schwartz, LR Shum, JC Rittenberger, DR Lower, S Giannoutsos, MA Virji, and MD Krasowski

Abstract

Introduction—*p*-Chloroaniline is more potent at producing methemoglobin than aniline in animal models. This case highlights the clinical presentation of an inhalation exposure to *p*-chloroaniline and associated laboratory analysis. An in-vitro study evaluating the metabolism of *p*-chloroaniline in human hepatocytes was undertaken to evaluate the metabolic fate more closely.

Case Presentation—A 20 year-old man was working at a chemical waste plant when he developed dizziness, abdominal pain, and nausea. The exam was remarkable for coma, tachycardia, cyanosis and pulse oximetry of 75%. Arterial blood gases showed a pH 7.38, pCO₂ 41 mmHg, pO₂ 497 mmHg, bicarbonate 24 mEq/L and methemoglobin 69%. Methylene blue administration led to complete recovery without sequelae. *p*-Chloroaniline was later identified as the chemical involved. He denied direct contact with the chemical, but was not wearing a dust mask or respirator. GC/MS confirmed *p*-chloroaniline and metabolites in the patient's urine.

Methods—Human hepatocytes were incubated with 100 μM *p*-chloroaniline for 24 hours, in both rifampicin- and vehicle only-treated cells. The cell culture medium was collected for GC/MS analysis for *p*-chloroaniline metabolites.

Results—Similar to the patient sample, both *p*-chloroaniline and *p*-chloroacetanilide were identified by GC/MS in hepatocytes incubated with *p*-chloroaniline. Neither *p*-chloroaniline incubated in empty cell culture nor direct GC/MS injection of *p*-chloroaniline generated any *p*-chloroacetanilide via non-enzymatic degradation.

Discussion/Conclusion—The seemingly innocuous dermal and inhalation exposure to *p*-chloroaniline dust can lead to life-threatening methemoglobinemia. The diagnosis can be confirmed with GC/MS analysis of the patient's urine, searching for *p*-chloroaniline and its primary metabolite *p*-chloroacetanilide.

Introduction

Aniline dyes are well-known to cause methemoglobinemia. As a source of oxidative stress, *p*-chloroaniline is more potent at producing methemoglobin than aniline in animal models (1). Previous case reports from the Italian (2) and French (3) literature reported *p*-chloroaniline as a cause of severe methemoglobin in occupational workers from both dermal and inhalation exposures. The risks associated with *p*-chloroaniline exposure can be grave if appropriate occupational precautions are not taken since non-oral exposure routes can lead to significant toxicity. *p*-Chloroaniline is frequently found as a product in the dye, pharmaceutical and herbicide industries. The rare occupational exposure to this chemical has led to the under-recognized hazards associated with its use.

The metabolic fate of *p*-chloroaniline in humans has not been well described in the English literature. Yet, the Japanese literature describes a patient who was acutely poisoned with *p*-chloroaniline. That study also analyzed urine from the patient by gas chromatography/mass spectrometry (GC/MS) and identified five possible metabolites: 2-amino-5-chlorophenol, 2,4-

dichloroaniline, *p*-chloroformanilide, *p*-chloroacetanilide, and 4-chloro-2-hydroxyacetanilide (4).

This case highlights the clinical presentation of an inhalation exposure to *p*-chloroaniline and associated laboratory analysis. Interference of arterial blood gas analysis by methylene blue is also illustrated. An in-vitro study evaluating the metabolism of *p*-chloroaniline in human hepatocytes was undertaken to evaluate the metabolic fate of *p*-chloroaniline more closely.

Case Presentation

A 20 year-old man's routine duties at work included shoveling unknown hazardous waste onto a conveyer for incineration. While performing this duty, he developed the sudden onset of dizziness, abdominal pain, and vomiting. Paramedics were immediately summoned. The patient was found lethargic, cyanotic and in respiratory distress. His capillary blood glucose was normal and he was placed on high-flow oxygen en route to a local emergency department. His initial evaluation was remarkable for tachycardia, cyanosis and a pulse oximetry reading of 75% despite high-flow oxygen. He was intubated and transferred to a tertiary care hospital.

Upon arrival, he was still cyanotic and hypoxic. Initial vital signs were as follows: temperature 37.0 C, pulse 123 beats per minute, respirations 14 breaths per minute (assisted by ventilator), blood pressure 160/100 mmHg, and pulse oximetry of 83% on 100% FiO₂. The rest of his physical exam was significant for coma (despite no administration of sedative-hypnotic medications), clear lungs, soft abdomen, warm and diaphoretic skin, and diffuse cyanosis. Endotracheal tube placement was confirmed and despite ventilator adjustments, there was no change in his cyanosis and hypoxia. While obtaining an arterial blood sample, his blood was noted to be unusually dark in color. Arterial blood gases demonstrated: pH 7.38, pCO₂ 41 mmHg, pO₂ 497 mmHg, bicarbonate 24 mEq/L, and methemoglobin of 69%. Intravenous methylene blue was immediately administered at 2 mg/kg. Within 30 minutes, the cyanosis resolved, he became more awake and pulse oximetry increased to 92%. Repeat arterial blood gases demonstrated: pH 7.34, pCO₂ 41, pO₂ "unavailable", bicarbonate 21 mEq/L, and a decrease in methemoglobin concentration to 30%. The rest of his laboratory results from admission showed normal complete blood count (no signs of hemolysis), electrolytes, BUN, creatinine, and urinalysis. His chest x-ray was clear and an EKG was only remarkable for sinus tachycardia.

The patient was maintained under mechanical ventilation due to a relative hypoxia despite normalization of his mental status. Three hours after methylene blue administration, his arterial blood gases were: pH 7.37, pCO₂ 39 mmHg, pO₂ 97 mmHg, bicarbonate 22 mEq/L, and methemoglobin of 6%. He was on a FiO₂ of 100% and had clear lungs, making his anticipated paO₂ 450 mmHg. An additional 4 hours later, his paO₂ suddenly increased to 380 mmHg and he was extubated without complication.

The patient clarified the events leading to his hospitalization. He was shoveling the same "dirt-like" substance for several hours when he decided to take a break and remove his respirator. He continued to wear his goggles, rubber gloves, boots, and a Tyvek™ suit. After he completed the short break, he returned to his job, but had forgotten to re-apply his respirator. Though he denied direct contact with the substance, his head was at the opening of the 55-gallon drum for approximately 15 minutes while he reached into the bottom to remove the last of its contents when he became ill. A co-worker was working 10 feet away on a separate container with different contents and developed no ill effects. A comprehensive urine drug analysis using GC/MS demonstrated *p*-chloroaniline, *p*-chloroacetanilide, caffeine, midazolam, and propofol. The midazolam and propofol were the result of iatrogenic administration of medications in the Emergency Department.

During the initial treatment of the patient, the causative agent was not immediately known. It did not appear coincidental that the patient was working at a hazardous waste incinerator at the time of his illness, and a chemical agent appeared to be the most likely cause of his illness. The plant safety officer did not know the chemicals to which the patient was exposed at the onset of his illness. Since the contents of these drums were labeled for incineration, the exact products are not often known. Instead, waste products are sorted by health risk category. A list of chemicals received by the incinerator is available, but often the exact contents of a specific drum are not known.

The authors attempted to identify the chemical to be sure no other clinical effects from the unknown would require treatment. Therefore, GC/MS was used to identify any potential methemoglobin-producing agents in the patient's urine. A spectral search using a library of occupational chemicals (NIST/EPA/NIH Mass Spectral Library, 2002 version, Gaithersburg, MD) detected *p*-chloroaniline and *p*-chloroacetanilide. Later, *p*-chloroaniline was confirmed in the drum with which the patient was working. *p*-Chloroaniline has since been identified as a frequent product listed for incineration at this facility and a frequent potential for worker exposure.

Methods

Chemicals

p-Chloroaniline, *p*-chloroacetanilide, 4-aminophenol, 2,4-dichloroaniline, 2-amino-5-chlorophenol, barbitol, dimethyl sulfoxide, and rifampicin were obtained from Sigma-Aldrich (St. Louis, MO). Methylene chloride was purchased from Fisher Scientific.

Gas chromatography/mass spectrometry

The patient's urine was analyzed by GC/MS in the Toxicology and Therapeutic Drug Monitoring section of the University of Pittsburgh Medical Center's Division of Clinical Chemistry laboratory, using a protocol designed to identify a broad range of therapeutic and illegal drugs in urine. For the extraction, 5 mL of urine was used and the pH adjusted to less than 6 with 1 *N* HCl. Following this, 5 mg of decolorizing activated charcoal was added. The sample was vortexed for 1 min and then centrifuged for 5 min at 3000 rpm. The supernatant was transferred to another tube (leaving behind a charcoal pellet). The tube with the charcoal pellet was vortexed for 15 sec, and 5 mL of methylene chloride with 1 µg/mL barbitol (internal standard) was added to each of the two tubes (i.e., the tube containing the supernatant and the tube containing the charcoal pellet). Both tubes were then vortexed for 1 min and then centrifuged for 5 min at 3000 rpm. For the tube containing the charcoal, the methylene chloride supernatant was carefully pipetted off the precipitated charcoal and transferred to another tube; the left-over charcoal was discarded.

In the tube containing the original supernatant, the top aqueous layer and interface layer were carefully pipetted off and discarded. The remaining liquid (methylene chloride extract) was then combined with the methylene chloride extract from the charcoal tube and then placed under a nitrogen evaporator and evaporated to dryness. The residue was then dissolved in 25 µL methanol for analysis by GC/MS. A Hewlett-Packard 5973 mass spectrometer combined with a 6890 gas chromatograph was used for the GC/MS analysis under the following conditions: injection mode, splitless; column, Agilent 19091J-433 capillary column (5% phenyl methyl siloxane, 30 m × 0.25 mm × 0.25 µM); column oven temperature, programmed from 80°C to 325°C at 8°C/min; injection temperature, 250°C; carrier gas, helium; column pressure, 9.38 psi; ionization method, electron impact; ionization energy, 70 eV; ionization current, 300 µA; accelerating voltage, 2.3 kV; ion source temperature 230°C.

Human hepatocyte studies

Human hepatocytes were obtained through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA), which is funded by National Institutes of Health Contract #N01-DK-9-2310. Human hepatocytes were prepared by the three-step collagenase perfusion technique as previously described (5). Hepatocytes were plated on Falcon twenty-four-well culture plates, previously coated with rat-tail collagen in Williams' E medium (Cambrex BioScience, Walkersville, MD) supplemented with 0.1 μ M insulin, 0.1 μ M dexamethasone (Cambrex BioScience), 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B, and 10% bovine calf serum (Invitrogen, Carlsbad, CA).

After the cells attached for 4 hours, the medium was replaced with serum-free medium containing all the supplements described above. All subsequent steps utilized serum-free media. Cells were maintained in culture at 37°C in atmosphere containing 5% CO₂ and 95% air. After 24 hour in culture, unattached cells were removed by gentle agitation and the medium changed every 24 hours. After plating for 48 and 72 hours, cells were exposed to either rifampicin (10 μ M; Sigma-Aldrich, St. Louis, MO) or carrier solvent alone (0.1% dimethylsulfoxide; Sigma-Aldrich). At 96 hours, the medium was removed and replaced with medium containing *p*-chloroaniline (100 μ M). After 24 hours of *p*-chloroaniline exposure, the medium was collected and stored at 4°C until further analysis.

Results

Analysis of the patient's urine by GC/MS following a methylene chloride-based extraction revealed the presence of compounds with electron impact spectra close to those previously reported for *p*-chloroaniline (Figure 1B) and *p*-chloroacetanilide (Figure 1E) (4). The retention times and spectra from analysis of the patient sample also matched GC/MS spectra and retention times obtained from direct injection of methanolic solutions of *p*-chloroaniline (Figure 1A) and *p*-chloroacetanilide (Figure 1D). Guided by data from published investigations on the metabolism of *p*-chloroaniline (4), spectra were also obtained from other possible *p*-chloroaniline metabolites by direct injections of methanolic solutions of 2,4-dichloroaniline and 2-amino-5-chlorophenol; however, these additional compounds were not identified in the patient's urine. Other unrelated compounds detected in the patient's urine by GC/MS included caffeine, midazolam, and propofol. Analysis of twenty randomly selected urine samples from other patients submitted to the Toxicology and Therapeutic Drug Monitoring laboratory did not reveal any presence of *p*-chloroaniline or *p*-chloroacetanilide, indicating that the presence of these compounds is unlikely to be an environmental contaminant. In addition, GC/MS analysis of direct injections of *p*-chloroaniline did not reveal *p*-chloroacetanilide showing that generation of *p*-chloroacetanilide was not an artifact of the gas chromatography separation conditions and mass spectrometry analysis. Given the toxicity of *p*-chloroaniline, the authors opted to study its metabolic fate on primary human hepatocytes in a cell culture model, rather than risk exposure to human volunteers. This in-vitro evaluation is not only safer, but should provide equally valid results. Primary hepatocytes were incubated with 100 μ M *p*-chloroaniline for 24 hours.

Half of the studies used hepatocytes that had been treated with 10 μ M rifampicin for 48 hours to increase the expression of hepatic drug-metabolizing enzymes; the remainder were treated with the vehicle control (0.1% v/v dimethyl sulfoxide) for 48 hours. Similar to the patient sample, both *p*-chloroaniline (Figure 1C) and *p*-chloroacetanilide (Figure 1F) was identified by GC/MS in hepatocytes incubated with 100 μ M *p*-chloroaniline, both in the rifampicin- and vehicle only-treated cells. To verify that *p*-chloroacetanilide was produced by liver cell metabolism and not non-cell mediated chemical reactions, 100 μ M *p*-chloroaniline was also incubated in empty cell culture wells under the same media, temperature, and atmosphere as

the hepatocyte cultures. Under these conditions, no *p*-chloroacetanilide was generated showing that the hepatocytes did indeed metabolize *p*-chloroaniline to *p*-chloroacetanilide.

Discussion

This case highlights the clinical presentation of an inhalation exposure to *p*-chloroaniline. The diagnosis of methemoglobinemia was made promptly by the clinical findings and the classic arterial blood gas findings at the time of presentation. This allowed for rapid treatment and recovery with the use of intravenous methylene blue. The paucity of data in the literature on the metabolic fate of *p*-chloroaniline prompted us to test the metabolism of this compound by primary human hepatocytes. Incubation of primary human hepatocytes also resulted in formation of *p*-chloroacetanilide demonstrating that this metabolite can be formed by liver cells. These results indicate that GC/MS may be used to detect the presence of *p*-chloroaniline and at least one of its metabolites. A previous study of acute human poisoning by *p*-chloroaniline published in the Japanese literature (4) identified five possible metabolites: 2-amino-5-chlorophenol, 2,4-dichloroaniline, *p*-chloroformanilide, *p*-chloroacetanilide, and 4-chloro-2-hydroxyacetanilide. That study used diethyl ether-based extractions at pH 1.0 and 10.0, treatments which likely hydrolyzed conjugates, possibly accounting for differences in detection between our study and theirs. Nevertheless, both studies demonstrate that *p*-chloroaniline can be converted to *p*-chloroacetanilide.

The mechanism by which *p*-chloroaniline mediates methemoglobinemia in humans is not well understood. Animal studies of aniline toxicity implicate nitroso and hydroxylamine metabolites as contributing significantly to methemoglobinemia (6,7). Although our study and others (4) did not detect nitroso or hydroxylamine metabolites of chloroaniline (see Fig. 2 for chemical structures), such metabolites would be predicted to be potent mediators of methemoglobinemia. Oxidation of *p*-chloroaniline to the hydroxylamine with subsequent redox cycling between the hydroxylamine and nitroso metabolites would place undue oxidant stress on hemoglobin (8). The structurally similar compound, aniline, has been shown to redox cycle in this fashion with the concurrent formation of methemoglobin (9). Due to the similarity of *p*-chloroaniline to aniline, one would expect similar mechanisms for methemoglobin formation.

Dapsone also forms methemoglobin similarly. As seen with dapsone use, patients with the poor ability to acetylate xenobiotics would be at a greater risk for oxidant stress from *p*-chloroaniline (10). Thus, one may theorize that the relatively innocuous exposure resulting in severe methemoglobinemia in the case patient may be due to his poor ability to acetylate. However, the patient's acetylation status had not been tested. Unfortunately, the ability of acetylated metabolites such as *p*-chloroacetanilide to produce methemoglobinemia is unknown either. The acetylation pathway may simply be a detoxifying pathway but further research is needed. Arterial whole blood was analyzed on an ABL_700 series blood gas analyzer (Radiometer Medical A/S, Denmark). The optical determination of methemoglobin on the instrument shows interference from methylene blue because of the overlap in their absorbance spectra at the 630 nm wavelength used for determination of methemoglobin concentration in blood (11). There can also be interference in O₂ content due to high methylene blue concentration. This led to the falsely low O₂ content measured after methylene blue treatment. This interference would be expected in most other manufacturers' blood gas analyzers due to the oxygen electrode used to measure O₂ content. Therefore, methemoglobin values and O₂ content obtained after initiation of therapy with methylene blue need to be interpreted with caution until methylene blue is eliminated from circulation.

After methylene blue is used to treat methemoglobinemia, a clinical evaluation of the patient's ability to oxygenate is necessary since the O₂ content will be falsely low in these scenarios. In our case, the patient remained intubated for several hours longer than necessary due to this

interference. There is no certain recommendation to alleviate this problem for all cases, but if repeat doses of methylene blue were necessary, this interference would have persisted for a longer duration. Most commercial blood gas analyzers will be affected by this problem, and therefore, there is no simple solution. The patient's clinical status will provide the most useful information about their ability to oxygenate. The oxygen saturation (measured via cooximetry) is a more reliable laboratory evaluation of oxygenation status due to its lack of interference with methylene blue. Certainly, it is well known the oxygen saturation (measured via the pulse oximeter) is not reliable after a methylene blue injection.

To our knowledge, only one case report on toxicity of *p*-chloroaniline has been published in the English language (12). Neonates were exposed to *p*-chloroaniline when chlorhexidine was accidentally used instead of sterile water in their incubators. Chlorhexidine was discovered to decompose to *p*-chloroaniline rapidly when exposed to the heat from the incubator. This was made known by GC/MS analysis of the solution in the incubators.

Conclusion

The seemingly innocuous dermal and inhalation exposure to *p*-chloroaniline dust and aerosol can lead to life-threatening methemoglobinemia (2,3,12). The oxidant stress rapidly reverses with methylene blue and the diagnosis can be confirmed with GC/MS analysis of the patient's urine, searching for *p*-chloroaniline and its primary metabolite *p*-chloroacetanilide.

Acknowledgements

M.D.K. is supported by a physician-scientist career development grant K08-GM074238 from the National Institutes of Health (NIH). M.D.K. gratefully acknowledges advice regarding primary human hepatocyte studies from Dr. Stephen Strom of the University of Pittsburgh, Department of Pathology. J.C.R. is supported by Grant Number 1 KL2 RR024154-02 from the National Center for Research Resources (NCRR), a component of the NIH, and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. J.C.R. is also supported by an unrestricted grant from the National Association of EMS Physicians/Zoll EMS Resuscitation Research Fellowship. The authors also gratefully acknowledge Dr. Jesse T. Mann and Dr. Alex C. Mangili for providing detailed translations of the non-English literature.

References

1. Nomura A. Formation of sulfhemoglobin using various drugs. *Nippon Yakurigaku Zasshi* 1975;71:351–365. [PubMed: 1237450]
2. Scotti P, Tomasini M. On a case of grave acute poisoning by parachloraniline with intense methemoglobinemia and transitory electrocardiographic changes. *Med Lav* 1966;57:662–666. [PubMed: 5985056]
3. Faivre M, Armand J, Evreux JC, Duverneuil G, Colin C. Toxic methemoglobinemia caused by aniline derivatives: parachloroaniline and paratoluidine (2 cases). *Arch Mal Prof* 1971;32:575–577. [PubMed: 5123553]
4. Yoshida T, Hirata M, Tabuchi T, Miyajima K. Identification of urinary metabolites in a patient of acute poisoning by *p*-chloroaniline. *Sangyo Igaku [Jap J Indust Heath]* 1991;33:501–508.
5. Komoroski BJ, Parise RA, Egorin MJ, Strom SC, Venkataramanan R. Effect of the St. John's wort constituent hyperforin on docetaxel metabolism by human hepatocyte cultures. *Clinical Cancer Research* 2005;11:6972–6979. [PubMed: 16203790]
6. Harrison JH Jr, Jollow DJ. Contribution of aniline metabolites to aniline-induced methemoglobinemia. *Mol Pharmacol* 1987;32:423–431. [PubMed: 3670278]
7. Singh H, Purnell ET. Aniline derivative-induced methemoglobin in rats. *J - Environ Pathol Toxicol Oncol* 2005;24:57–65. [PubMed: 15715509]
8. Golly I, Hlavica P. N-Oxidation of 4-chloroaniline by prostoglandin synthase. *Biochem J* 1985;260:803–809. [PubMed: 2985047]

9. Bus JS, Popp JA. Perspectives on the mechanism of action of the splenic toxicity of aniline and structurally-related compounds. *Food Chem Toxicol* 1987;25:619–626. [PubMed: 3305247]
10. Tingle MD, Mahmud R, Maggs JL, Pirmohamed M, Park BK. Comparison of the metabolism and toxicity of dapsone in rat, mouse, and man. *J Pharmacol Exp Ther* 1997;283:817–823.
11. Haymond S, Cariappa R, Eby CS, Scott MG. Laboratory assessment of oxygenation in methemoglobinemia. *Clin Chem* 2005;51:434–444. [PubMed: 15514101]
12. Van der Vorst MMJ, Tamminga P, Wifburg FA, Schutgens RBH. Severe methaemoglobinaemia due to para-chloroaniline intoxication in premature neonates. *Eur J Pediatr* 1990;150:73. [PubMed: 2079085]

Figure 1.

GC/MS detection of *p*-chloroaniline and *p*-chloroacetanilide. (A) and (D) were determined following direct injections of methanolic solutions of *p*-chloroaniline and *p*-chloroacetanilide, respectively. (B) and (E) were determined from extractions of the urine sample from the patient. (C) and (F) were determined from extractions of cell culture media that had been used to incubate primary human hepatocytes with 100 μ M *p*-chloroaniline. The hepatocytes used for the data in (C) and (F) were from hepatocytes not stimulated with rifampicin although similar results were obtained with rifampicin-stimulated hepatocytes (not shown).

Figure 2. The chemical structures of (A) *p*-chloroaniline, the acetylated metabolite identified via GC/MS, (B) *p*-chloroacetanilide, and the metabolites suspected to cause methemoglobinemia, (C) 4-chlorophenyl hydroxylamine and (D) 4-chloronitroso benzene.