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Short communication

Gas chromatographic–mass spectrometric identification and quantification of aniline after extraction from serum and derivatization with 2,2,2-trichloroethyl chloroformate, a novel derivative

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Abstract

Aniline is widely used as an intermediate in the synthesis of dyes. It is also used in the manufacture of pharmaceuticals, photographic developers, shoe polish, etc. Exposure to aniline is toxic because it produces methemoglobin. In humans, blood methemoglobin levels are often measured as an index of exposure to aniline. Here a method is described for identification and quantification of aniline by gas chromatography–mass spectrometry after extraction from human serum and derivatization with 2,2,2-trichloroethyl chloroformate. Aniline, along with the internal standard *N*-methylaniline, were extracted from alkaline serum using chloroform. Aniline and the internal standard were derivatized with 50 μl 2,2,2-trichloroethyl chloroformate. After evaporating excess derivatizing reagent, the residue was reconstituted in 50 μl chloroform and injected into the gas chromatographic–mass spectrometry (GC–MS) system. A positive identification of derivatized aniline can be made by observing strong molecular ions at *m*/*z* 267 and 269. Similarly, the derivatized internal standard showed strong molecular ions at *m*/*z* 281 and 283. The within-run and between-run precisions of the assay were 3.61 and 5.92%, respectively, at an aniline concentration of 5 mg/l. The assay was linear for serum aniline concentrations of 0.5–25.0 mg/l. The detection limit was 0.1 mg/l. The assay was not affected by lipemia, hemolysis or high bilirubin concentration in serum.

Keywords: Derivatization, GC; Aniline; 2,2,2-Trichloroethyl chloroformate

1. Introduction

Aniline is a colorless aromatic liquid that tends to darken on exposure to light. It has a characteristic odor. Aniline is used as an intermediate in the synthesis of dyes. Commercial applications also include its use in the manufacturing of pharmaceuticals, photographic developers, shoe polish, resins, varnish, perfumes and synthesis of organic compounds [1]. The threshold limit value in the industrial atmosphere is currently 2 ppm. Exposure to aniline is commonly by inhalation of the vapor or by cutaneous absorption of the liquid. Probably the latter mode is of the greatest toxicological importance in industry [2]. Because aniline easily penetrates skin, it has been associated with many cases of accidental exposure and attempted suicide [3,4].

Acute or chronic exposure to aniline produces

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symptoms of headache, dizziness and nausea. Exposure to aniline produces methemoglobin in blood. A methemoglobin level of 15% is consistent with clinical cyanosis and levels exceeding 60% may be life threatening. A woman who ingested 80 ml of aniline developed a blood aniline level of 25 mg/l and a methemoglobin level of 50%, but survived the intoxication after hemodialysis and methylene blue administration. [5]. Exchange transfusion was used to treat a child who ingested 5 ml of aniline and developed a blood methemoglobin level of 77% [6]. Absorption of aniline from diapers marked with aniline-containing ink has resulted in cases of severe methemoglobinemia in newborn babies [7].

Less than 1% of absorbed aniline is excreted unchanged in urine. The major metabolite of aniline found in urine is *p*-aminophenol. Linch considered that a urinary *p*-aminophenol concentration of 10 mg/l is an indication of toxic exposure to aniline and a concentration of 20 mg/l indicates the need for medical attention [8]. Piotrowski demonstrated that the rate of urinary excretion of *p*-aminophenol in a timed urine specimen taken at the end of the exposure period can be used to estimate the amount of aniline absorbed by a subject [2]. The urinary *p*-aminophenol concentration also appears to be directly related to blood methemoglobin levels in workers exposed to aniline [9].

The concentration of aniline is usually not measured in blood. The concentration of *p*-aminophenol is measured in urine after acid hydrolysis of glucuronide and sulfate conjugates in order to determine aniline exposure [10]. One major limitation of this approach is that *p*-aminophenol is also a major metabolite of acetaminophen and phenacetin. Therefore, use of these common drugs may lead to a *p*-aminophenol concentration of 200 mg/l or more in urine, a concentration 10- to 20-fold higher than suspected from exposure to aniline. Therefore, use of these drugs has to be ruled out before measuring the *p*-aminophenol concentration in urine for determination of aniline exposure. Moreover, the concentration of unchanged aniline in urine is very low and cannot be used as a marker for aniline exposure.

In order to circumvent this problem, a method was developed to determine the concentration of aniline in blood. *N*-Methyl aniline was used as an internal standard. Lubash et al. determined the concentration of aniline in blood using the diazo reaction with *N*-(1-naphthyl) ethylenediamine [5]. However, this method is non-specific because diazotizable metabolites of aniline also react with the reagent. Aniline is a polar molecule and the amino group was derivatized prior to gas chromatographic–mass spectrometric analysis. The derivatizing agent used for this method was 2,2,2-trichloroethyl chloroformate which formed a carbamate with the amino group of aniline. This derivatization protocol is rapid and has never been described in the literature for analysis of aniline. Moreover, in this novel derivatization protocol the small molecule aniline with a molecular weight of 93 was converted to 2,2,2-trichloroethylaniline carbamate with a molecular weight of 267.

2. Experimental

Aniline, the internal standard *N*-methylaniline and the derivatizing agent 2,2,2-trichloroethyl chloroformate were purchased from Aldrich (Milwaukee, WI, USA). The HPLC-grade chloroform and sodium tetraborate decahydrate were also obtained from Aldrich.

A standard solution of aniline (1 mg/ml) was prepared in chloroform. A standard solution of *N*-methylaniline, the internal standard, was also prepared in chloroform (0.1 mg/ml). To 1 ml of serum supplemented with aniline, 50 μl of the internal standard solution was added. Then 1 ml of borate buffer (pH 9.8) was added. The borate buffer was prepared by dissolving 20 g sodium tetraborate decahydrate in 1 l deionized water. Aniline, along with the internal standard, were extracted with 5 ml chloroform. Then the bottom organic layer was separated from the top aqueous layer by centrifugation for 5 min at 1500*g*. The upper aqueous layer was discarded and the bottom organic layer was concentrated under nitrogen almost to dryness. Then 50 μl of the derivatizing agent (2,2,2-trichloroethyl chloroformate) was added to the concentrated extract and the reaction mixture was incubated at 80°C for 10 min. Then the excess derivatizing agent was evaporated almost to dryness and the residue was reconstituted in 50 μl ethyl acetate and 2 μl was injected into the GC–MS.

The GC–MS analysis was carried out using a Model 5890 gas chromatograph coupled to a 5970 series mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). The capillary column used was an Ultra-2 also available from Hewlett-Packard. Splitless injection was used for this study. The initial temperature of the oven was 175°C. After maintaining that temperature for 5 min, the oven temperature was increased at a rate of 20°C/min to reach a final oven temperature of 300°C. The final temperature was maintained for 1 min and the total run time was 12.8 min. Helium was used as carrier gas. The mass spectrometer was operated in the electron ionization scan mode (range, m/z: 50–350). Quantification of the peaks was based on peak area.

3. Results and discussion

3.1. Chromatographic properties of derivatized aniline and the internal standard

Chemical structures of the derivatives are given in Fig. 1.

A baseline separation between derivatized aniline (retention time 9.2 min) and the derivatized *N*-methylaniline, the internal standard (retention time 8.4 min), was observed. A typical total ion chromatogram showing the separation of two peaks is given in Fig. 2. Excellent peak shape was observed for both derivatized aniline and the internal standard. No interfering peak was observed in the chromatograms when lipemic, high-bilirubin-containing (liver disease) or hemolyzed specimens were supplemented with aniline and subsequently analyzed by the new GC/MS protocol.

3.2. Mass spectral characterization of derivatized aniline and the internal standard

In the electron ionization mass spectrum of 2,2,2-trichloroethylaniline carbamate (derivatized aniline) two strong molecular ions (due to the isotopic effect of chlorine) were observed at m/z 267 (relative abundance 88.3%) and m/z 269 (relative abundance 80.2%). Another strong peak at m/z 120 (relative abundance 84.1%) was observed due to the $[M–O–CH_2–CCl_3]^+$ fragment. The base peak was observed

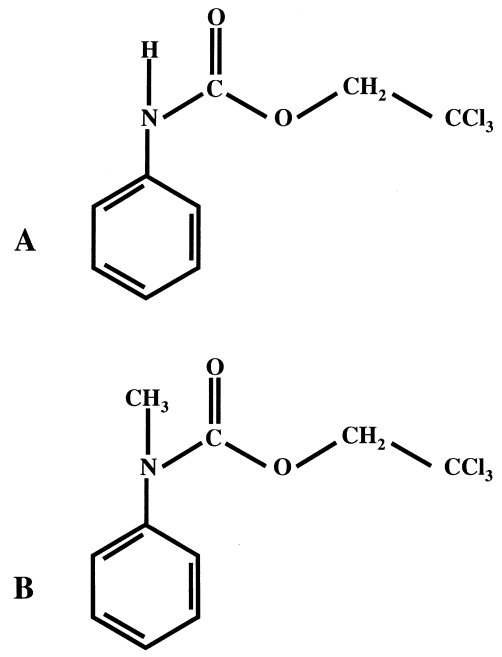

Fig. 1. Chemical structures of the derivatives. (A) 2,2,2-Trichloroethylaniline carbamate, (B) 2,2,2-trichloroethyl *N*-methylaniline carbamate.

at m/z 92 due to very stable $C_6H_5NH^+$ (Fig. 3). The electron ionization mass spectrum of 2,2,2-trichloroethyl *N*-methylaniline carbamate showed two strong molecular ions at m/z 281 (relative abundance 57.4%) and m/z 283 (relative abundance 50.4%). Another strong peak (relative abundance 40.0%) was

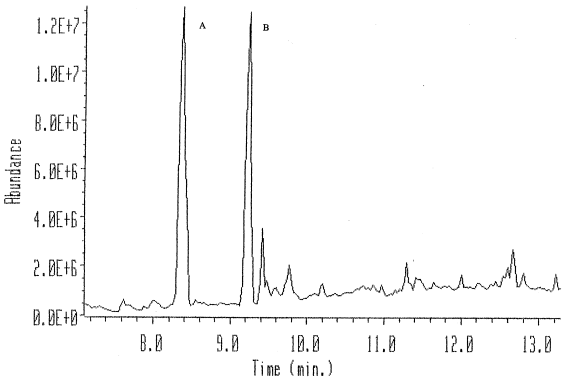

Fig. 2. Total-ion chromatogram showing the separation between derivatized aniline (peak B) and derivatized internal standard (peak A). The concentration of aniline in serum was 5 mg/l. The concentration of the internal standard was also 5 mg/l.

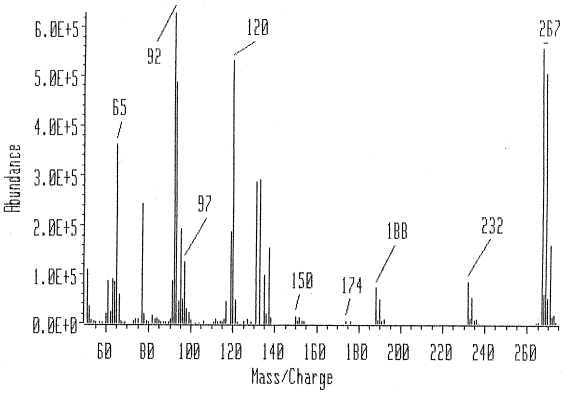

Fig. 3. Electron ionization mass spectrum of 2,2,2-trichloroethylaniline carbamate.

observed due to the loss of the $O–CH_2–CCl_3$ group from the derivatized internal standard molecule. The base peak was observed at m/z 106 due to $C_6H_5NCH_3^+$. Another strong peak was observed at m/z 77 (relative abundance 48.1%) due to $C_6H_5^+$ (Fig. 4).

3.3. Precision, linearity and detection limit

The within-run and between-run precisions of aniline assay were determined using a serum standard containing 5 mg/l aniline. The within-run CV was 3.61% (mean 4.98, SD 0.18, $n=8$). The between-run precision was 5.92% (mean 4.90, SD 0.29, $n=8$). The assay was linear for a serum aniline concentration of 0.5–25.0 mg/l. Using the x-axis as the target serum aniline concentration and the y-axis as the observed serum aniline concentration, the following regression equation was observed in the linearity study:

$$Y = 0.97X + 0.53 \quad (r = 0.99)$$

The detection limit of the assay was 0.10 mg/l of serum aniline concentration.

Lubash et al. reported an aniline concentration of 25 mg/l in a critically ill woman [5]. Therefore, in the linearity study, standards were prepared up to 100 mg/l aniline, in order to cover the very high and very low end of blood aniline concentration that may occur due to exposure. However, deviation from linearity was observed at an aniline concentration of >25 mg/l. Therefore, serum samples containing >25 mg/l should be diluted prior to analysis.

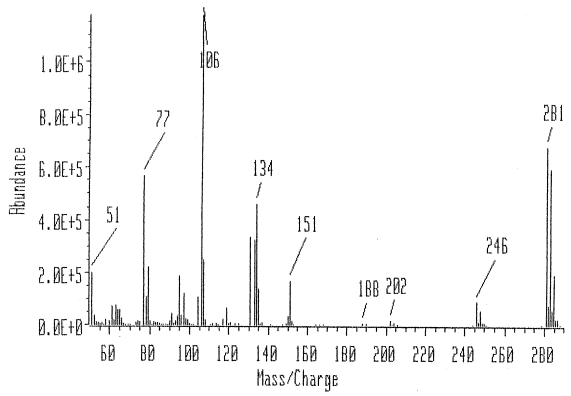

Fig. 4. Electron ionization mass spectrum of 2,2,2-trichloroethyl *N*-methylaniline carbamate.

3.4. Carry-over

A blank serum containing no aniline, supplemented with the internal standard only and after extraction and derivatization, was injected into the GC–MS just after analyzing a serum specimen containing 25 mg/l aniline. No peak for aniline was observed in the chromatogram, indicating that the assay had no carry-over problem. In another experiment, 2 μl ethyl acetate was injected into the GC–MS after analyzing a serum specimen containing 25 mg/l aniline. Again, no peak was observed. Therefore, the assay is free from carry-over.

3.5. Analysis of samples with high triglycerides, bilirubin and hemolysis

The potential interference from lipemic (high triglycerides), high-bilirubin-containing and gross hemolyzed specimens in the GC–MS assay was studied. No potentially interfering peak was observed for any such specimen. Moreover, a good correlation was observed between the target concentration and the observed concentration, indicating that the assay is applicable to these specimens (Table 1).

3.6. Application of the assay

Because aniline is metabolized to *p*-aminophenol, a direct measurement of aniline in urine is not useful for the evaluation of exposure. Unfortunately, *p*-

Table 1
Target and observed concentrations of aniline in lipemic, high-bilirubin-containing and hemolyzed specimens

Specimen	Bilirubin	Triglyceride	Aniline concentration (mg/l)	
			Target	Observed
Lipemic	0.6	443	4.0	4.3
	0.5	374	8.0	8.1
High bilirubin	4.5	158	4.0	3.8
	7.3	179	8.0	8.3
Hemolyzed	ND	ND	4.0	4.1
	ND	ND	8.0	8.2

aminophenol is the major metabolite of acetaminophen, a common over-the-counter analgesic. Therefore, recent use of acetaminophen has to be ruled out before *p*-aminophenol in urine can be used as a marker for aniline exposure in a patient. This problem can be circumvented by directly measuring aniline in serum. This GC–MS protocol using a novel derivatization method is specific and sensitive for aniline. However, further study is required to investigate whether this method can also measure aniline released from hemoglobin in whole blood.

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