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Short communication

Traces of phosgene in chloroform: Consequences for extraction of anthracyclines

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Abstract

Chloroform is commonly used to extract anthracyclines from various biological matrices. However, their determination can be seriously compromised by phosgene traces present as a result of failing stabilization of chloroform. Out of the three varieties in which chloroform exists (not stabilized, stabilized with an alcohol and stabilized with a hydrocarbon) only the ethanol stabilized type minimizes chances on creating artifacts. Chromatographic separation after extraction of four anthracyclines (doxorubicin, epirubicin, daunorubicin and idarubicin) and two metabolites (13-*S*-dihydrodoxorubicin and 13-*S*-dihydroepirubicin) with chloroform under various conditions indicate that the appropriate choice of stabilizer in this extraction solvent is highly relevant.

Keywords: Chloroform; Stability; Phosgene; Ethanol; Amylene; 2-Methyl-2-butene; Anthracyclines; Liquid–liquid extraction

1. Introduction

Chloroform exists in three varieties: without stabilizer, stabilized with an alcohol like ethanol, and stabilized with a hydrocarbon (e.g. amylene, cyclohexene, 2-pentene).

Without stabilization, chloroform degrades to form small amounts of free radicals, hydrochloric acid and phosgene, which is an extremely toxic substance [1,2]. Therefore, a stabilizer such as ethanol is usually added at levels of about 0.5–1% to inhibit the formation of phosgene by interfering in the free radical chain reactions. Moreover, it converts phosgene to ethyl chloroformate (Fig. 1) [3,4]. On the other hand, levels of hydrocarbons are generally between 0.002 and 0.02%. These stabilizers supposedly act as hydrochloric acid scavengers rather than as true stabilizers [5–8].

Over the years, a number of publications pointed out that stabilization with a hydrocarbon was less effective in the prevention of phosgene formation than stabilization with ethanol [2–4,9]. Particularly amine containing compounds are greatly affected, since carbamoyl chloride and carbamate artifacts can readily arise if phosgene is present [3,4,9–16].

Anthracyclines belong to the most frequently used anticancer drugs. They are effective against a broad range of solid tumors and haematological malignancies [17]. Although the use of chloroform is currently under debate due to its toxicity, it still is a common solvent to extract anthracyclines from various biological matrices. It is used as eluting agent in SPE-procedures [18–20] as well as liquid–liquid extracting agent [21–27]. Unfortunately, no details were reported on the stabilization of chloroform. However, Beijnen et al. [21] did state that the recoveries were strongly dependent on the quality of chloroform used for the extraction, but did not provide a rationale for this observation.

This paper focuses on the importance of stabilizing agent when chloroform is applied for the extraction of anthracyclines together with some of their biologically active metabolites (Fig. 2). It demonstrates the consequences of improper stabilization of chloroform under certain conditions.

2. Experimental

2.1. Instrumentation

All experiments were carried out on a LaChrom HPLC system from Merck-Hitachi (Tokyo, Japan) consisting of a L-7612 solvent degasser, a L-7100 pump with low pressure gradient

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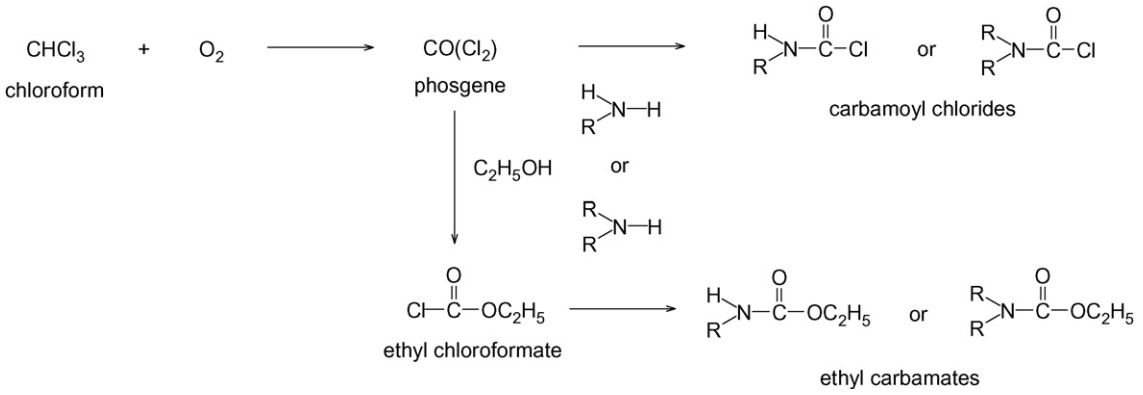

Fig. 1. Reaction mechanism of phosgene generation and interaction with amines and ethanol (reproduced with permission from Ref. [3]).

accessory, a L-7200 autosampler, a L-7360 column oven, a L-7485 fluorescence detector and a D-7000 interface. All data were acquired and analyzed using the Multi-HSM software. Mixing of the samples was performed by a rotary mixer from Labinco (Breda, The Netherlands) and a vortex mixer from LabLine (Melrose Park, IL, USA). Centrifugation of the samples was performed in a MSE Mistral 2000 centrifuge (Breda, The Netherlands). Evaporation under nitrogen was conducted in a TurboVap LV evaporator from Zymark (Hopkinton, MA, USA).

2.2. Chemicals and reagents

Analytical reference standards of 13-*S*-dihydrodoxorubicin hydrochloride, 13-*S*-dihydroepirubicin hydrochloride, daunorubicin hydrochloride and idarubicin hydrochloride were a kind gift from Dr. Antonino Suarato of Pharmacia Italia S.p.A. (Nerviano, Italy). Doxorubicin hydrochloride and epirubicin hydrochloride were purchased from LGC Promochem (Molsheim, France).

Chloroform HPLC-grade was purchased from several brands. Merck (Darmstadt, Germany) delivered chloroform Suprasolv (stabilized with ethanol, article number 1.02432.1000) and chloroform Lichrosolv (stabilized with amylene, article number 1.02444.1000), while chloroform Chromasolv Plus stabilized with ethanol (article number 650471-1L) and chloroform Chromasolv Plus stabilized with amylenes (article number 650498-1L) were obtained from Sigma (Bornem, Belgium). The chloroform qualities from Biosolve (Valkenswaard, The Netherlands) were chloroform HPLC stabilized with ethanol (article number 03480601) and chloroform HPLC stabilized with amylene (article number 03080601).

Water and ethanol, both HPLC-grade, as well as gradient grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Merck also provided formic acid, acetic acid, 47% aqueous potassium hydroxide solution, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium acetate and aqueous 25% ammonia solution. Diphenylamine and *p*-dimethylaminobenzaldehyde were from Sigma (Bornem, Belgium), while a 20% phosgene solution in toluene was from Fluka (Bornem, Belgium). All these chemicals were reagent grade or higher.

A 1 M phosphate buffer pH 7.0 was prepared by weighing 8.35 g potassium dihydrogen phosphate and 6.73 g dipotassium hydrogen phosphate and adding water up to a volume of 100 mL. Then, the eventual small deviation from the desired pH was corrected by adding an aqueous 47% potassium hydroxide solution. A 1 M ammonium buffer pH 9.0 was prepared by weighing 5.01 g ammonium acetate and 2.11 g ammonia solution and adding water up to a volume of 100 mL. Acetic acid adjusted the pH to 9.0.

A chromogenic reagent was prepared by dissolving 10% (w/v) of a mixture of equal parts of *p*-dimethylaminobenzaldehyde and diphenylamine in ethanol.

2.3. Stock solutions

Individual primary stock solutions of doxorubicin hydrochloride, epirubicin hydrochloride, daunorubicin hydrochloride and idarubicin hydrochloride at a concentration of 500 μg/mL and of 13-*S*-dihydrodoxorubicin hydrochloride and 13-*S*-dihydroepirubicin hydrochloride at a concentration of 100 μg/mL were prepared in methanol. All primary stock solutions were stored in polypropylene flasks in the dark at −20 °C until use. A secondary stock solution was prepared by mixing the individual primary stock solutions and dilution with methanol up to a concentration of 10 μg/mL (calculated as free base). This stock solution was also protected from light and stored in a polypropylene flask at −20 °C. A working solution was prepared by dilution with methanol up to a concentration of 3 μg/mL. This solution was stored in a polypropylene flask in the dark at 4 °C.

2.4. Sample preparation

Standard solutions were prepared by adding 50 μL of the working solution to 1450 μL of HPLC 'starting' eluent. This eluent consists of 0.1% formic acid in water–0.1% formic acid in acetonitrile (75:25, v/v). As a result, this solution contains 100 ng/mL of each compound. Fifty microliters was injected into the chromatographic system.

For extraction experiments a volume of 50 μL working solution was added to 1350 μL water in polypropylene tubes.