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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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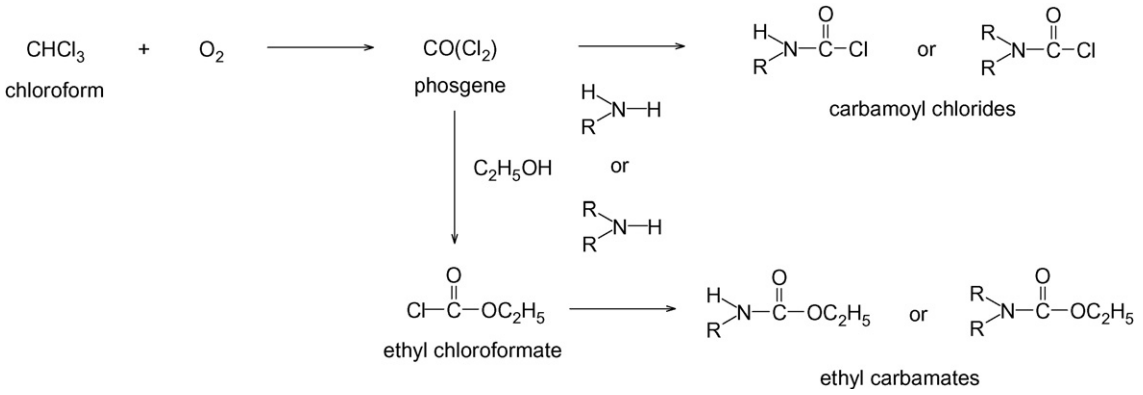


Fig. 1. Reaction mechanism of phosgene generation and interaction with amines and ethanol (reproduced with permission from Ref. [3]).

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.

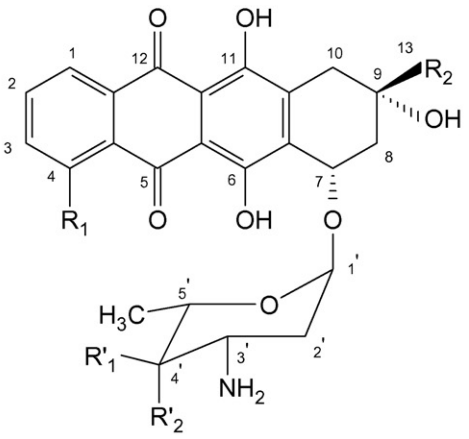
2.3. Stock solutions

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.



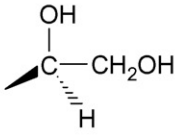
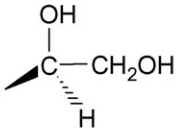
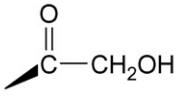
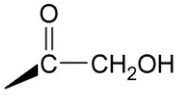
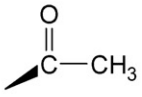
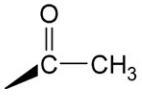
Compound	R_1	R_2	R'_1	R'_2
13-*S*-dihydrodoxorubicin	$-OCH_3$		-H	-OH
13-*S*-dihydroepirubicin	$-OCH_3$		-OH	-H
doxorubicin	$-OCH_3$		-H	-OH
epirubicin	$-OCH_3$		-OH	-H
daunorubicin	$-OCH_3$		-H	-OH
idarubicin	-H		-H	-OH

Fig. 2. Chemical structures of the studied anthracyclines.

The resulting solution was vortexed for 5 s. Instantaneously 7.5 mL of chloroform was added, followed by 150 μL buffer solution (1 M phosphate buffer pH 7.0 or 1 M ammonium buffer pH 9.0). The sample was extracted for 10 min on a rotary mixer, immediately followed by centrifugation for 5 min at $1500 \times g$. The upper aqueous layer was instantly removed and discarded. The lower organic layer was transferred to another tube. After addition of 50 μL of a 10% formic acid in acetonitrile solution, vortexing for 5 s and centrifugation for 2 min at $1500 \times g$, the resulting solution was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 1.5 mL of HPLC starting eluent. Samples were thoroughly vortexed and sonicated for 10 min. Finally, the samples were centrifuged for 2 min at $1500 \times g$ before injection of 50 μL into the chromatographic system.

2.5. Liquid chromatography–fluorescence detection

Chromatographic separation was performed on a Merck Purospher Star RP-18 endcapped column (5 μm, 150 mm × 4.6 mm), fitted with a Merck Purospher Star RP-18 endcapped guard column (5 μm, 4 mm × 4 mm) (Darmstadt, Germany). Gradient elution was applied with a mixture of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 1.0 mL/min. The initial gradient conditions were set at 24% solvent B, increased to 29% solvent B in 8 min and to 49% solvent B in another 8 min, and to a final composition of 95% B in 1 min. Afterwards the column was flushed for 5 min at 95% B at 1.5 mL/min and immediately re-equilibrated at 24% solvent B for 4 min at a flow rate of 1.5 mL/min. After 2 min at the initial gradient conditions, the next sample was injected. The temperature of the oven was set at 30 °C. Detection was performed at an excitation wavelength of 480 nm and an emission wavelength of 555 nm.

2.6. Recovery

The recovery of each analyte was determined by the peak area ratio of an extracted sample and the standard solution. All experiments were carried out in quintuplicate. Recovery was expressed by the mean (%) and the S.D. (%).

2.7. Linearity

Calibration curves with standard solutions containing 20, 40, 60, 80 and 100 ng/mL of all compounds were constructed. As a test, linearity was also evaluated for all compounds after extraction with one type of chloroform (Merck Suprasolv ethanol stabilized) at pH 7 and 9.

3. Results and discussion

3.1. Selection of chloroform

HPLC-grade chloroform was examined from three different suppliers (Merck, Sigma and Biosolve), each of them in two varieties (ethanol and amylene stabilized). All bottles were well within the indicated shelf-life, stored sheltered from light and opened just before analysis. Ethanol concentrations from the different manufacturers vary between 0.5 and 1.0% (w/w). The amylene (synonym: 2-methyl-2-butene) concentration in Merck chloroform Lichrosolv is about 0.002% (w/w) [personal communication, Sales Support Chemicals Division of VWR International (Haasrode, Belgium) and Life Science & Analytics Product Management Solvents Division of Merck (Darmstadt, Germany)]. However, the level of amylenes (a mixture of amylene and 2-pentene) in Sigma chloroform is approximately 0.0165% (w/w) [personal communication, Technical Service Department of Sigma–Aldrich Chemie BV (Bornem, Belgium)], while the amylene content in Biosolve chloroform is about 0.004% (w/w).

Table 1
Recovery results

Compound	Recovery (mean ± S.D. (%); $n = 5$)							
	Merck Suprasolv ethanol stabilized	Merck Lichrosolv amylene stabilized	Sigma Chromasolv Plus ethanol stabilized	Sigma Chromasolv Plus amylenes stabilized	Biosolve HPLC ethanol stabilized	Biosolve HPLC amylene stabilized	Merck Lichrosolv amylene stabilized +1% EtOH (24 h)	Sigma Chromasolv Plus amylenes stabilized + 1% EtOH (24 h)
Extraction at pH 7.0								
13-*S*-Dihydrodoxorubicin	15.2 ± 0.3	n.d.	12.0 ± 0.3	n.d.	17.7 ± 0.6	12.8 ± 0.8	21.2 ± 1.8	14.8 ± 1.4
13-*S*-Dihydroepirubicin	27.2 ± 0.9	n.d.	21.3 ± 0.8	n.d.	30.1 ± 0.9	21.1 ± 1.3	32.7 ± 2.5	26.0 ± 1.7
Doxorubicin	71.6 ± 2.4	n.d.	68.8 ± 2.7	n.d.	74.7 ± 4.9	78.6 ± 2.9	64.0 ± 4.2	65.9 ± 1.7
Epirubicin	74.5 ± 2.8	n.d.	73.4 ± 2.6	n.d.	76.5 ± 4.4	82.6 ± 3.1	68.6 ± 3.5	74.7 ± 1.6
Daunorubicin	74.0 ± 2.6	n.d.	67.2 ± 5.3	n.d.	75.2 ± 10.4	84.9 ± 4.4	57.9 ± 7.2	58.9 ± 3.7
Idarubicin	71.5 ± 2.2	1.9 ± 0.1	66.0 ± 5.3	2.1 ± 0.1	74.2 ± 9.8	83.8 ± 4.0	51.0 ± 6.3	54.2 ± 3.3
Extraction at pH 9.0								
13-*S*-Dihydrodoxorubicin	62.6 ± 2.9	26.8 ± 0.6	71.4 ± 1.9	34.7 ± 4.1	73.2 ± 5.1	71.4 ± 9.1	36.4 ± 4.2	55.3 ± 0.9
13-*S*-Dihydroepirubicin	63.3 ± 2.8	28.5 ± 0.7	73.9 ± 2.1	33.8 ± 4.4	76.6 ± 5.0	72.6 ± 9.5	34.3 ± 4.6	58.3 ± 1.2
Doxorubicin	65.0 ± 3.3	24.8 ± 0.9	65.0 ± 3.6	23.0 ± 4.3	67.4 ± 6.4	64.0 ± 7.3	44.5 ± 6.5	46.1 ± 1.5
Epirubicin	59.4 ± 3.9	20.5 ± 1.2	57.2 ± 3.8	20.5 ± 3.5	61.9 ± 5.8	55.6 ± 6.2	44.2 ± 6.3	48.1 ± 1.8
Daunorubicin	74.2 ± 2.7	18.0 ± 0.9	80.4 ± 3.3	15.8 ± 3.7	78.3 ± 6.9	81.2 ± 11.0	47.9 ± 7.5	45.3 ± 2.5
Idarubicin	68.9 ± 2.2	19.0 ± 0.8	79.6 ± 2.6	16.5 ± 3.6	78.5 ± 6.0	82.9 ± 9.8	43.5 ± 7.0	42.6 ± 2.4

n.d.: Not detected.

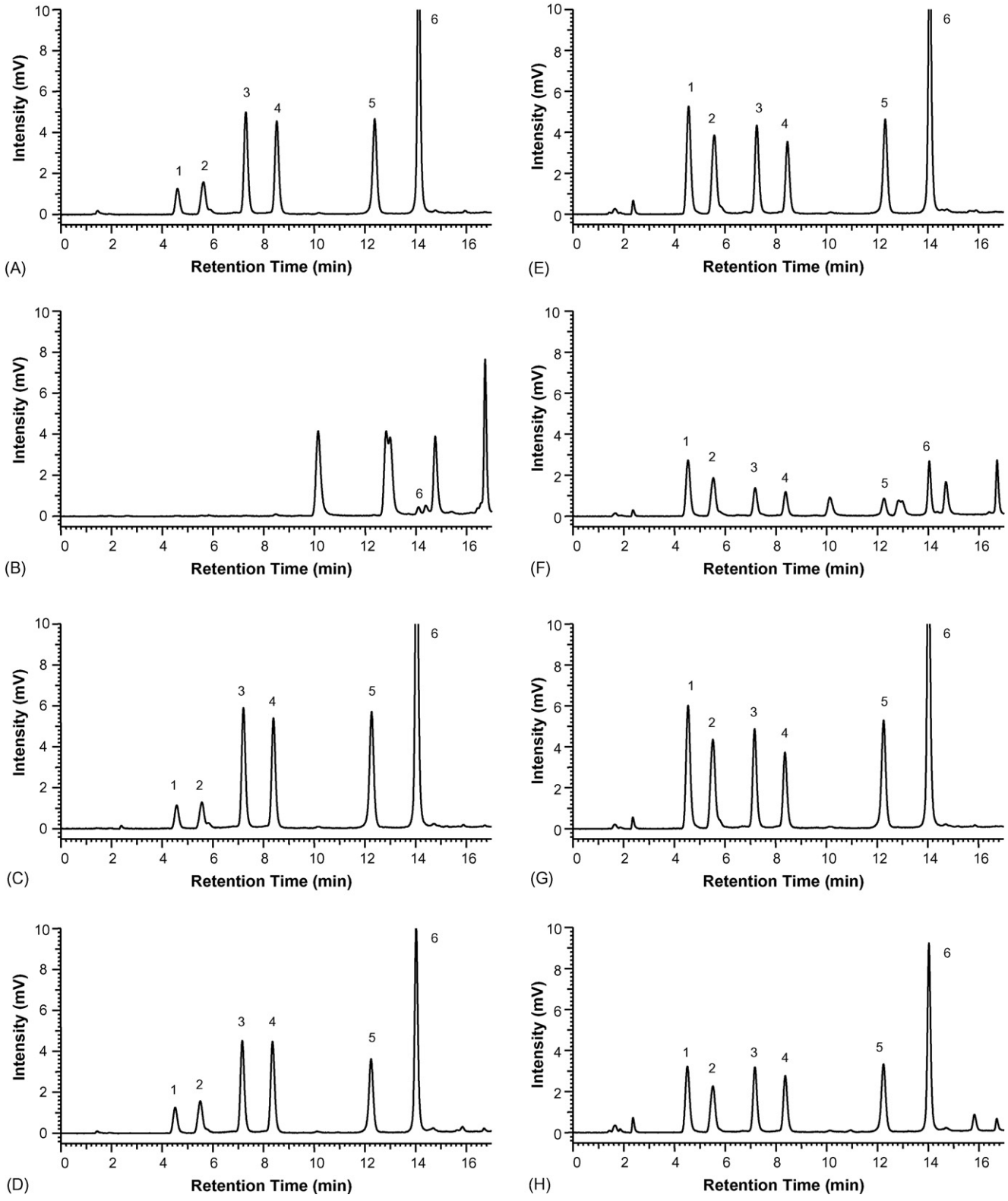


Fig. 3. Chromatograms obtained after extraction with chloroform. Compounds: 13-*S*-dihydrodoxorubicin (1, t_R: 4.5 min), 13-*S*-dihydroepirubicin (2, t_R: 5.5 min), doxorubicin (3, t_R: 7.2 min), epirubicin (4, t_R: 8.4 min), daunorubicin (5, t_R: 12.3 min), idarubicin (6, t_R: 14.0 min). (A) Chromatogram obtained after extraction at pH 7.0 with chloroform stabilized with ethanol. (B) Chromatogram obtained after extraction at pH 7.0 with amylene stabilized chloroform that contains traces of phosgene. (C) Chromatogram obtained after extraction at pH 7.0 with amylene stabilized chloroform without traces of phosgene. (D) Chromatogram obtained after extraction at pH 7.0 with amylene stabilized chloroform that contains traces of phosgene, 24 h after addition of 1% ethanol (w/v). (E) Chromatogram obtained after extraction at pH 9.0 with chloroform stabilized with ethanol. (F) Chromatogram obtained after extraction at pH 9.0 with amylene stabilized chloroform that contains traces of phosgene. (G) Chromatogram obtained after extraction at pH 9.0 with amylene stabilized chloroform without traces of phosgene. (H) Chromatogram obtained after extraction at pH 9.0 with amylene stabilized chloroform that contains traces of phosgene, 24 h after addition of 1% ethanol (w/v).

3.2. Chloroform stabilized with ethanol

Comparable recoveries between the suppliers were obtained (Table 1). The lower recovery of the two metabolites, 13-*S*-dihydrodoxorubicin and 13-*S*-dihydroepirubicin, after extraction at pH 7.0 was due to the chemical properties of these compounds. Typical chromatograms obtained after extraction are shown in Fig. 3(A and E). No artifacts were present.

3.3. Chloroform stabilized with amylene

Large differences in recoveries amongst the manufacturers were observed (Table 1). After extraction at pH 7.0, a (nearly) complete loss of the compounds was noticed and artifacts were seen with two brands. Therefore, presence of phosgene was suspected. A colorimetric test was conducted with a chromogenic reagent described by Turk [2], based on a mechanism postulated by Qureshi et al. [28]. The chromogenic reagent was prepared by dissolving 10% (w/v) of a mixture of equal parts of *p*-dimethylaminobenzaldehyde and diphenylamine in ethanol. Two hundred microliters of this reagent was added to 10 mL of each of the six varieties chloroform, as well as to a control sample consisting of 10 mL of unsuspected chloroform stabilized with amylene spiked with 2 μL of a 20% phosgene in toluene solution. Both the two suspected solvents and the control sample readily developed an intense yellow color. All other samples remained colorless. However, it is impractical to perform this test before each use of chloroform. It should be noted that a higher amount of stabilizer did not provide a more efficient stabilization. If no artifacts were present, extraction yields were comparable to chloroform stabilized with ethanol (Table 1). Typical chromatograms are shown in Fig. 3 (B and C).

After extraction at pH 9.0 with phosgene contaminated chloroform only a partial loss of the compounds of interest occurred and lower amounts of artifacts were observed. A complete destruction was probably avoided due to competition with an excess of the ammonium buffer. It should be noted, though, that artifact formation could not be prevented by addition of a competing nitrogen source. Recoveries after extraction with phosgene-free chloroform were again similar to ethanol stabilized varieties (Table 1). Typical chromatograms are shown in Fig. 3(F and G).

In a final experiment 1% ethanol (w/v) was added to phosgene contaminated chloroform. After 24 h, extraction experiments were carried out. Within the detection window, only small amounts of artifacts were present (Fig. 3(D and H)). However, recoveries were generally substantially lower as compared to ethanol stabilized chloroform (Table 1). This could be explained by the formation of more apolar ethyl carbamate artifacts. Indeed, as demonstrated by Cone et al. [3], ethanol reacts with phosgene to form ethyl chloroformate, which still has the ability to react with amine containing compounds (Fig. 1). Therefore, addition of an alcohol to phosgene contaminated chloroform should not be applied to circumvent the artifact problem.

3.4. Linearity

Calibration curves with standard solutions containing 20, 40, 60, 80 and 100 ng/mL of all compounds were constructed. All coefficients of determination (r^2) were higher than 0.999 (data not shown).

As a test, linearity was also evaluated for all compounds after extraction with one type of chloroform (Merck Suprasolv ethanol stabilized) at pH 7 and 9. All coefficients of determination were higher than 0.98, except for daunorubicin and idarubicin after extraction at pH 9 (r^2, respectively, 0.939 and 0.941) (data not shown).

4. Conclusion

Ineffective stabilization of chloroform can cause phosgene formation, which leads to artifacts in combination with amine containing compounds. Therefore, if chloroform is applied for the extraction of anthracyclines, ethanol stabilized varieties should be preferred.

Once phosgene is present in chloroform, addition of an excess of ammonia during extraction cannot overcome artifact formation. Moreover, addition of an alcohol in contaminated chloroform removes phosgene, but cannot prevent artifact formation.

As a general rule, one should always pay attention to the shelf-life and existence of stabilizers in solvents. In addition, authors should mention which stabilizer is present in the applied solvents.

Conflict of interest statement

The authors declare not to have a conflict of interest.

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