Biometals (2012) 25:231–235
DOI 10.1007/s10534-011-9489-3

# Arsenic trioxide versus tetraarsenic oxide in biomedical research: misunderstandings and misinterpretations

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Received: 20 June 2011 / Accepted: 17 August 2011 / Published online: 28 August 2011


**Abstract** This work presents an analytical chemist's view on the sometimes unconscious use of arsenic trioxide in (bio)medical research. Arsenic trioxide is a frequently used chemical in cancer treatment research and its action to various malignant cells has been extensively studied and published. Unfortunately some research articles show trivial errors with regards to background knowledge of the chemical, handling the chemical, experimental design and interpretation of results like e.g. in a range of articles comparing advantages of tetraarsenic oxide over arsenic trioxide (dimeric/monomeric) although the dissolution of both yields the same active compound ($HAsO_2$). To fully understand the implications of these errors we will highlight some of them with the intent to harmonize future work in this field.

**Keywords** $As_2O_3$ · $As_4O_6$ · Tetras · Concentration units · Redox conversion · Cell cultures

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## Background

Mineral arsenicals have been used in traditional medicine for thousands of years and in some cases they are also the best choice available in modern days (Liu et al. 2008). After the first description and explanation of the effect of arsenic trioxide on the treatment of acute promyelocytic leukemia (APL) by Chen et al. (1996), the number of research articles on the subject increased exponentially (1,700 in total to date). The American Food and Drug Administration approved in September 2000 the use of TRISENOX® (Cell Therapeutics, USA), a sterile injectable solution of arsenic trioxide, for treatment of APL (Trisenox 2000). The molecular formula of the drug substance in the solid state is $As_2O_3$ (molecular weight of 197.84 g $mol^{-1}$) and at present contains a concentration of 1.0 mg $ml^{-1}$ in 10 ml ampoulas for single use. Inactive ingredients are sodium hydroxide (1.2 mg $ml^{-1}$), which is added to increase the solubility of $As_2O_3$ (poorly soluble in pure water) and hydrochloric acid, which is used to adjust the pH to 7.5–8.5.

Arsenic trioxide is a white solid compound existing in two forms, arsenolite (cubic crystal structure) and claudetite (monoclinic crystal structure) (Wiberg et al. 2001; Henke and Hutchison 2009). Under normal conditions (room temperature and atmospheric pressure) solid arsenic trioxide is present in the form of $As_4O_6$ (dimeric $As_2O_3$, sometimes named tetraarsenic oxide) and only above

800°C dissociates into monomeric $As_2O_3$. Upon dissolution of arsenic trioxide in aqueous media (like e.g. in the case of TRISENOX®) both $As_2O_3$ and $As_4O_6$ are converted into the same arsenic species, $HAsO_2$ and/or $AsO_2^-$, depending on the pH.

Besides use of arsenic trioxide in medical treatment it is also used in metabolic studies (determination of arsenic metabolites in body fluids and tissues of patients treated with arsenic trioxide) and biomedical/genetic studies (unravelling of mechanisms related to arsenic in target cells/organs, mostly conducted on cell cultures). Although valuable research data have been obtained we feel that a more insightful approach might even further advance the (bio)medical research field, especially with regards to setting up and evaluating experiments from an analytical chemistry perspective. Below some commonly made misunderstanding and misinterpretation mistakes are given to illustrate the need to refine the way of working.

## Misunderstandings and misinterpretations

### Definition of concentrations

In many papers concentration units are poorly defined and often various units are used in one and the same paper with more interpretations possible. An example is given to illustrate this problem: Shen et al. (1997) dissolved arsenic trioxide to prepare a solution containing 1 mg $ml^{-1}$ $As_2O_3$ (supposedly containing 0.758 mg $ml^{-1}$ As) and presented the same experimental data in graph and table form, with concentration units in μmol $l^{-1}$ As and μmol $l^{-1}$ $As_2O_3$, respectively, implying an arsenic concentration difference of a factor two. Although the research yielded very interesting results, the unfortunate mishmash of units frustrates the reader and potentially leads to mistakes in follow-up research by other research groups. To make the relationships between the various units unambiguous, in Fig. 1 the μmol $l^{-1}$ ↔ μg $l^{-1}$ conversions for $As_2O_3$ and $As_4O_6$ are given.

A special case of this concentration unit confusion can be found in some articles on the advantage of tetras (tetraarsenic oxide, $As_4O_6$) over arsenic trioxide ($As_2O_3$) (e.g. Park et al. 2003, 2009; Woo et al. 2005). Not surprisingly, tetraarsenic oxide was found to be more effective than arsenic trioxide when the same concentrations, expressed in μmol $l^{-1}$, were used (again implying a factor of 2 difference concentration of arsenic). However, this "novel" arsenic compound for anticancer intervention must be regarded as a misunderstanding as in dissolved form tetras and arsenic trioxide are one and the same arsenic species. Unfortunately, some reviews (e.g. Wondrak 2009) have proclaimed tetraarsenic oxide to be an alternative for arsenic trioxide and a patent appeared on the subject as well (Bae et al. 2003). Speculation on the different behaviour of the "two compounds" (Kim et al. 2005; Chang et al. 2007) must be taken with a grain of salt and are probably associated with concentration unit confusion as described above or other imperfections in experimental design. Such problems are not limited to the arsenic field only as Filella and Williams (2010) identified similar issues for studies on antimony in cell culture media.

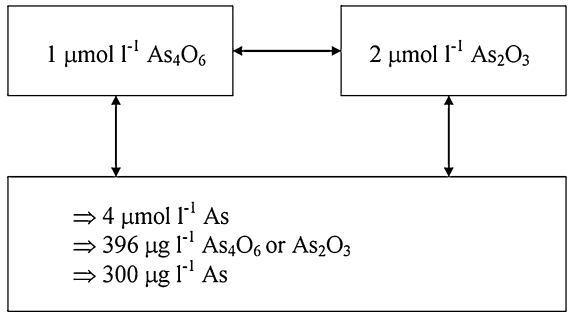


**Fig. 1** Conversion of concentration units (μmol $l^{-1}$ ↔ μg $l^{-1}$) for $As_2O_3$ and $As_4O_6$

### Redox conversion

Another topic which is almost never touched in medical research (especially in cell cultures studies) is the possibility of redox conversion between arsenic species (Bertolero et al. 1987; Zelenik et al. 2010). Besides the occurrence of $HAsO_2$ and $AsO_2^-$ (commonly named arsenite or trivalent arsenic) unwanted pentavalent arsenic species ($H_3AsO_4$, $H_2AsO_4^-$, $HAsO_4^{2-}$ and $AsO_4^{3-}$, commonly named arsenate or pentavalent arsenic) might be generated as a result of redox conversion (see Fig. 2). The possibility of this redox conversion occurring depends on the pH and the so-called reduction potential Eh of the solution, as defined by the composition of the solution and its redox chemistry. Whether or not this

actually happens in practice depends also on the kinetics of the processes involved and thus the timescale of the experiment.

Different cell culture growth media should have rather uniform pH (optimally 7.4) and Eh (optimally ca. +75 mV for many cell lines) values (Griffiths 2000), although Eh values can vary considerably even when tried to be kept constant (Meneses et al. 2000). Redox potential is not only affected by oxygen, pH and medium composition, but to a significant extent also by the rate of generation of reductants by cells (Pluschkell and Flickinger 1995–96). Under the optimal conditions for cell culture growth mentioned above it can be calculated (Van Elteren et al. 2002) that upon spiking the culture with arsenite the thermodynamic equilibrium distribution of arsenic species is as follows: 0.3% $HAsO_2$, 41.9% $H_2AsO_4^-$ and 57.8% $HAsO_4^{2-}$ (i.e., 0.3% trivalent As and 99.7% pentavalent As). However, this is a distribution pattern at equilibrium; since the kinetic parameters are unknown the actual rate towards equilibrium is unpredictable. Small deviations from ideal growth conditions might result in prevailing trivalent arsenic species at lower Eh and pentavalent arsenic species at higher Eh. Cells on their own have a broad range of Eh values from $\approx -240$ mV (in proliferation) to $\approx -170$ mV (in apoptosis) (Schafer and Buettner 2001) and as such provide more chances for arsenic to stay in trivalent form. Toxicities of tri- and pentavalent arsenic species are notably different, as are their mechanisms of uptake and action in human cells.

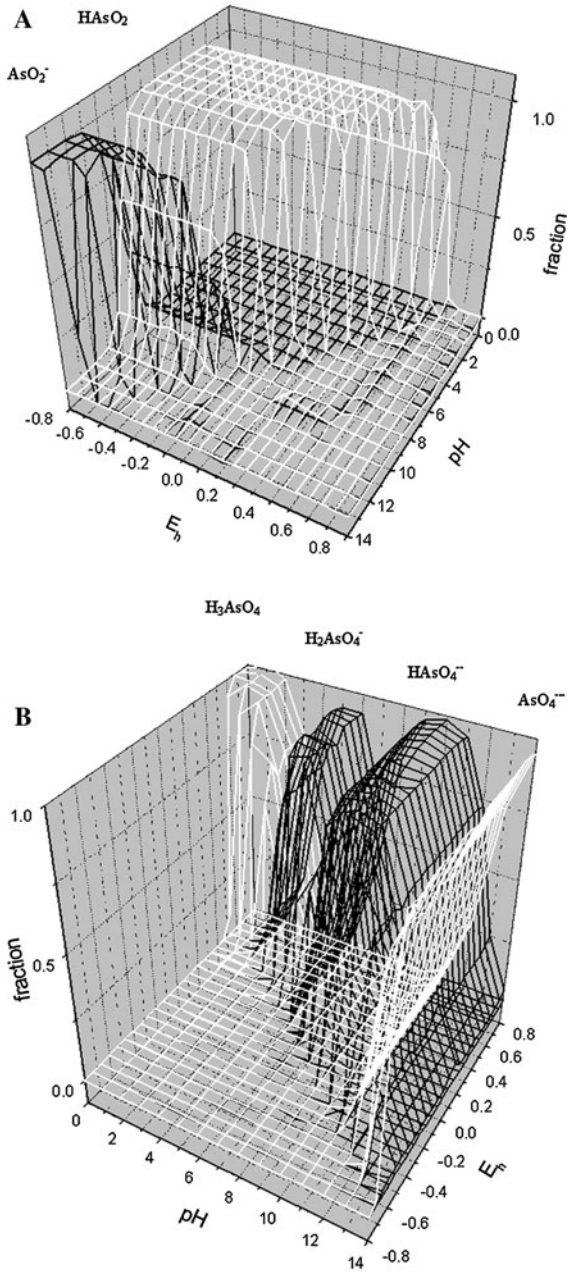


**Fig. 2** Equilibrium arsenite species (**a**) and arsenate species (**b**) species distribution according to the thermodynamic equilibria given in the text. It should be noted that for better visualization the orientation of Figures **a** and **b** is different; the pH-Eh planes are rotated for 90°

The integrity of arsenite in stock and working solutions has to be controlled regularly since redox conversion is possible in these solutions as well. Oxidation of arsenite to arsenate in stock solution (mg $ml^{-1}$ range) is neither very common nor very fast although it can happen, but oxidation of arsenite in working solutions (μg $ml^{-1}$ range) and final diluted solutions (ng $ml^{-1}$ range) might be (very) fast (in the order of days).

## Precision in conducting of experiments

Laboratory work requires a high level of awareness and accuracy, especially when preparing stock solutions and standards, in this case of arsenic trioxide. Real-life experiences from working with students showed us that preparation of a defined concentration of arsenite in solution was sometimes off more than 20% when compared to an arsenite reference standard. Since weighing is a very precise operation reasons might be found in the use of damp hygroscopic $As_2O_3$ as a starting compound or incomplete

dissolution of arsenic trioxide. It should be highlighted that dissolution in a simple buffer might not yield the expected dissolved concentration as arsenic trioxide is sparingly and extremely slowly soluble in cold water (O'Neil 2006). Much larger errors in arsenic concentrations were found in final cell culture media with deviations up to 50% from the nominal value. Imprecise micropipetting of tiny amounts of arsenic stock solution was found as a reason for this error. Micropipettes can be extremely accurate but need to be checked and calibrated on a regular basis.

## Conclusions

Misunderstandings and misinterpretations on the use of arsenic trioxide in (bio)medical research have been highlighted from an analytical chemist's point of view with the intention to come to a more conscious use of this chemical and interpretation of the experimental data obtained.

## References


Bae I-J, Kim J-B, Eun C-K, Song S-K, Suh B-S, Lee K-H, Doo M-S, Kwak J-H, Song B-D, Yoon T-J, Kang T-B, Park C-H (2003) Anticancer therapy agent of arsenic hexoxide ($As_4O_6$) of a natural chemical substance and its pharmaceutical compositon, US Patent 6,589,567 B2, 8 July 2003

Bertolero F, Pozzi G, Sabbioni E, Saffiotti U (1987) Cellular uptake and metabolic reduction of pentavalent to trivalent arsenic as determinants of cytotoxicity and morphological transformation. Carcinogenesis 8:803–808

Chang HS, Bae SM, Kim YW, Kwak SY, Min HJ, Bae IJ, Lee YJ, Shin JC, Kim CK, Ahn WS (2007) Comparison of diarsenic oxide and tetraarsenic oxide on anticancer effects: relation to the apoptosis molecular pathway. Int J Oncol 30:1129–1135

Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY, Jin XL, Tang W, Li XS, Xong SM, Shen ZX, Sun GL, Ma J, Zhang P, Zhang TD, Gazin C, Naoe T, Chen SJ, Wang ZY, Chen Z (1996) In vitro studies on cellular and molecular mechanisms of arsenic trioxide ($As_2O_3$) in the treatment of acute promyelocytic leukemia: $As_2O_3$ induces NB4 cell apoptosis with downregulation of bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood 88:1052–1106

Filella M, Williams PA (2010) Antimony biomethylation in culture media revisited in the light of solubility and chemical speciation considerations. Environ Toxicol 25:431–439

Griffiths B (2000) Scaling-up of animal cell cultures. In: Masters JRW (ed) Animal cell culture, 3rd edn. Oxford University Press Inc, New York USA, pp 19–68

Henke KR, Hutchison A (2009) Arsenic chemistry. In: Henke KR (ed) Arsenic, environmental chemistry, health threats and waste treatment. John Wiley & Sons Ltd, Chichester, UK, pp 9–68

Kim J, Bae SM, Lim DS, Kwak SY, Lee CK, Lee YS, Bae IJ, Yoo JY, Lee YJ, Kim CK, Ahn WS (2005) Tetraarsenic oxide-mediated apoptosis in a cervical cancer cell line, SiHa. Cancer Res Treat 37:307–312

Liu J, Lu Y, Goyer RA, Waalkers MP (2008) Mineral arsenicals in traditional medicines: orpiment, realgar, and arsenolite. J Pharmacol Exp Ther 326:363–368

Meneses A, Gomez A, Ramirez OT (2000) Feedback control of redox potential in hybridoma cell culture animal cell technology, products from cells, cells as products, chapter I, pp 23–29. doi:10.1007/0-306-46875-1_4

O'Neil MJ (ed) (2006) The Merck index: an encyclopedia of chemicals, drugs, and biologicals, 14th ed. Merck, Rahway

Park IC, Park MJ, Woo SH, Lee HC, An S, Gwak HS, Lee SH, Hong SI, Bae IJ, Seo KM, Rhee CH (2003) Tetraarsenic oxide induces apoptosis in U937 leukemic cells through a reactive oxygen species-dependent pathway. Int J Oncol 23:943–948

Park SG, Jung JJ, Won HJ, Kang MS, Seo SK, Choi IW, Eun CK, Ahn KJ, Park CW, Lee SW, Lew YS, Bae IJ, Choi IH (2009) Tetra-arsenic oxide (tetras) enhances radiation sensitivity of solid tumors by anti-vascular effect. Cancer Lett 277:212–217

Pluschkell SB, Flickinger MC (1995–1996) Improved methods for investigating the external redox potential in hybridoma cell culture. Cytotechnology 19:11–26

Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30:1191–1212

Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL, Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ, Chen Z, Wang ZY (1997) Use of arsenic trioxide ($As_2O_3$) in the treatment of acute promyelocytic leukemia (APE). 2. Clinical efficacy and pharmacokinetics in relapsed patients. Blood 89:3354–3360

Trisenox (2000) FDA approves trisenox for second-line treatment of APL. Oncol news Int 9. http://www.cancernetwork.com/display/article/ 10165/90478

Van Elteren JT, Stibilj V, Šlejkovec Z (2002) Speciation of inorganic arsenic in some bottled Slovene mineral waters using HPLC–HGAFS and selective coprecipitation combined with FI-HGAFS. Water Res 36:2967–2974

Wiberg E, Wiberg N, Holleman AF (eds) (2001) Holleman–Wiberg inorganic chemistry, 1st Engl edn. Academic Press, London

Wondrak GT (2009) Redox-directed cancer therapeutics: molecular mechanisms and opportunities. Antioxid Redox Signal 11:3013–3069

Woo SH, Park MJ, An S, Lee HC, Jin HO, Lee SJ, Gwak HS, Park IC, Hong SI, Rhee CH (2005) Diarsenic and tetraarsenic oxide inhibit cell cycle progression and bFGF- and

VEGF-induced proliferation of human endothelial cells. J Cell Biochem 95:120–130

Zelenik A, Šlejkovec Z, Falnoga I, Tušek-Žnidarič M, Zajc I (2010) Transformation of $As_2O_3$ in cell culture studies. In: 17th young investigators' seminar on analytical chemistry, Venice, 29 June–1 July 2010. Book of abstracts: YISAC, p. 51