Chemosphere 173 (2017) 452–459

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

In vitro cytotoxic effects of DEHP-alternative plasticizers and their primary metabolites on a L929 cell line

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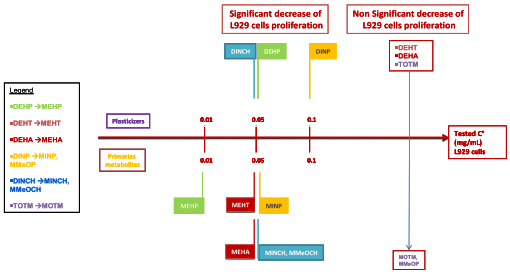
HIGHLIGHTS

- All plasticizers induce an action on cell proliferation at 0.1 mg/ml.
- **DEHP**, **ATBC** and **DINCH** are considered as potentially toxic in the standard EN 10993-5.
- All plasticizers primaries metabolites cause a decrease in cell viability except **MOTM**.
- **MEHT**, **MINP**, **MINCH** reduce significantly the cell proliferation at 0.1 mg/ml.
- **MINCH** causes a very high inhibition of cell proliferation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 4 July 2016
Received in revised form
3 December 2016
Accepted 4 January 2017
Available online 8 January 2017

Handling Editor: Frederic Leusch

Keywords:
Plasticizers

ABSTRACT

Phthalic acid esters have been widely used to improve the plasticity of PVC medical devices. They carry a high exposure risk for both humans and the environment in clinical situations. Our study focuses on the cytotoxicity of alternative plasticizers. Postulated primary metabolites were synthesized, not being commercially available. Cytotoxicity assays were performed on L929 murine cells according to the ISO-EN 10993-5 standard design for the biocompatibility of medical devices. The tested concentrations of plasticizers (0.01, 0.05 and 0.1 mg/ml) covered the range likely to be found in biological fluids coming into direct contact with the medical devices. **DEHP**, **DINP** and **DINCH** were cytotoxic at the highest concentration (0.1 mg/ml) for 7 days of exposure. Their corresponding metabolites were found to be more cytotoxic, for the same concentration. By contrast, **TOTM** and its corresponding metabolite **MOTM** were not found to be cytotoxic. **DEHA** showed no cytotoxicity, but its corresponding monoester (**MEHA**)

Abbreviations: ARMED®, Assessment and Risk Management of Medical Devices in Plasticized Polyvinylchloride; **DEHA**, diethylhexyladipate; **DEHP**, diethylhexyl phthalate; **DEHT**, di-ethylhexylterephthalate; **DINCH**, diisononyl cyclohexane-1,2-dicarboxylate; **DINP**, di-isononylphthalate; **MEHP**, monoethylhexyl phthalate; **MMeOP**, mono(4-methyloctyl)phthalate; **MMeOCH**, mono(4-methyloctyl) cyclohexane-1,2-dicarboxylate; **MEHT**, monoethylhexylterephthalate; **MINCH**, monoisononyl cyclohexane-1,2-dicarboxylate; **MINP**, monoisononylphthalate; **MOTM**, monooctyltrimellitate; **SCENIHR**, Scientific Committee on Emerging and Newly-Identified Health Risks; **TOTM**, trioctyltrimellitate.

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http://dx.doi.org/10.1016/j.chemosphere.2017.01.026
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Metabolite synthesis
Cytotoxicity
DEHP-alternative plasticizers
Phthalates

produced a cytotoxic effect at 0.05 mg/ml. In clinical situations, medical devices can release plasticizers, which can come into contact with patients. *In vivo,* the plasticizers are quickly transformed into primary metabolites. It is therefore important to measure the effects of both the plasticizers and their corresponding metabolites. Standard first-line cytotoxicity assays should be performed to ensure biocompatibility.

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1. Introduction

PVC (polyvinyl chloride) is widely used in medical devices, in particular to produce flexible tubing such as in infusion sets or extracorporeal circulation lines. To confer and maintain flexibility, plasticizers are added to the PVC matrix. However, it is now generally agreed that plasticizers can migrate from the PVC matrix into infused drug solutions or biological fluids, and thereby come into contact with the patient. Some of these chemicals are likely to be hazardous for patients, as demonstrated for diethylhexyl phthalate (**DEHP**), now classified as CMR 1B (carcinogenic, mutagenic or toxic to reproduction) under the CLP Regulation (European Union, R 2008). It is also well known that the monoethylphthalate **MEHP**, produced *in vivo* by enzymatic hydrolysis of the plasticizer, is even more toxic than the plasticizer itself (ECB, 2004; CRHER, 2005; SCENIHR, 2015). The use of **DEHP** in PVC medical devices was therefore challenged by the European authorities (European Union, R 2007), and in 2012 a French law banned the use of **DEHP** in plasticized PVC medical tubing in neonatology and maternity services as from July 1, 2015 (French law, 2012). Supported by physical and chemical properties, and some animal toxicology data, manufacturers turned to trioctyltrimellitate (**TOTM**), diethylhexylterephthalate (**DEHT**), diisononyl cyclohexane-1,2-dicarboxylate (**DINCH**), di-isononylphthalate (**DINP**) and diethylhexyladipate (**DEHA**), to replace DEHP in PVC medical devices (Fig. 1). However, data on the migration of these additives from medical devices, and on their potential human toxicity are still insufficient.

To be marketed, medical devices have to meet certain essential requirements. These mainly concern their performance, safety, toxicity and biocompatibility. The last three properties concern interactions between tissues in contact with the materials, but other tissues may also be affected. The evaluation of a device has to take into account not only the substances released from it, but also those derived from their degradation. Lastly, the duration of contact with the body must be considered. To study toxicity and biocompatibility, *in vivo* and *in vitro* tests are proposed in the standard EN ISO 1993. Devices have to undergo tests prescribed according to the level of risk to which patients are exposed. The cytotoxicity test described in EN ISO 10993-5 is mandatory, regardless of the medical device: the authors of the standard consider that the toxicity assessment of a medical device, material or additive on a cell model can be predictive of *in vivo* toxicity. An apparent toxicity in this model does not necessarily demand rejection of the product concerned, but calls for further investigation. Recommended tests depend on the final use of the medical device. For example, an infusion set will not need to meet the same requirements as a central catheter coming directly into contact with blood. Manufacturers mostly test the medical device itself and not the separate materials used in its composition. Secondary materials can include bonding substances and accessories such as fittings. Most often no data is available on the additives used for either the main material or secondary ones. Many additives serve to facilitate the manufacture or use of the medical device: for example, manufacturers often use plasticizers, lubricants or dyes. The biocompatibility of these additives, which affect the ergonomic properties of medical devices, is poorly evaluated. We studied the cytotoxicity of plasticizers added to PVC medical devices using the MTT test, and the cell line (L929 cell line) described in EN 10 993-5. The concentrations studied were those found in biological fluids during direct contact with the medical devices (Takahashi et al., 2008; Kambia et al., 2011; Scenirh, 2015; Eckert et al., 2016).

Information is lacking on the toxicity of the primary metabolites of **DEHP**-alternative plasticizers. The aim of our study was to assess the toxicity of several such alternative plasticizers and their primary metabolites, with cytotoxicity assays performed according to the EN 10993-5 standard using the MTT assay. The primary metabolites of **TOTM**, **DEHT**, **DEHA**, **DINCH**, **DINP** and **DEHA** are not commercially available, so had to be chemically synthesized. The production of pure metabolites enabled us to study their respective cytoxicities, and to compare them with that of **MEHP** in the same conditions.

2. Materials and methods

2.1. Chemicals, biochemical and reagents

Unless otherwise stated, all manipulations were performed under argon; all reagents were purchased from the following commercial suppliers: Sigma-Aldrich, Acros Organics, Carlo Erba, TCI Europa, and Alpha Aesar. Anhydrous DMF and anhydrous triethylamine were purchased from Acros Organics. THF was distilled over benzophenone and sodium. Dichloromethane was distilled over calcium hydride. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AC-200 instrument operating at 200 MHz and 50 MHz for ^{1}H NMR and ^{13}C NMR, respectively. All ^{1}H NMR spectral peaks are reported in δ units, parts per million (ppm), and the coupling constants are indicated in hertz (Hz). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and br = broad. The chromatographic system consisted of an HTC PAL autosampler, and a Transcend TLX-1 HTLC System (Thermo Fisher Scientific, San Jose, United States), The HRMS analysis was performed using a Thermo Exactive benchtop Orbitrap® instrument. TLC was performed on pre-coated silica gel sheets (POLYGRAM® 60F254 plates) and visualized under UV light (254 nm). Column chromatography was performed using silica gel normal phase (35–70 μm). Uncorrected melting points (Mp) were recorded on an Electrothermal IA9300 apparatus. Infrared spectra (IR) were recorded on a Bruker FT Vector 22 instrument.

DEHP (Ref: D201154, CAS: 117-81-7), **TOTM** (ref: 538140, CAS: 3319-31-1), **DEHA** (ref: 524197 CAS: 103-23-1), **DINP** (ref: 376663, CAS: 28553-12-0), and **DEHT** (ref: 525189, CAS: 6422-56-2) were purchased from Sigma Aldrich, France. **DINCH** (CAS: 166-412-78-8) was supplied by BASF, France. The primary metabolites **MEHP**, **MEHT**, **MINP**, **MINCH**, **MEHA, MMeOP, MMeOCH** and **MOTM** were synthesized and characterized by the UMR 990 team, Clermont-Ferrand, France.

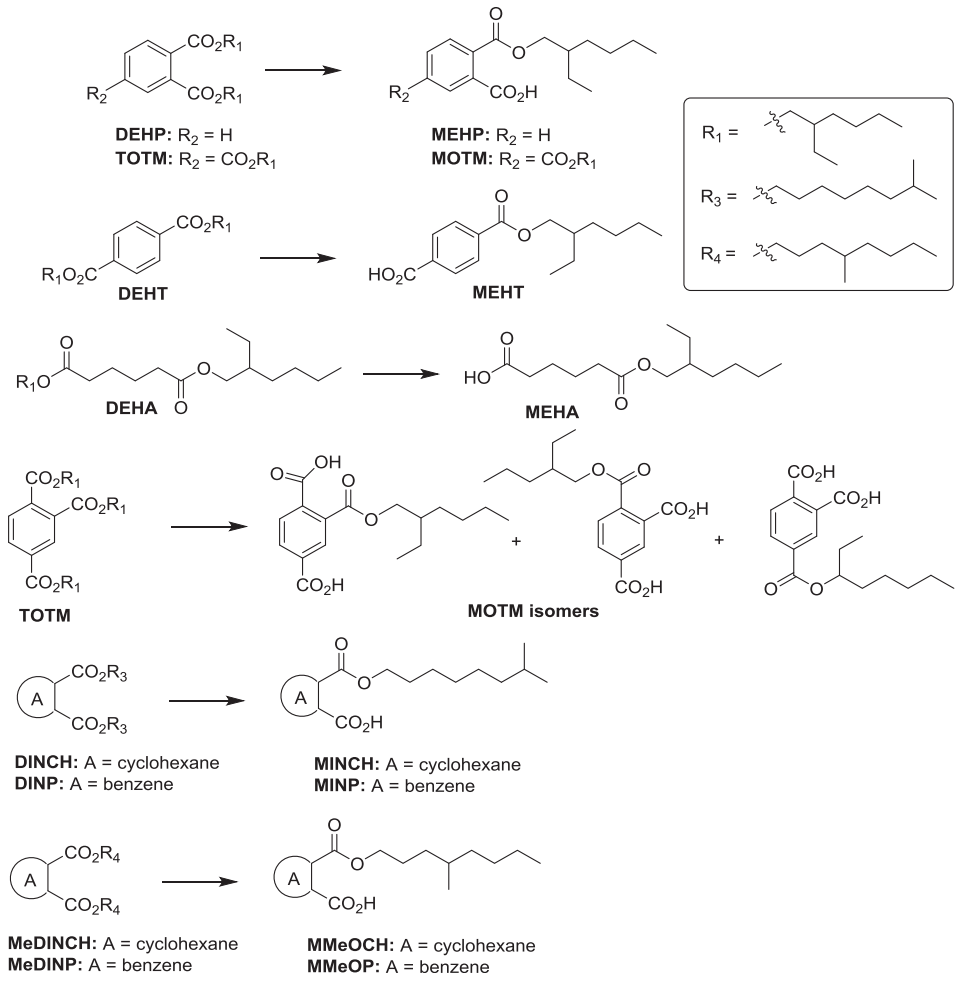

Fig. 1. Plasticizers most often used in medical devices and their corresponding primary metabolites.

Dimethylsulfoxide DMSO (ref: D8418 CAS: 67-68-5), Mouse fibroblasts L929 (ref: 85011425 lot: 10L019), MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, ref: M2128) were also purchased from Sigma Aldrich. Dulbecco's modified Eagle Medium (DMEM) fetal calf serum (SVF), phosphate saline buffer (PBS), penicillin (P), streptomycin (S), and actinomycin D were purchased from Life technologies SAS, France.

2.2. Methods for the synthesis of primary metabolites

Metabolites **MEHP (3)**, **MOTM (5)**, **MEHA (8)**, **MINCH (19)**, **MINP (21)**, **MeMINCH (30)** and **MeMINP (31)** were synthesized from the appropriate anhydrides **(1a, b), (7), (18) or (19)** and the appropriate alcohol derivatives 2-ethylhexanol **(2)**, 7-methyloctanol **(17)** or 4-methyloctanol **(29)** as previously described (Nuti et al., 2005). Scheme 1S, 2S, 3S, 5S and 6S depict the synthesis of the primary metabolites listed above (See Supporting Information).

Briefly, to a solution of the appropriate anhydride **(1a, b), (7), (18) or (19)** (1 eq.) dissolved in dry pyridine, was added 2-ethylhexanol **(2)**, 7-methyloctanol **(17)** or 4-methyloctanol **(29)** (1 eq.). The resulting mixture was stirred at 125 °C (external temperature) until the anhydride derivative had disappeared (TLC monitoring). The mixture was quenched with cold water (50 mL) and extracted with diethyl ether (8 × 40 mL). The combined organic layer was washed with a 10% solution of hydrochloric acid (100 mL). Finally, the mixture was extracted with 0.4 M K_2CO_3 (100 mL). The aqueous basic layer was acidified to pH 1 with a solution of hydrochloric acid (1M) and then extracted with Et_2O (4 × 100 mL). The combined organic layer was washed with brine (150 mL), dried over $MgSO_4$ and evaporated under reduced pressure.

The metabolite **MEHT (12)** was obtained by esterification of the terephthalate derivative **(11)** and 2-ethylhexanol **(2)** (See Supporting Information, scheme 4S).

2.3. Biocompatibility assays

2.3.1. Solubilization of the plasticizers

All the plasticizers and their metabolites were solubilized in DMSO (stock solutions) at 1% concentration (10 mg/mL). The final concentrations of the respective stock solutions were checked by GC-MS (Supporting Information, Table S1). Work solutions of each plasticizer were obtained by dilution of the stock solutions to the concentrations 0.01 mg/ml (0.1%); 0.05 mg/ml (0.5%) and 0.1 mg/ml (1%) in the culture medium (DMEM).

2.3.2. Cells cultures

L929 cells were cultured in 96-well plates (without plasticizers) with DMEM supplemented with 10% of SVF, 100 IU/mL of penicillin and 100 μg/mL of streptomycin (complete DMEM) at 37 °C with 5% of CO_2.

2.3.3. MTT assay procedure

The MTT reagent was used at a concentration of 5 mg/mL in PBS. At the end of the cell cultures, the media were removed, and 30 μL of the MTT reagent was added to each well. Cells were then incubated for 3 h at 37 °C with 5% of CO_2. The MTT reagent was removed, and 200 μL of dimethyl sulfoxide was added to each well (multiwell plates without plasticizers). After 1 h of formazan dissolution, the optical density (OD) of each well was measured at 570 nm (spectrophotometer VICTOR™ Multilabel HTS Counter PerkinElmer).

2.3.4. Specificity of the MTT assay

L929 cells were seeded in triplicate at 10^4 cells/well. After 24 h, the medium was replaced by 200 μL of the complete DMEM (control) or 200 μL of the complete DMEM supplemented with 0.1% of actinomycin D. Cells were then cultured for 5 days with a medium change every day. MTT assays were performed every day for each condition. Cell viability percentage was calculated using the formula: ((OD test − OD control) / OD control).

2.3.5. Linearity of the MTT assay

L929 cells were seeded in triplicate at increasing numbers of cells per well (5×10^3–10×10^3–50×10^3–100×10^3–150×10^3–200×10^3–250×10^3–300×10^3–350×10^3) in 96-well plates. After 8 h of adherence, the MTT assay was performed as described above.

2.3.6. Viability assay for the DMSO solvent

According to the work plasticizer solutions, L929 cells were cultured in complete DMEM supplemented with 0.1%, 0.5%, 1%, 2.5% and 5% of DMSO. Cells were seeded in triplicate at 10^4 cells/well. After 24 h, the medium was replaced every day by 200 μL of the complete DMEM for the control and 200 μL of the complete DMEM supplemented with DMSO at the selected concentrations. Cells were cultured for 7 days. An MTT assay was performed every day according to the MTT assay procedure.

2.3.7. Viability assays for the plasticizers

For the viability assay of the plasticizers, cells were seeded in triplicate at 10^4 cells/well. After 24 h, the media were replaced by 200 μL of the followings solutions: (i) control conditions with complete medium, (ii) control DMSO conditions with the complete medium supplemented with the same concentration of DMSO as the plasticizer work solutions, and (iii) work solutions (0.1%, 0.5% and 1%) of the plasticizers. Cells were cultured for 7 days with a medium change every day. MTT assay and quantification of the viability percentage were carried out every day.

2.3.8. Statistical analysis

The experiments performed in triplicate were repeated at least twice, and the outcomes of one representative data set of each plasticizer are reported. Differences in viability percentages between the control conditions and the test conditions were analyzed using the Mann-Whitney non-parametric test. A p value less than 0.05 was considered significant.

3. Results

3.1. Synthesis of metabolites

Primary metabolites **MEHP (3)** (Nuti et al., 2005), **MOTM (5)**, **MEHA (8)**, **MINCH (19)**, **MINP (21)**, **MMeOCH (30)** and **MMeOP (31)** were synthesized by esterification of the appropriate anhydride (**(1a, b)**, **(7)**, **(18)** and **(20)**) by the appropriate alcohol: 2-ethylhexanol **(2)**, 7-methyloctanol **(17)** or 4-methyloctanol **(29)** (Fig. 2) as previously described (Nuti et al., 2005). For **MOTM**, a condensation of anhydride **(1b)** and **(2)** yielded a mixture of two isomers, which was not separated. **MEHA (8)** was straightforwardly obtained from anhydride **(7)** (Cisneros et al., 2012) and alcohol **(2)** with good yield. We also synthesized the **MEHT** metabolite. Starting material terephthalic acid **(9)**, after protection with benzyl bromide, then treatment with thionyl chloride followed by an esterification with **(2)**, and a final catalytic hydrogenation, afforded **MEHT (12)**.

MINCH and **MINP** were obtained by condensation of 7-methyloctan-1-ol **(17)** and dihydro-2H-pyran-2,6-(3H)-dione **(18)** or hexahydroisobenzofuran-1,3-dione **(20)**, respectively. Compound 7-methyloctan-1-ol **(17)** was commercialized, but was very costly. We therefore synthesized it in four steps as described in Scheme 5S. **MMeOCH** and **MMeOP** were obtained by a similar procedure with 4-methyloctan-1-ol **(29)** (Scheme 6S. All intermediates and final compounds were analyzed by IR, ^1H and ^{13}C NMR, and HRMS. For all details, see Supporting Information. The purity of all our synthesized metabolites and their corresponding intermediates exceeded 95%.

3.2. Biocompatibility assay

3.2.1. Specificity of MTT assay

Fig. 3 shows the optical density obtained after MTT assay on L929 cells cultured in complete medium with and without actinomycin D for 5 days. The optical density of control wells gradually increased from D1 to D5; L929 cells proliferated normally. The optical density in the wells cultured in the presence of actinomycin D fell significantly from D1 compared with the control, and tended to zero at D3. The apoptosis-inducting effect of actinomycin D (Kleeff et al., 2000) time-dependently caused cell death, which was close to 100% at D3 in the MTT assay.

3.2.2. Linearity of MTT assay

Fig. 4 shows the linearity of the MTT assay. A linear correlation ($r^2 = 0.9812$) was found between the number of seeded cells and the optical density obtained at the end of the MTT assay after 8 h of adhesion.

3.2.3. DMSO solvent effect on cell viability

Fig. 5 shows the proliferation of L929 cells cultured in complete medium with and without DMSO (0.1%, 0.5%, 1%, 2.5% and 5%) for 7 days. There was no significant difference in cell proliferation between control (DMEM) and the cells cultured in DMEM supplemented with 0.1% and 0.5% of DMSO. Cells cultured in DMEM supplemented with 1% of DMSO proliferated for the 7 days of culture, but their proliferation was significantly lower than that of the control. Beyond 1% of DMSO, L929 cells did not proliferate.

3.2.4. Viability of cells when exposed to the plasticizers and their primary metabolites

Fig. 6 shows the results of viability tests, at day 7 (D7), performed on L929 cells cultured in the presence of three concentrations of plasticizers after 7 days of contact. Plasticizers were not toxic to the cells at the concentration of 0.01 mg/ml compared with the control containing DMSO at the same concentration. A decrease in cell viability was observed for **DEHP** and **DINCH** from the concentration of 0.05 mg/mL. For these two plasticizers, this decrease in viability was higher than 50%. **DINP** reduced cell proliferation from a concentration of 0.1 mg/mL (40%). For the other plasticizers (**TOTM**, **DEHA** and **DEHT**), there were no significant differences compared with DMSO at any of the three concentrations tested.

Fig. 7 depicts the results of the viability tests on primary metabolites of the plasticizers. From 0.01 mg/mL, only **MEHP** had an

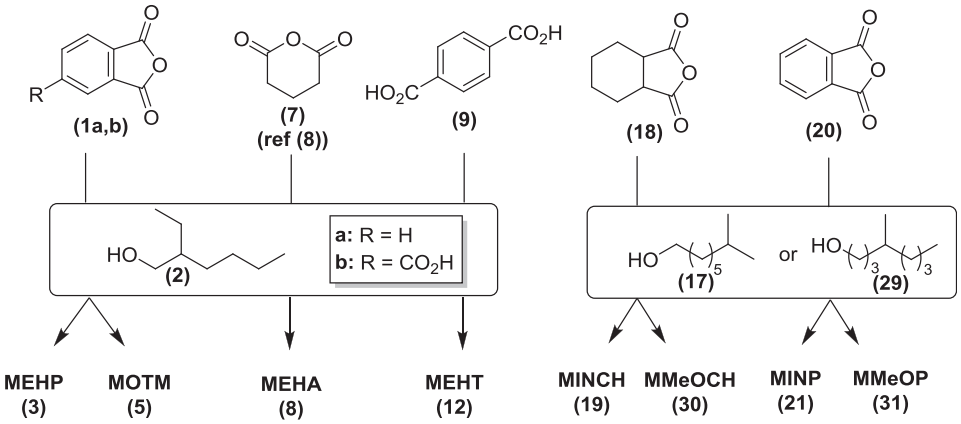

Fig. 2. Access to **MEHT**, **MEHA**, **MOTM**, **MINCH, MINP**, **MMeOCH** and **MMeOP** from 2-ethylhexanol **(2)**, 7-methyloctanol **(17)** or 4-methyloctanol **(29).**

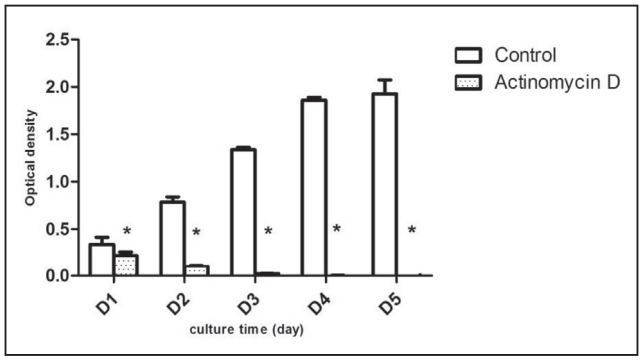

Fig. 3. Specificity of the MTT assay. Optical density of the control wells *vs.* the optical density of the actinomycin D wells. L929 cells were seeded at 10^4 cells/well, and cultured in Complete DMEM with and without actinomycin D (0.1%) for 5 days. MTT assays were carried out every day for each condition. $^*p < 0.05$ control *vs.* test (DMEM + %DMSO).

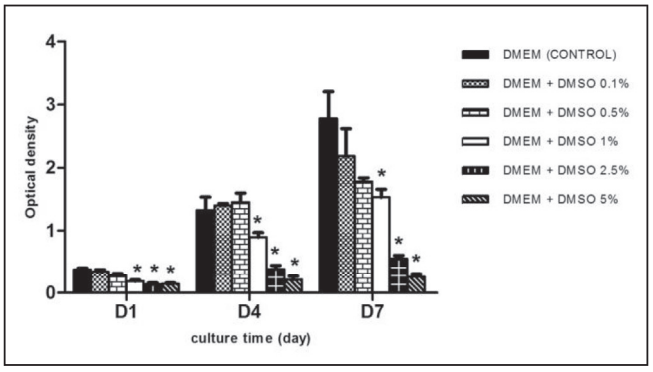

Fig. 5. Viability assays of the solvent DMSO. L929 cells were seeded at 10^4 cells/well, and cultured in complete medium with and without DMSO (0.1%, 0.5%, 1%, 2.5% and 5%) for 7 days. MTT assay was carried out every day. $^*p < 0.05$ control *vs.* test (DMEM + %DMSO).

impact on cell growth. **MEHP** expressed a cytotoxicity of 50% and 70% respectively at 0.05 mg/mL and 0.1 mg/ml. Compared with **MEHP**, the effect on the L929 proliferation was different for all the other primary metabolites studied except for **MINCH** and **MEHT**: at 0.05 mg/ml, these latter were as cytotoxic as **MEHP** (about 50% cell death). From 0.1 mg/ml, they were much more cytotoxic than **MEHP** (98% and 99% of cell death observed for **MEHT** and **MINCH**, respectively versus 70% for **MEHP**). For **MMeOCH**, a structural isomer of **MINCH**, results were close to those obtained for **MINCH** from 0.05 to 0.1 mg/mL. By contrast, the **MMeOP**, a structural isomer of **MINP**, never significantly decreased cell viability at any of the concentrations tested. For **MOTM**, no significant decrease in proliferation was observed compared with the DMSO control at any of the concentrations tested. **MEHA** was weakly toxic, its cytotoxicity lying in the range 20–30% at 0.05 and 0.1 mg/mL, respectively.

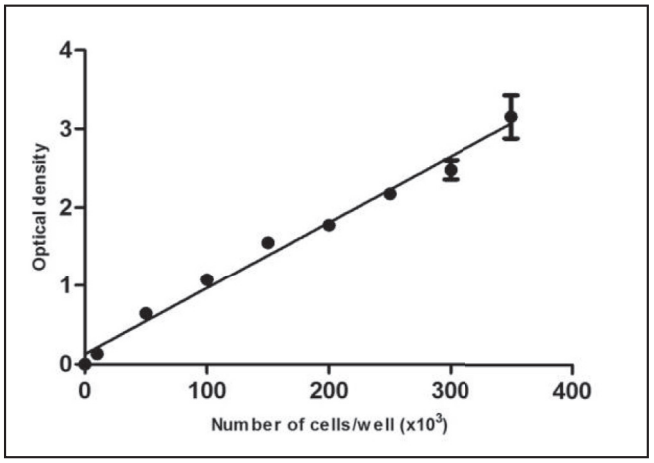

Fig. 4. Linearity of MTT assay. L929 cells were seeded at increasing numbers of cells per well (10×10^3–50×10^3–100×10^3–150×10^3–200×10^3–250×10^3–300×10^3–350×10^3) in complete medium. After 8 h of adherence, the MTT assay was performed for each well.

In summary, we observed that **MOTM** and **MMeoP** were not cytotoxic at any of the concentrations tested. **MEHA** was weakly cytotoxic. **MEHP**, **MEHT**, **MINP**, **MINCH** and **MMeOCH** were cytotoxic with a concentration effect.

4. Discussion

Since the plasticizer **DEHP** was classified as CMR 1B, manufacturers have used various alternative plasticizers with high molecular weights or different solubility properties, such as other phthalates, trimellitates, citrates, alkyl sulfonic phenyl esters (ASEs), C10-21-alkanes, acetylated monoglycerides of hydrogenated castor oil (COMGHAs), or acetates. In PVC medical devices, the plasticizers most commonly added are **TOTM** and **DINCH**, but **DINP** and **DEHA** are also frequently found. **DEHT** is seldom used in MDs as the main plasticizer, but it is also a contaminant of **TOTM**

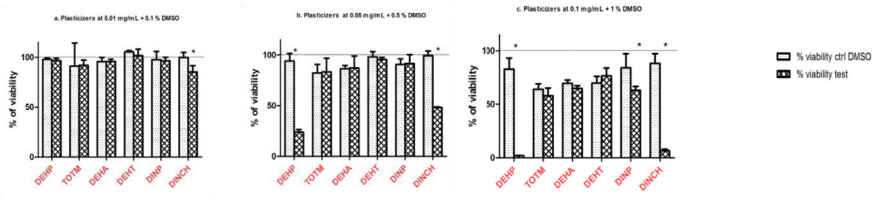

Fig. 6. Cytotoxicity test of plasticizers at 0.01 mg/mL (a), 0.05 mg/mL and 0.1 mg/mL, day 7. Outcomes of the cell viability analysis using MTT assay. L929 cells were seeded at 10^4 cells/well and cultured for 7 days in three conditions with medium change every day: control (complete medium), control DMSO (complete medium supplemented with DMSO 0.1%, 0.5% and 1%) and test (work solutions of plasticizer in complete medium at 0.1% (0.01 mg/mL), 0.5% (0.05 mg/mL) and 1% (0.1 mg/mL). MTT assay and quantification of the viability percentage were carried out every day. $^*p < 0.05$ DMSO control *vs.* Test.

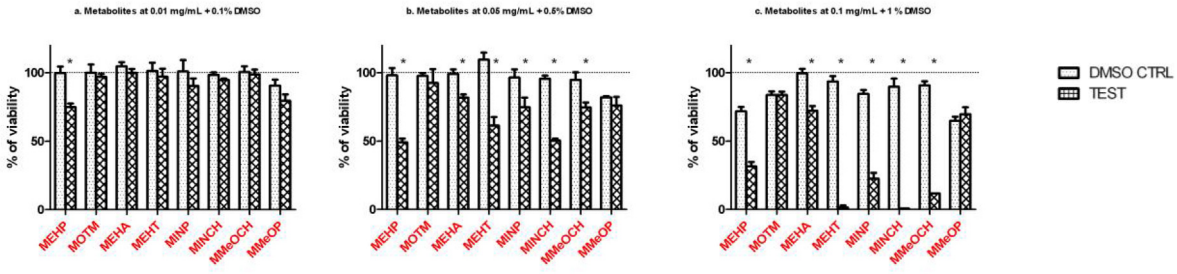

Fig. 7. Cytotoxicity test of primary metabolites at 0.01 mg/mL (a), at 0.05 mg/mL and at 0.1 mg/mL, day 7. Outcomes of the cell viability analysis using MTT assay. L929 cells were seeded at 10^4 cells/well and cultured for 7 days in three conditions with medium change every day: control (complete medium), control DMSO (complete medium supplemented with DMSO 0.1%, 0.5% and 1%) and test (work solutions of plasticizer in complete medium at 0.1% (0.01 mg/mL), 0.5% (0.05 mg/mL) and 1% (0.1 mg/mL). MTT assay and quantification of the viability percentage were carried out every day. $^*p < 0.05$ DMSO control *vs.* Test.

(Gimeno et al., 2014; Bourdeaux et al., 2016). Here we focus on plasticizers commonly used in medical devices. Special attention was of course paid to all phthalate substitutes because of their endocrine-disrupting and reprotoxic properties. Except for **TOTM**, there are many studies on alternative PVC plasticizers used by manufacturers. For **DEHA**, carcinogenic properties were observed toward female B6C3F1, and a disturbance of the estrous cycle and increased ovarian follicle atresia were detected in rats (Miyata et al., 2006). For **DEHT**, no effects were reported on reproductive tissues, organs, kidneys, liver hepatocytes or peroxisomes, which are known targets of **DEHP**-toxicity (Wirnitzer et al., 2011; Deyo, 2008). **DINP** and **DINCH** have been studied more thoroughly. Levels of **DINP** exposure are far below those that have no observed adverse effects in animals, and also below health-based exposure guidance values set by regulatory authorities and other authoritative bodies as acceptable (Borch et al., 2004; Patynaa et al., 2006; Kransler et al., 2012). **DINCH** has been studied by Schütze and coworkers**. DINCH** is neither a reproductive toxicant nor an endocrine disruptor in rodents. Thyroid hyperplasia and signs of renal toxicity were only observed at relatively high dose levels in animals of either sex (Schütze et al., 2014, 2015).

Until now, all these numerous studies have estimated the kinetics of metabolism of **DEHP** from the urinary excretion of its main primary and secondary metabolites, or the parent substance, after administration of the PVC plasticizer (Fromme et al., 2016; Schütze et al., 2012, 2014; Anderson et al., 2011; Koch et al., 2011, 2012, 2013). For other studies, analyses were conducted using deuterated plasticizers (D2 or D4-DEHP, D2 or D4-DINCH, D2 or D4-DINP): after administration to mice, urine samples were analyzed by LC-MS to measure metabolite concentrations (Anderson et al., 2011; Koch et al., 2005; Koch and Angerer, 2007).

Besides these results, the literature reports that plasticizers are metabolized *in vivo*. In a first metabolism step, they are cleaved into monoesters (or diester for **TOTM**), which are further oxidized in various ways into alcohol, ketone or acid derivatives. These secondary metabolites are recovered in urine (Martis et al., 1987). These metabolites are not all currently available on the market, and several were produced after oral or intravenous administration of the plasticizers to animals and collected as glucuronated derivatives. Primary and secondary metabolites were obtained by hydrolysis of these derivatives. The low quantities obtained by these methods do not always allow full cytotoxicity studies. To overcome this limitation, we undertook here for the first time the total synthesis of the postulated primary metabolites of these plasticizers, namely **MOTM, MEHA, MEHT, MINP, MINCH, MMeOP** (isomer of MINP) and **MMeOCH** (isomer of MINCH) as they are described in the literature.

All the primary metabolites were successively synthesized (for details, see Fig. 2 and supplementary material). The synthesized metabolites were chemically conformant (purity 95%), and so the results of the cytotoxicity assays can be considered as reflecting the synthesized substance and not impurities. The objective of our preliminary work was to compare the effect of these plasticizers and their metabolites on the in vitro cell culture models used to evaluate the biocompatibility according to the EN 10993 (NF, 2009). However, authors report difficulties applying the EN 10993-5 (NF, 2009) standard for plasticizers: these substances are hydrophobic, and cannot be used as they are for direct contact in a culture medium. It is therefore necessary to use a solvent for these materials, which is itself non-cytotoxic toward the cells, to perform the tests. DMSO was used for this purpose (Wang et al., 2012). Solubility tests showed that the plasticizers were soluble up to a concentration of 1% in DMSO (data not shown). We validated the DMSO maximum concentrations that can be used in the presence of L929 according to our experimental protocol (7 days of cell culture at an initial cell density of 10^4 cells/well). DMSO could thus be used up to a maximum concentration of 1%. To avoid bias, all tests were compared with a control DMSO (medium supplemented with DMSO at the same concentration).

All the plasticizers and their corresponding primary metabolites

were cultured directly with murine L929 fibroblasts at three different concentrations (0.01, 0.05 and 0.1 mg/mL) for 7 days. Concerning cytotoxicity of plasticizers, no effect was observed at 0.01 mg/mL. **DEHP** and **DINCH** caused a decrease in L929 cell proliferation from the concentration of 0.05 mg/mL, and their effect was massive at the highest concentration, with total cell death. **DINCH** was as toxic as **DEHP** for the L929 fibroblast cells. These results were unexpected because the chemical structures of the two substances are widely different. **DEHP** is a phthalate with two ethylhexanyl chains, whereas DINCH has no aromatic scaffold, and two more lipophilic chains grafted on the ester function. Except for **DINP,** which caused cell proliferation inhibition (20–30%) from 0.1 mg/mL, all the other plasticizers (e.g. **TOTM**, **DEHA** and **DEHT**) showed no significant differences compared with DMSO at any of the three concentrations tested. By contrast, **TOTM**, one of the most widely used alternative plasticizers, though banned in food applications, presented a favorable toxicity profile, If we consider the cytotoxicity threshold described in the standard EN 10993-5, **DEHP** and **DINCH** were equivalent in terms of cytotoxicity (viability threshold < 70%).

These results show a clear difference in the cytotoxicities of the plasticizers evaluated. Köksal et al. studied the cytotoxicity of cyclohexyl butyl phthalate (BHP) with the MTT assay on L929 cell lines. They found an ICC at 0.29 μg/ml for BHP. (Köksal et al., 2016). Alternative plasticizers evaluated in our study showed significantly lower cytotoxicities. The most cytotoxic (except for DEHP) did not lower cell viability at a concentration of 0.01 mg/ml, 30 times higher than the IC50 of BHP.

Concerning the primary metabolites of the PVC plasticizers, their effects on L929 cell viability were very different when they were tested at the same three concentrations. From 0.05 mg/mL, all the metabolites were cytotoxic except for **MOTM**. **MEHT** and **MINCH** were as toxic as **MEHP**. Their corresponding parent plasticizers (**DEHT** and **DINCH**) had no effect at this concentration. At 0.1 mg/mL, **MEHT** and **MINCH** were as toxic as **DEHP** and more toxic than **MEHP**. **MINP** was weakly toxic at 0.05 mg/mL, but cell viability fell sharply at 0.1 mg/mL to reach 80% cell death, i.e. more toxic than **MEHP** and with an effect similar to that observed with **DEHP**. The isomer of **MINCH** (**MMeOCH**) had a similar cytotoxic activity at 0.05 mg/mL (15% cell death), but was also more toxic than **MEHP** and behaved like **DEHP** at 0.1 mg/mL.

The metabolization of plasticizers increases their cytotoxicity. For example, in the literature, **MEHP**, the **DEHP** bioactive metabolite (Frederiksen et al., 2007), was shown to be 10 times more potent than **DEHP** (Huber et al., 1996). **MEHP** is a well-known activator of the PPAR family of nuclear receptors (Hurst and Waxman, 2003; Maloney and Waxman, 1999). The toxicities of the other metabolites are not well known. A recent study reports that **MINCH** (50, 100 μM) like **MEHP** (50 μM), is also a potential PPAR-α agonist and a metabolic disruptor, able to induce SVF preadipocyte differentiation, which may interfere with the endocrine system in mammals (Campioli et al., 2015). The implications of primary metabolite toxicities higher than that of plasticizers themselves depend on the *in vivo* metabolism and species. This information is not available, and so there is a need to perform *in vivo* animal toxicity studies on primary and secondary metabolites identified for each plasticizer. Koch et al. (2013) have measured the amount of **MINCH** excreted in urine after oral absorption of **DINCH**. However, they do not report the absorption percentage of **MINCH**, its metabolism rate, or the rate of metabolization of **MINCH** to its secondary metabolites (**Cx-MINCH**, **OH-MINCH** and **OXO-MINCH**). The value of 1% for **MINCH** measured in urine is therefore not predictive of the amount of **MINCH** formed and excreted from the vascular system. The literature shows that absorption of plasticizers after oral exposure is very low in rodents (50% for **DEHP** (Anderson et al., 2011))

5. Conclusion

The main finding of our study is that **TOTM** showed lower toxicity on L929 cells than the other plasticizers. Its primary metabolite, **MOTM,** did not display any toxicity compared with the other metabolites. This finding is important because we recently demonstrated a predominance of **TOTM** in 32 PVC medical devices, accompanied by some **DEHP** (<0.1% w/w), **DEHT**, and sometimes **DEHA** (Bourdeaux et al., 2016). Under conditions appropriate for obtaining biosimilar model results, **TOTM** thus presents a favorable profile as a plasticizer for medical devices. Our results still have to be interpreted and correlated with plasticizer extraction tests performed in clinical conditions of use. An infusion set used once in the life of a patient for 24 h presents a lower risk than a hemodialysis circuit used for 4 h three times a week. Hence to assess patient exposure risk, it will be necessary to determine the amount of plasticizer extracted per unit mass of PVC in clinical conditions. Such work is particularly necessary for medical devices used for vascular access. One objective of the ARMED project is to propose a migration simulation model (Bernard et al., 2015): the SCENIHR, 2015 reported different migration profiles among plasticizers. Given the primary metabolite toxicities of several DEHP alternatives (**MEHT**, **MINP**, **MINCH** and **MMeOCH**), it will also be necessary to look for the presence of their secondary metabolites, and study their toxicities.

Declaration of interest

This study is a part of the ARMED® project, and received financial support from the French National Agency for the Safety of Medicines and Health Products (ANSM).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.01.026.

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