Review

Assessment of genotoxicity of herbal medicinal products: Application of the “bracketing and matrixing” concept using the example of Valerianae radix (valerian root)

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A R T I C L E   I N F O

Article history:
Received 3 October 2013
Received in revised form 12 February 2014
Accepted 2 April 2014

Keywords:
Genotoxicity
Herbal medicinal products
Valerian root
Bracketing and matrixing
HMPC
Ames-test

A B S T R A C T

An assessment of genotoxicity is a precondition for marketing authorization respectively registration of herbal medicinal products (HMPs), as well as for inclusion into the ‘Community list of herbal substances, preparations and combinations thereof for use in traditional herbal medicinal products’ established by the European Commission in accordance with Directive 2001/83/EC as amended, and based on proposals from the Committee on Herbal Medicinal Products (HMPC).

In the ‘Guideline on the assessment of genotoxicity of herbal substances/preparations’ (EMEA/HMPC/107079/2007) HMPC has described a stepwise approach for genotoxicity testing, according to which the Ames test is a sufficient base for the assessment of genotoxicity in case of an unequivocally negative result. For reducing efforts for testing of individual herbal substances/preparations, HMPC has also developed the ‘guideline on selection of test materials for genotoxicity testing for traditional herbal medicinal products/herbal medicinal products’ (EMEA/HMPC/67644/2009) with the aim to allow testing of a standard range of test materials which could be considered representative of the commonly used preparations from a specific herbal drug according to a ‘bracketing/matrixing’ approach.

The purpose of this paper is to provide data on the practical application of this bracketing and matrixing concept using the example of Valerianae radix, with the intention of facilitating its inclusion in the “Community list”. Five extraction solvents, representing the extremes of the polarity range and including also mid-range extraction solvents, were used, covering the entire spectrum of phytochemical constituents of Valerianae radix, thereby including polar and non-polar constituents. Extracts were tested in the Ames test according to all relevant guidelines. Results were unequivocally negative for all extracts. A review of the literature showed that this result is in accordance with the available data, thus demonstrating the lack of a genotoxic potential.

In conclusion the two guidelines on genotoxicity provide a practically applicable concept. Valerianae radix has no genotoxic potential, supporting its use in HMPs and its inclusion in the Community list.

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Introduction

The frame for the regulation of HMPs in the European Union (EU) is given by directive 2001/83/EC as amended by directive 2004/24/EC. According to regulation (EC) No 726/2004, the committee for herbal medicinal products (HMPC) is responsible for preparing the view of the European Medicines Agency (EMA, formerly EMEA) on herbal medicines (Knöss and Chinou 2012). According to the guideline EMEA/HMPC/32116/2005, for many herbal substances and preparations, used in well-established or traditional herbal medicinal products (HMPs), a safety profile in accordance to modern standards is supported or at least partially substituted by their documented history of medicinal use. Thus, only if a safety concern is recognized or suspected, supporting non-clinical investigations may be needed (EMA, 2008). In general, the combination of documented experience gained during
long-standing use with bibliographic data is the main basis of the non-clinical assessment of traditional and well-established HMPs. Therefore, particular attention should be paid to effects that are difficult or even impossible to detect clinically. They include genotoxicity, carcinogenicity, and toxicity to reproduction. Especially the lack of data from genotoxicity testing may present a safety concern. Thus there is a need for the assessment of the genotoxic potential of herbal preparations. For many active substances data on genotoxicity are missing or data described in the literature are inadequate. If an adequate assessment cannot be made, further genotoxicity testing is required (EMEA, 2006b).

Guidelines on genotoxicity testing of pharmaceuticals, primarily directed to the assessment of new chemically defined active ingredients, have been established by ICH since the 1990s (ICH S2) and have been adopted as EMA guideline (EMA/CHMP/ICH/126642/2008). This guideline describes a battery approach of genotoxicity testing, in which pro-and eukaryotic test systems in vitro and in vivo are employed (EMEA, 2008). The basic requirement is to assess genotoxicity initially in a bacterial reverse mutation test, followed by tests in mammalian cells in vitro and a mandatory test in a mammalian model in vivo.

For HMPs, the HMPC has established a stepwise approach, in order to address both scientific aspects of genotoxicity testing and the special needs of HMPs within the current regulatory framework. This approach also includes the documented history of medicinal use, with the guideline EMEA/HMPC/32116/2005 setting the frame and the guideline EMEA/HMPC/107079/2007 giving detailed guidance. The basic assessment is a bacterial reverse mutation test, using a battery of different strains as well as metabolic activation. For technical information on how to perform this test (Ames-test; Salmonella typhimurium mutation assay) the OECD guideline no. 471 (OECD, 1997a) is referred to. Only in cases of equivocal or positive results, which cannot be explained sufficiently or clearly attributed to specific constituents with a well-known safety profile (e.g. the ubiquitous quercetin or other flavonoids common also in food), additional in vitro tests, e.g. mouse lymphoma cell assay, and, if necessary, also in vivo studies are required.

Usually, European community herbal monographs established by the HMPC for well-established and/or traditional use cover a range of herbal preparations. In order to alleviate the manufacturers task of testing their own specific preparations, a distinct guidance was developed offering a strategy to reduce the number of test materials (EMEA, 2009). This approach suggests testing a representative range of preparations of an herbal drug rather than conducting individual tests as is otherwise required – the so-called ‘bracketing/matrixing’ approach (Wiesner and Knöss, 2010).

The main objective of this guideline is to achieve consensus on a standard range of test materials which could be considered representative of the commonly used preparations of a herbal drug, with the intention to facilitate providing data necessary for allowing their entry to the Community list established by decision of the European Commission (2008/911/EC).

This guideline mentions the option to extrapolate the results obtained with a specific preparation to closely related preparations. Only in case of extracts prepared with ethanol/water mixtures of substantially different concentrations, the demonstration of the phytochemical similarity of the test materials may be required (EMEA, 2009). This test design also assumes that the genotoxic potential of any intermediate preparation is represented by the results of the extremes tested.

Recently, the conduction of a joint project for testing genotoxicity of selected extracts using this “bracketing and matrixing” concept for more than 30 herbal drugs has been reported (Gaedicke et al., 2009; Kelber et al., 2012). The present paper shows the results of the practical application of the “bracketing and matrixing” concept on a specific herbal drug, Valeriana radix. The community herbal monograph from October 2006 on Valeriana radix lists a wide range of preparations, covering extracts prepared with ethanol/water (ethanol max. 40–70% (V/V)), dried valerian root (powdered herbal substance), aqueous dry extracts, valerian (ethanolic) tincture, expressed juice and valerian root oil (EMEA, 2006a).

### Materials and methods

#### Selection of extracts

Extracts from valerian root used as active substances in HMPs authorized or registered within the EU were provided by pharmaceutical manufacturers. The extracts had been produced and characterized analytically in compliance with specifications approved by regulatory agencies and the respective regulatory guidelines. Extract characteristics have been documented in accordance with the respective certificates of analysis. Table 1 shows five different extracts that were used in the genotoxicity assessments. They were selected according to the principles of the guideline EMEA/HMPC/67644/2009 (EMEA, 2008) and represent the complete range of extraction solvents including water as the most polar and heptane as the most apolar solvent. They also included extracts of intermediate strength, prepared with ethanol 40% (V/V) and 70% (V/V) as well as an oily macerate prepared on the basis of ethanol 96% (V/V). Thus they are assumed to cover the whole phytochemical range of preparations of valerian root (Table 1).

#### Reverse mutation test

The Salmonella typhimurium histidine (his) reverse mutation test is a microbial assay which measures his+ → his0, the reversion induced by chemicals which cause base changes or frameshift mutations in the genome of this organism (Ames et al., 1973, 1975). Only bacteria that have reverted to histidine-independence (either spontaneously or by the action of the test chemical) will continue to divide and to form colonies, thereby indicating a concentration-related mutagenic effect of the test item. The testing of the extracts from valerian root was conducted by LPT (Laboratory of

### Table 1

Test materials used for genotoxicity testing of Valeriana officinalis L., radix.

<table>
<thead>
<tr>
<th>Extraction medium</th>
<th>DER</th>
<th>Native extract</th>
<th>Excipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water1</td>
<td>3–6:1</td>
<td>70%</td>
<td>30%</td>
</tr>
<tr>
<td>Ethanol 40% (V/V)2</td>
<td>4–7:1</td>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td>Ethanol 70% (V/V)2</td>
<td>3–6:1</td>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td>Ethanol 96% (V/V)2</td>
<td>1:10</td>
<td>n.a. (oily macerate)</td>
<td>Rapseed oil</td>
</tr>
<tr>
<td>Heptane3</td>
<td>167:1</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

n.a. not applicable; DER drug extract ratio.

1 Extract contained in a HMP registered or authorized within the EU.
2 Extract especially prepared (by two-step mazeration with stirring at 45 °C) for coverage of the non polar extractables, not contained in an HPM registered or authorized within the EU, and containing valeptelates (isovaltrate 1.35%, valtrate 0.76%, measured by HPLC).

### Table 2

Chemicals used as positive control items.

<table>
<thead>
<tr>
<th>(a) Without metabolic activation</th>
<th>(b) With metabolic activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium azide in H2O (10 µg/plate)</td>
<td>TA 1535, TA 100</td>
</tr>
<tr>
<td>2-Nitro-fluorene in DMSO (10 µg/plate)</td>
<td>TA 98, TA 1537</td>
</tr>
<tr>
<td>9-Aminoacridine in ethanol abs. (100 µg/plate)</td>
<td>TA 102</td>
</tr>
<tr>
<td>Methyl-methane sulfonate (MMS) in DMSO</td>
<td>TA 98, TA 102, TA 1537</td>
</tr>
<tr>
<td>(1300 µg/plate)</td>
<td>Cyclophosphamide in aqua ad injectabilia (1500 µg/plate)</td>
</tr>
</tbody>
</table>
Pharmacology and Toxicology, Hamburg, Germany) according to the relevant OECD guideline, No. 471, and in compliance with the EC directive on methods for the determination of toxicity–mutagenicity (European Commission, 2000), the ICH guideline S2 (R1) (EMA, 2012), and to good laboratory practice (GLP) regulations [German Chemicals Act and OECD, 1998]. Also the US FDA GLP regulations (FDA, 2012) and the Japanese guidelines for non-clinical studies of drugs manual (Japanese Ministry of Health and Welfare, 1995) were considered.

According to these guidelines, 5 strains of Salmonella typhimurium obtained from Dr. Bruce N. Ames were used, after check on genetic identity. These strains were TA 98 and TA 1537, which primarily respond to frameshift mutagens, and TA 100, TA 102 and TA 1535, which respond to base-pair substitution mutagens. These strains contain, in addition to the mutation in the histidine operon, several other mutations which increase their sensitivity against mutagens. Extracts were tested in amounts of 100, 316, 1000, 3160 and 5000 µg native extract per plate, with half-logarithmic intervals between concentrations and with triplicates for each concentration and experiment. Two independent experiments, each with and without metabolic activation, were conducted: the first experiment was carried out with the standard plate incorporation method, the second one with the pre-incubation method (OECD, 1997a).

Solvents, reference items, metabolic activation system

Shortly before use, extracts were dissolved in dimethylsulfoxide (DMSO). The vehicle served as negative control. Preliminary to the main test, a cytotoxicity test was carried out as a plate incorporation test without metabolic activation, using strain TA 100 and the procedure described below. In the main test, the 5 different concentrations mentioned above were tested. Positive control items were selected according to guidelines (Table 2).

Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (Maron and Ames, 1983). S9 was collected from 20 to 30 rats, characterized regarding protein and P 450 content (Lowry et al., 1951; Mazel, 1971) and stored in liquid nitrogen. The S9 mix was freshly prepared on the day of the test (Maron and Ames, 1983), containing 5%

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**Table 3**

<table>
<thead>
<tr>
<th>Range of spontaneous reversion frequencies (negative reference item). Spontaneous reversion frequencies may be slightly different on plates with S9 mix and vary slightly from experiment to experiment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 98</td>
</tr>
<tr>
<td>TA 100</td>
</tr>
<tr>
<td>TA 102</td>
</tr>
<tr>
<td>TA 1535</td>
</tr>
<tr>
<td>TA 1537</td>
</tr>
</tbody>
</table>

---

**Table 4**

Preliminary cytotoxicity testing with root extracts from Valeriana officinalis L. in S. typhimurium strain TA 100. Values are given as revertants per plate. Significantly increased values compared to NR point to a potential enhancement of revertant rates, reduced values to cytotoxicity.

<table>
<thead>
<tr>
<th>Extraction medium µg/plate</th>
<th>Water</th>
<th>Ethanol 40%</th>
<th>Ethanol 70%</th>
<th>Ethanol 96%/oily maizere</th>
<th>Heptane</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>181.5</td>
<td>166.0</td>
<td>151.5</td>
<td>119.0</td>
<td>124.0</td>
</tr>
<tr>
<td>3160</td>
<td>168.5</td>
<td>159.5</td>
<td>153.0</td>
<td>150.5</td>
<td>115.5</td>
</tr>
<tr>
<td>1000</td>
<td>165.5</td>
<td>156.0</td>
<td>188.5</td>
<td>131.0</td>
<td>119.0</td>
</tr>
<tr>
<td>316</td>
<td>189.5</td>
<td>140.0</td>
<td>177.0</td>
<td>159.5</td>
<td>122.0</td>
</tr>
<tr>
<td>100</td>
<td>156.5</td>
<td>141.5</td>
<td>160.5</td>
<td>121.0</td>
<td>139.5</td>
</tr>
<tr>
<td>31.6</td>
<td>156.0</td>
<td>142.5</td>
<td>168.5</td>
<td>123.5</td>
<td>133.5</td>
</tr>
<tr>
<td>10.0</td>
<td>156.0</td>
<td>160.5</td>
<td>123.5</td>
<td>128.5</td>
<td>125.5</td>
</tr>
<tr>
<td>3.16</td>
<td>167.0</td>
<td>164.5</td>
<td>141.0</td>
<td>143.5</td>
<td>134.5</td>
</tr>
<tr>
<td>1.0</td>
<td>150.5</td>
<td>166.0</td>
<td>154.5</td>
<td>119.0</td>
<td>115.0</td>
</tr>
<tr>
<td>0.316</td>
<td>136.5</td>
<td>156.0</td>
<td>155.0</td>
<td>136.5</td>
<td>154.5</td>
</tr>
<tr>
<td>NR 178.0</td>
<td>158.5</td>
<td>174.0</td>
<td>124.0</td>
<td>155.0</td>
<td></td>
</tr>
</tbody>
</table>

NR = negative reference item(solvent control: DMSO, 100 µl/plate.}

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**Table 5**

Overview of the results of mutagenicity testing of root extracts from Valeriana officinalis L., in five strains of S. typhimurium. All native extracts were tested up to 5000 µg/plate, the highest concentration according to current guidelines. The respective positive controls showed mutagenicity as expected.

<table>
<thead>
<tr>
<th>Extraction medium</th>
<th>Water</th>
<th>Ethanol 40% (V/V)</th>
<th>Ethanol 70% (V/V)</th>
<th>Ethanol 96% (V/V)</th>
<th>Heptane</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 98</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>TA 100</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>TA 102</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>TA 1535</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>TA 1537</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

S9, MgCl2, KCl, glucose-6-phosphate, NADP and phosphate buffer (pH 7.4), and filter-sterilised by using a 0.45 µm filter.

**Plate incorporation and pre-incubation methods**

For plate incorporation, the test components were mixed with the soft agar and then immediately poured onto a coded minimal glucose agar plate. Plates were inverted and placed in a dark 37°C incubator for 48–72 h. The revertant colonies on the test and control plates were counted with a colony counter (Biocount 2000, Biosys), and the presence of the background lawn resulting from the trace of histidine added to the top agar was confirmed on all plates.

In the pre-incubation method, the test item/test solution was pre-incubated with the test strain for 20 min at 37°C prior to mixing with the overlay agar. The remaining steps were the same as described for the plate incorporation method.

Toxicity was evidenced by a reduction in the number of spontaneous revertants, a clearing or diminution of the background lawn or by the degree of survival of the treated cultures. Insolubility of the test item was defined as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The precipitate should not interfere with the scoring. Test items that were cytotoxic already below 5 mg/plate or 5 µl/plate were tested up to a cytotoxic concentration.

**Evaluation**

While the statistical evaluation of the results of the Ames test is under discussion (Kim and Margolin, 1999; Mortelmans and Zeiger, 2000) and depends also from the historical data achieved in the individual testing site, the responsible laboratory considered a test item showing a positive response, if the number of revertants was significantly increased (p ≤ 0.05, U-test according to Mann and Whitney, Colquhoun, 1971) (ranges see Table 3) to at least 2-fold of the respective solvent control for TA 98, TA 100 and TA 102 and 3-fold of the solvent control for TA 1535 and TA 1537 in both of the independent experiments. In addition, a significant
mutation of root extracts from Valeriana officinalis L. in the bacterial reverse mutation test (Ames) Test. Test: strain TA 98, TA 100, TA 102, TA 1537, TA 1535, and TA 1537. Test for genotoxicity was not noted (Table 4).

The main study, five concentrations of each extract ranging from 100 to 5000 μg/plate were tested in independent experiments, each carried out with and without metabolic activation. Signs of cytotoxicity were not noted in both the plate incorporation test and the pre-incubation test up to the concentration of 5000 μg/plate. A reduction in the number of colonies by slightly more than 50% compared with the solvent control was noted in strain TA 1537 without metabolic activation in the pre-incubation test (aqueous extract) and plate incorporation test (extract with ethanol 40% (V/V)), and with metabolic activation in the pre-incubation test (aqueous extract) in higher concentrations, but was rated by the laboratory as being coincidental, due to very low absolute numbers of colonies and lack of a dose dependency.

A mutagenic effect (increase in revertant colony numbers as compared to control counts) was not observed for any of the valerian extracts tested up to the concentration of 5000 μg/plate in any of the 5 test strains in two independent experiments with and without metabolic activation (plate incorporation and pre-incubation test, respectively), even if common regulatory thresholds of 2 (for strains TA 98, TA 100, TA 1535 and TA 1537) or 1.5 (for strain TA 102) are taken into consideration. A summary of the results is given in Table 5. The mean revertant rates are listed in Table 6.

According to guideline EMEA/HMPC/676/4/2009 (EMEA, 2009), the results cover the entire spectrum of phytochemical constituents of valerian root, including polar and non-polar constituents, and can therefore be considered representative of the commonly used herbal substances/preparations prepared from it, including the entire herbal drug. A negative test result fulfils the genotoxicity testing requirements of directive 2001/83/EC and guideline EMEA/HMPC/107079/2007 for including an herbal substance or preparation in the Community list of herbal substances, preparations and combinations thereof if there are no other reasons withstanding.

Discussion

The study follows current EMEA guidelines which aim to provide a general framework and practical approaches on how to assess or test the potential genotoxicity of herbal substances/preparations and how to interpret the results, thereby representing a pragmatic approach to address both scientific aspects of genotoxicity testing and the special needs of HMPs within the current regulatory framework applicable to these products.

Due to the use of extraction solvents representing the extremes of the polarity range and including also mid–range extraction solvents, the entire spectrum of phytochemical constituents, including polar and non-polar constituents, is covered. This allows also the transfer of the results to further extracts with different drug extract ratios, as relevant differences in the analytical profile can be assessed to be only due to a limited solubility of specific constituents in the respective extraction solvent. The “bracketing and
matrixing concept" warrants that also in case of such constituents an optimal solvent has been included, thus making them accessible to genotoxicity testing.

As the Ames tests were unequivocally negative (EMEA, 2008), on the basis of the HMPC nonclinical guideline no further genotoxicity testing of valerian root preparations is required (EMEA, 2006b). The results may therefore facilitate the inclusion of valerian root and preparations thereof in the Community list of herbal substances.

The lack of genotoxicity of valerian root seen in this study is supported also by the available literature. This includes the ESCOP monograph (ESCAP, 2003) as well as the assessment report of the HMPC (EMEA, 2007), which do not contain information pointing to any relevant mutagenic properties of valerian root or preparations thereof. Within the latter, four publications have been assessed. In two earlier publications, data on alkylating, cytototoxic and mutagenic effects have been presented for valepatriotes and their degradation products (Bos et al., 1998; von der Hude et al., 1986). However, these substances either are not detectable in valerian root extracts contained in HMPS or are found only in very low amounts (Wagner and Jurcic, 1980). A further test was conducted in mice with a dietary product of undefined quality. It showed weak effects in the bone marrow micronucleus test in a concentration of about 4 g/kg (Al-Majed et al., 2006), which is much higher than the recommended dosage in humans (6-fold of the average human single dose calculated via human equivalent dose for a 60 kg human) and is 2-fold of the highest dose level applicable for non-toxic substances in the mammalian erythrocyte micronucleus test according to OECD (OECD, 1997b). A somatic mutation and recombination test in Drosophila melanogaster did not show any genotoxic effects of an infusion of valerian root. As this drug had been purchased from a local health food store, questions concerning the quality of the herbal substance in relation to pharmaceutical properties and the dose used in therapy are open. In addition, this test procedure is not accepted as a standard method for the investigation of genotoxicity (Romero-Jimenez et al., 2005).

Besides these publications, further studies are accessible via relevant data bases (including Medline and Toxline). They include an in vitro study with valepatriotes in a human endothelial cell line (ECV304): With very high concentrations a moderate degree of DNA damage could be demonstrated in the Comet assay (Hui-Lian et al., 2003). However, this proves to be proven to be not due to mutagenic properties of the substance, but to ROS-induced epigenetic mechanisms caused by the very high concentrations of test substance used. In addition, the test model is not yet validated. As being caused by epigenetic mechanisms and related to valepatriotes, which are not present in valerian preparations used in HMPS, the results do not point to mutagenic properties relevant in therapy. In a further publication an herbal combination was assessed, which included a valerian dry extract and extracts from Crataegus oxyacantha and Passiflora incarnata (Tabach et al., 2009). Both the results of the Ames and the Micronucleus test did not indicate a genotoxic potential.

Altogether, the literature data are in accordance with a lack of genotoxic properties of preparations of Valeriana officinalis L., and thereby also underline the validity of the genotoxicity assessment conducted according to the HMPC guidelines EMEA/HMPC/340719/2005 and EMEA/HMPC/67644/2009.

This study therefore allows the conclusion that the framework given by HMPC in its guidelines mentioned above is apt to successfully generate data which may support the inclusion of herbal substances or preparations thereof in the Community list of herbal substances as well as regulatory applications resp. registrations of preparations from the respective herbal drugs. According to EMA/ICh-Guidelines the results have to be discussed when used in marketing authorization procedures (EMEA, 2008).

**Perspectives**

The application of the “Bracketing and Matrixing” approach to further herbal substances/preparations seems to be very promising for simplifying regulatory applications of HMPS in traditional or well established medicinal use in Europe.

**Conflict of interest**

Kooperation Phytopharmaka GbR is a scientifically independent organization with 75 member companies.

The data presented may extend the data available in public domain and stimulate scientific discussion. The manuscript is reflecting the views of the authors and shall not be quoted as representing the position of BFArM.

**Acknowledgements**

The authors thank J. Leuschner and A. Winkler from LSR Laboratory of Pharmacology and Toxicology GmbH & Co. KG, Hamburg, Germany, who have conducted the tests and were helpful in discussing questions arising during the preparation of this manuscript, D. Long, Darmstadt, Germany, for help in preparing the tables for publication and for administration of the literature and S. N. Okpanny, Wiesbaden, Germany, for discussions and proof reading.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2014.04.003.

**References**


German Chemicals Act, Good Laboratory Practice Regulations of the EC enacted in Germany in the 'Chemikaliengesetz' [German chemicals act], current edition.


