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Concerns:
Data package 3, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it

The principle of the method had been published in 1989, and a first collaborative validation study in 1991. In these first trials the stimulation of the lymph nodes, i.e. cell proliferation, was measured by ^{3}H-Thymidin incorporation. In 1999 the principle of the LLNA had been stated as valid alternative to guinea pig assays by the ICCVAM, although the need for further modifications was also noted. Concerns focused on false positive results caused by strong irritants or negative results based on the use of aqueous formulations.

In 2002 the method has been published in guideline OECD 429, and 2003 in EPA guideline OPPTS 870.2600 as a stand-alone test. Corresponding to the concerns mentioned above the use of "wholly aqueous vehicles are to be avoided.". As published by Ryan et al. in 2002 1% Pluronic PE 9200 (L92) may be chosen for using aqueous vehicles in the Local Lymph Node Assay [Ref.3.1.]. As can be taken from the information in this paper it is possible to achieve positive results by the addition of this surfactant to aqueous formulations of test items. However, the cut-off concentrations (EC3 values) increased significantly compared to vehicles recommended in the guidelines. Apart from that the data impressively show the influence of vehicles on the cut-off concentrations determined by the LLNA exemplary illustrated by Table 1 (primordial Table 3 in the paper of Ryan et al.).

Table 1 (taken from publication Ryan et al., 2002)

Effect of vehicle on the relative skin sensitization potency of DNBS, formaldehyde, potassium dichromate and nickel sulfate

Chemical	Vehicle	EC3 Value
DNBS	Water	16%
	1% L92	6.4%
	DMSO	2.0%
	DMF	<1.0%
Formaldehyde	Water	14.5%
	1% L92	4.2%
	DMSO	<1.0%
	DMF	<1.0%
Potassium dichromate	1% L92	0.17%
	DMSO	0.05%
	DMF	0.0327%
Nickel sulfate	1% L92	2.5%
	DMSO	4.8%
	DMF	>5.0%

To examine the use of surfactants on the ability to test aqueous formulations in the Local Lymph Node Assay we started with aqueous formulations of HCA. The test item was formulated immediately before each administration in Pluronic PE 9200 / 0.9% NaCl solution, 1% v/v or Cremophor / 0.9% NaCl solution, 2% v/v [cf. also Ref 3.3.].

In a first trial we compared HCA in different vehicles with 2% Cremophor. Results are shown in the Table below (Table 2).

Table 2: Modified LLNA using NMRI and HCA as positive control. Cut-off cell count index is set to 1.4, i.e. EC1.4 should be used [Ref. 3.2.].

HCA					Statist. Signific.		
	Vehicle	**3%**	**10%**	**30%**		**EC1.4**	**Potency***
	MEK	1,22	1.42	1.99	*	9.3	moderate
	AOO (4:1)	1.15	1.28	1.79	*	14.7	weak
	DMF	0.87	1.13	1.77	*	18.4	weak
	PEG400	0.81	1.04	1.69	*	21.1	weak
	Cremophor	0.71	0.98	1.37		(31.5)	(weak)

* Potency classification according to ECETOC technical Report No. 87, 2003

Although an improvement, addition of Cremophor alone did not reach the EC values between 5% and 20% as normally determined with standard (guideline) vehicles. Therefore, we included an additional infrared irradiation (about 20 min. before treatment) of the animals to enhance the blood flow in the skin and by this enhance penetration. This additional treatment by infrared irradiation caused indeed higher, and statistically significant stimulation indices as can be taken from the Table below.

Vehicle	3%	10%	30%		EC1.4	Potency#
Cremophor (2%)	0.71	0.98	1.37		(31.5)	(weak)
Cremo. (2%) + IR	0.82	1.34	1.45	*	20.9	weak

*: Statistically significant

#: Potency classification according to ECETOC technical Report No. 87, 2003

Similar studies were then conducted with L92 and infrared irradiation in combination with aqueous HCA formulations. In each case HCA has been classified by this method as weak sensitizer within a range of EC values comparable to those obtained with other (guideline) vehicles. Such positive control studies with aqueous formulations are done in regular intervals in our lab (Bayer HealthCare AG, Immunotoxicology) since years. Results of these studies are also included in the Excel file attached to this data package [Ref. 3.3.].

It has to be mentioned here that based on all our experiences so far with Cremophor or Pluronic it seems that Pluronic (L92) enhances the intrinsic irritant properties of test compounds while Cremophor does not! This property of L92 may be problematic for correct classification of test items when radioactive labeling without discrimination of irritation and sensitization is used for measuring cell proliferation. One example of such a positive control study report with HCA in 1% Pluronic is attached as Ref. 3.4., which is equal to data of Ref. 3.3., "Tabelle 4, 2005/2".

Because sponsors did not want us to submit data with aqueous formulations all we can provide are data from a pre-validation study with HCA as positive controls and three aqueous formulations (A-C) from which one had been tested positive in GPMT before (A as weak sensitizer; B unknown; C tested negative before). The results are given in Ref. 3.5. including all controls with 2% Cremophor or 1% L92 plus infrared irradiation.

The overall conclusion from these studies is that stimulation index induced by formulation A at the highest concentration (50%) just reached the cut-off level of EC1.4, statistically significant. Hence, formulation A would be classified as a weak sensitizing formulation while the other two formulations turned out to be negative.

Conclusions:

--- There is some differences in stimulation indices obtained with various vehicles. EC value may vary by a factor of +/- 2 of overall mean. A change in classification of potency by this factor is possible [cf. also review article by McGarry, 2007; Ref. 3.6.].

--- Aqueous formulations may be tested by adding 1% L92 or 2% Cremophor to the formulation to increase adherence to the skin. Skin irradiation with infrared will accessorily improve the outcome, i.e. test sensitivity.

--- By this modifications (surfactant + infrared irradiation) it is possible to test aqueous formulations with nearly the same sensitivity as with vehicles recommended in the guidelines.

--- However, there is no profound validation study of the LLNA or a modification of it with aqueous formulations or mixtures down to the present day.

--- It seems as if Pluronic enhances the irritant properties of test compounds applied, and by this increase the non-specific activation of lymph node cells which may be a problem for classification according to potency by radioactive methods.

Kind regards,

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