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# Alternative Methods for the Safety Evaluation of Chemicals

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To evaluate the toxicity of chemicals, sometimes the alternative methods instead of prescribed methods are very useful. As the alternative methods have their own sensitivity to distinguish chemical toxicity, we have to consider the detection principle and the sensitivity of the methods before use. Many alternative methods are developing now. It is desirable that the detection sensitivity and the results consistency between the alternative and the prescribed methods will be increased by the improvement of the methods and/or the ingenious way of using. In this review, we describe the public situation, trend, and our examination of the alternative methods to detect genotoxic, skin irritating or skin sensitizing potential of chemicals.

This paper is translated from R&D Report, "SUMITOMO KAGAKU", vol. 2005-II.

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## Introduction

There are various toxicity tests available today to evaluate the safety of chemical products. Among them, when handling chemicals in plants, or selecting candidates among many new chemicals during the initial developmental stage, the minimum toxicities to be evaluated are as follows: genotoxicity; acute toxicity; skin and eye irritation; and skin-sensitization. Based on these toxicities, we can determine how to handle or develop the chemicals.

The most widely used screening test method for genotoxicity is a reverse mutation test using bacteria, also known as the Ames test. Of all toxicity tests, the Ames test is one of the simplest and cheapest. However, for screening chemical candidates in the early developmental stage, an improved test method that can simultaneously evaluate a greater number of samples and bring results in a shorter time using fewer amount of samples is desirable.

To determine acute toxicity, skin and eye irritation and skin-sensitization, the guidelines require conducting tests using animals. However, the prescribed methods are time-consuming and require substantial costs, thus it is desirable to develop alternative methods to animal testing, which consequently leads to improved animal welfare.

This paper will introduce the current status and chal-

lenges in the development of alternative methods for the safety evaluation of chemicals, with a focus on our trials (e.g., the umu test for genotoxicity, the skin 3D-model test for skin irritation, the LLNA (Local Lymph Node Assay) test and the peptide-binding assay for skin-sensitization).

## Genotoxicity (umu Test)

### 1. Trends in Regulations and Existing Test Methods

Genotoxicity is the potential of a chemical substance to cause damage to DNA, which is of course a genetic material. If the damaged DNA is not repaired to its original state, gene mutations or chromosomal aberrations may occur. These abnormalities may initiate cellular carcinogenesis. Therefore, it is very possible for a substance possessing a genotoxicity to be carcinogenic. Furthermore, a substance that causes gene mutations or chromosomal aberrations may induce heritable disease in the next generation. Since animal tests to determine carcinogenicity and heritable effects upon the next generation require substantial time and cost, it is extremely difficult to conduct such animal testing for all chemical substances as they are developed, one after another. For this reason, when handling a chemical for which the presence of carcinogenicity and heritable defects is unknown, its genotoxicity is a toxicity that should be evaluated

early in the chemical-development stage.

To detect the different kinds of genetic damage that can be caused by diverse mechanisms, several *in vitro* and *in vivo* genotoxicity tests have been designed to date (see **Table 1**).

**Table 1** A List of Mutagenicity Tests

Materials	Categories of Mutagenicity Tests		
	Gene Mutation	Chromosomal Aberration	DNA Damage & Repair
Bacteria	•Ames Test •HGPRT Gene Mutation Test	***** •Chromosomal Aberration Test	•Rec-Assay
Mammalian Cells	•Mouse Lymphoma Assay	•Sister Chromatid Exchange Assay	•Unscheduled DNA Synthesis Assay
Animals	•Spot Test •Gene Mutation Assay in Transgenic Mice	•Micronucleus Test •Chromosomal Aberration Test •Sister Chromatid Exchange Assay	•Unscheduled DNA Synthesis Assay

It has been confirmed that most genotoxic agents can be detected by combining some of these test methods. Therefore, according to the international and domestic guidelines for agricultural chemicals and pharmaceuticals, in order to make a registration for a particular chemical it is mandatory to conduct and obtain a comprehensive evaluation from the results of the following three tests: the Ames test, the chromosomal aberration test using mammalian cells and the micronucleus test using rodents. On the other hand, for general chemicals (whose numbers far exceed the above types of chemicals), the “Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances” stipulates the Ames test and the chromosomal aberration test using mammalian cells as mandatory tests for genotoxicity. Additionally, the “Industrial Safety and Health Law” specifies the Ames test as mandatory. In particular, the Ames test has been empathically recognized as important because of the following reasons: the Ames test is conducted to detect the potential for mutagenesis; although it uses bacteria, the mechanism behind bacterial mutagenesis is basically the same as that for higher organisms; the test method is relatively simple; and the results can be obtained in a short time period at relatively low cost.

The Ames test uses the particular strains of *Salmonella typhimurium*, which cannot synthesize the amino acid histidine, required for their growth. These

strains of *Salmonella typhimurium* have been developed by Ames *et al.*<sup>1)</sup> In the Ames test the bacteria treated with the test substance is transferred to a culture medium that does not contain histidine, and then genotoxicity is determined by counting the emerged colonies that have become able to synthesize histidine due to a reverse mutation occurring in the genes for histidine synthesis.

In our laboratory, we have also been conducting chemical screening using the Ames test, not only for the evaluation of pharmaceuticals and agricultural chemicals in the early development phase, but also for the responsible handling of general chemicals and for worker safety at manufacturing sites. Accompanying the accelerating speed of chemical development in recent years, the number of the Ames test conducted has been increasing rapidly.

The major shortfall of this test is that most of its procedures rely on manpower, which thereby limits to only one or two, the number of chemicals that can be handled by one person per day. Moreover, although the test method is relatively simple and the testing period is rather short, a sample amount of at least 100–200mg is required. Furthermore, at least three days are required for colony growth. Therefore, an alternative screening method, which can test a greater number of samples using a lesser amount of test substances at greater speed, is strongly desired.

## 2. Applications of the Umu Test and Future Challenges

The umu test is a method developed in 1985 by Oda *et al.*<sup>2)</sup> While the Ames test detects the mutation of genes for histidine synthesis by growing a mutant colony that is a phenotype of such mutation, the umu test detects damage to DNA by measuring the expression level of the *umu* gene product, which is one of the DNA repairing enzymes that is induced as soon as damage occurs to the DNA through the SOS response, which bacteria possess as an original property. The principle behind the umu test is as follows: The bacterial strain used for the umu test is *Salmonella typhimurium* TA1535, which is also used for the Ames test, carrying the plasmid pSK1002, which bears an *umuD* gene including a promoter and an *umuC* gene fused with *lacZ*, the structural gene for  $\beta$ -galactosidase. The activity of  $\beta$ -galactosidase induced by the genotoxic chemical via the SOS response can easily be measured with chromogenic substrates, colorless sub-



shows strong genotoxicity, or by eliminating candidate chemicals that show strong genotoxicity during a fairly early stage, in which the candidate chemicals have not yet been defined. Regarding future challenges, in order to apply the umu test more widely, we believe it is necessary to improve the testing system so that the detection rate for Ames-positive chemicals can be increased.

## Skin Irritation/Corrosion (Skin 3D-model)

### 1. Trends in Regulations and Existing Test Methods

Irritation is an inflammatory reaction caused by a chemical to which the skin or eyes have been exposed. When the skin is exposed to a chemical, necrosis of the epidermis or dermal cells, or erythema and swelling due to inflammatory cytokines, can be observed. When the eye is exposed to a chemical, corneal opacity can be observed due to changes to the corneal surface, redness and swelling on the conjunctiva. Additionally, although it is rare, skin inflammation that reaches to the dermis or strong corneal clouding can be observed. In some cases these damages do not improve at all. If that is the case, it is defined as “corrosion,” which is an irreversible damage.

For agricultural chemicals, the skin/eye-irritation test is mandatory for the registration application. (It is occasionally mandatory for pharmaceuticals as well, depending upon the application route.) The OECD, EPA, EC and the guidelines stipulated by the Ministry of Agriculture, Forestry and Fisheries of Japan recommend animal testing using rabbits. Additionally, a step-by-step test scheme has been proposed. For example, if the chemical is a strong alkali ( $\text{pH} \geq 11.5$ ) or a strong acid ( $\text{pH} \leq 2$ ), or if the chemical has been recognized as corrosive from the structural-activity relationship, no irritation testing is necessary. In the event that corrosion or severe irritation is detected on the skin, the eye-irritation test will be omitted. It is believed that the attitude of animal welfare comes into play in the proposal of such a step-by-step testing scheme, given that irritation testing can cause a great deal of pain to the animals being used. There is a trend (mainly in Europe) to reduce the pain caused to experimental animals, as well as to reduce the number of animals used for such irritation testing.

The development of an alternative method for irritation testing began in the 1980s. For skin irritation testing, human 3-D skin model validation tests were

conducted in Europe from 1996 to 2000, mainly through the ECVAM (European Center for the Validation of Alternative Methods).<sup>5), 6)</sup> Subsequent to the improvement of the test protocol and several catch-up validation tests, human 3-D skin model testing was accepted into the OECD guidelines in 2004 as an *in vitro* skin corrosion test method for the screening of skin irritation.<sup>7)</sup> In the U.S. as well, a similar evaluation test was conducted by the ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) in 2002.

In Japan, neither the “Law Concerning the Examination and Regulation of Manufacture, Etc., of Chemical Substances” nor the “Occupational Safety and Health Act” have stipulated any regulations for the evaluation of skin/eye irritation. Thus, the company must evaluate irritation potential of intermediate products on workers independently. Currently, our company has obtained skin/eye irritation data for both finished and intermediate products, for the purpose of ensuring worker safety and protecting workers from potential irritation caused by these products. The number of tests conducted to date exceeds 100 per year. We believe it is important for us to have an alternative method for these irritation-evaluation tests, not only from the perspective of animal welfare but also for the purpose of reducing costs and obtaining test data at an earlier stage. Therefore, we initially examined the possibility to introduce human skin 3D-model testing as an *in vitro* skin-corroding property test, which is a screening test in terms of skin corrosion.

### 2. Human Skin 3-D Model Test (Skin-Corroding Property Screening Test)

Fig. 2 depicts an outline of the human skin 3D-model.

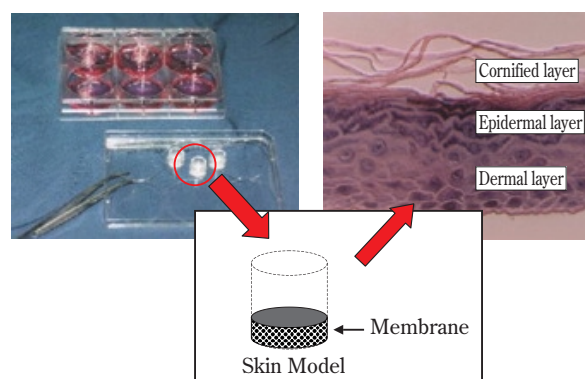


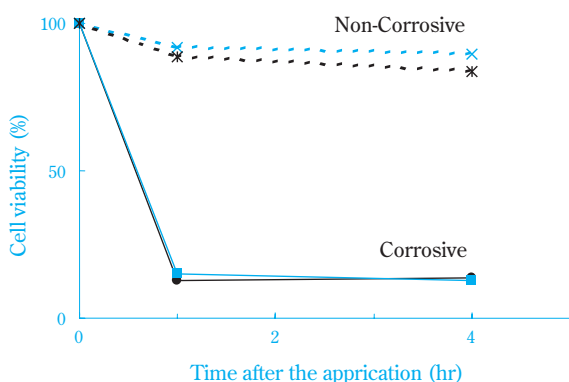
Fig. 2 Human skin 3D-model (EpiDerm™)

The model shown in Fig. 2 is a 3-D cell culture system composed of a three-dimensional structure that includes a basal lamina, granular layer and cornified layer. Since the model possesses metabolic ability, the human skin 3D-model test can be considered as the test method that more precisely reproduces the vital reaction of the skin. We have exposed test chemicals to the models and evaluated their skin-corroding property using the cell survival rate as an index.<sup>8)</sup> Table 4 depicts a summary of the test methods. EpiDerm™ and EPISKIN™ are the skin 3D-models currently available on the market.

In Japan, with respect to human skin 3D-model testing (skin-corroding property screening test), a small-scale validation test using 12 chemicals was conducted among several laboratories in 2004. Our company participated in this validation test. We are planning to release the results of this domestic validation separately. Our company also conducted human skin 3D-model testing using our own chemicals. Given these chemicals, we have successfully distinguished

**Table 4** Human skin 3D-model (EPISKIN™, EpiDerm™) Test Methods (ICCVAM summary report)

	EPISKIN™	EpiDerm™ (EPI-200)
Dosing procedures	Liquids : 50 µL applied neat Solids : 20 mg + saline	Liquids : 50 µL applied neat Solids : 25 mg + 50 µL H <sub>2</sub> O
Exposure	3 minutes, 1 hour, 4 hours	3 minutes, 1 hour
Endpoint	Relative cell viability compared to concurrent negative control	
Negative and positive controls	Negative control : saline Positive control : glacial acetic acid	Negative control : water Positive control : 8.0 N KOH
Positive criteria	Relative cell viability : < 30% at any exposure duration	Relative cell viability : < 50% after 3 minutes, and/or < 15% after 60 minute



**Fig. 3** Result of Human skin 3D-model Test

between corrosive and non-corrosive chemicals, although there were not many examples (Fig. 3).

### 3. Future Challenges

There is a pressing need for our company to find alternative *in vitro* test methods to replace animal testing, not only from the perspective of animal welfare, but also because such alternative methods could reduce both the costs involved and the time needed for testing.

Regarding the aforementioned human skin 3D-model, validation testing is currently being conducted only for the screening of skin corrosion. However, the human skin 3D-model is considered to be the most promising alternative test method for skin irritation, since it possesses skin structure and metabolic ability but is not affected by solubility or properties of the subject chemical. If the human skin 3D-model is used for skin-irritation testing, the test period can be reduced significantly, from the 14 days required for animal testing to only two days. Significant cost reductions are not yet available, because the model cups used in the testing are expensive. However, in recent years less expensive model cups have been developed, which have the same shape and functionality as the model cups currently approved by the guidelines. It is therefore expected that once these new products have demonstrated proven reliability, they will replace the existing products and the cost of human skin 3D-model testing will become more affordable.

Contrastingly, although the human skin 3D-model can currently evaluate chemicals that cause corrosion *in vivo*, at this present stage it cannot be used to evaluate substances that are not water-soluble or chemicals that have weak irritation levels. Additionally, although the correlation between EC<sub>50</sub> (chemical concentration at which the cell viability reaches 50%) or ET<sub>50</sub> (chemical exposure time at which the cell viability reaches 50%) and irritation has been observed via the ECVAM and ICCVAM, no standards have yet been established.

Additionally, although it is quite rare, corrosion can be observed on a subject rabbit without being evident on a skin model. The cause of this phenomenon is considered to be the effects of inflammatory cytokines. Therefore, research is also being undertaken that focuses more upon cytokine secretion<sup>9)</sup> and changes in gene expression.<sup>10)</sup>

An alternative method of evaluating eye irritation has been developed and is currently being evaluated. This method uses the isolated eyes from domestic animals and poultry. Moreover, the EpiOcular™ test kit composed of human keratinocytes has been developed for eye irritation testing. The EpiOcular™ kit has a structure similar to that of the human cornea, as with the case of the skin 3D-model.<sup>11)</sup> The NICEATM (National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods) and the ICCVAM have evaluated various tests (Isolated Rabbit Eye Test, Isolated Chicken Eye Test and Hen's Egg Test – Chorionic Membrane Test), with both organizations having concluded that all these tests can be applied (some with specific conditions) in a tiered method that identifies corrosive chemicals and severe eye-irritating chemicals. The EpiOcular™ kit has not yet been evaluated in detail, such as to determine reliability and correlation with animal testing.

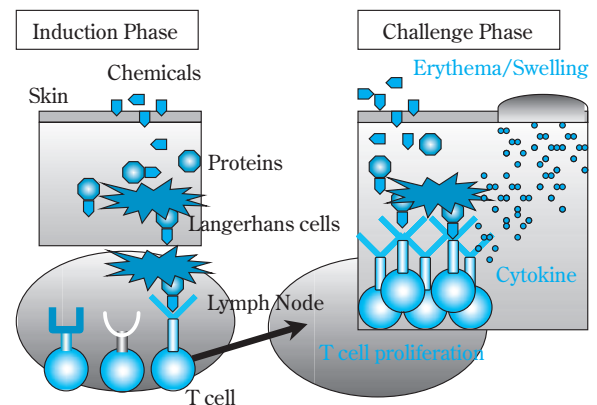
Our laboratory plans to continue examining and developing alternative test methods for skin irritation using the human skin 3D-model, while at the same time paying attention to emerging international trends. We are also planning to promote the replacement of conventional eye irritation test methods with *in vitro* tests, mainly by introducing testing methods that show the most promise.

## Skin-sensitization (LLNA and Peptide-binding assay)

### 1. Trends in Regulations and Existing Test methods

Skin sensitization is an allergic reaction. It results in a rash caused by repeated exposure to a chemical. From previous research, it is known that two phases are involved in the mechanism of skin sensitization: "induction" and "challenge." In the induction phase, a chemical penetrates into the skin after coming into contact with the skin. Then, the chemical reacts to proteins in the skin and becomes an antigen. The antigen is then presented by the Langerhans cells (LCs). When the antigen is recognized by a particular species of T-lymphocyte, it causes the T-lymphocyte proliferation. The challenge phase follows exactly the same steps as the induction phase, until the same chemical becomes antigen and presented by the Langerhans cells. However, because a large number of T-lymphocytes are already present in the skin, various

cytokines are released beneath the skin by those T-lymphocytes to cause the skin reactions of erythema and swelling<sup>12), 13)</sup> (Fig. 4).



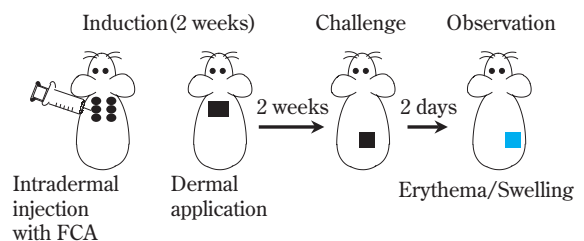
**Fig. 4** Mechanism of skin sensitization

Various test systems based on skin-sensitizing mechanisms have been evaluated to date. The Maximization Test (GPMT), which uses guinea pigs, is one test method that has been widely accepted in the registration applications for various chemicals.<sup>14)</sup> The GPMT contains both phases: induction and challenge. In the GPMT, the detection sensitivity is improved by adding an immunopotentiator during the induction phase (Fig. 5). Our company also uses the GPMT primarily for chemical evaluation when the registration applications have to be made to the Ministry of Agriculture, Forestry and Fisheries and under the Drugs, Cosmetics and Medical Instruments Act, as well as to the EPA/EU applications.

Meanwhile, in actual workplace-- even though there could be intermediate products which quite often are

#### Test property

- ① Confirmation of skin reaction (erythema / swelling)
- ② High sensitivity
- ③ Long test period (4 weeks)



- Reaction Score (0-6) : Score  $\geq 1$   $\rightarrow$  positive
- Sensitizing ratio = positive / total number of animals

**Fig. 5** Guinea Pig Maximization Test (GPMT)

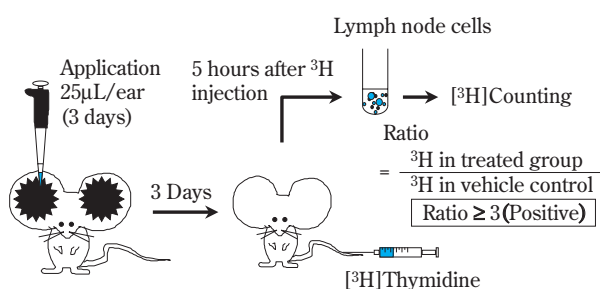
unstable and might potentially show strong skin sensitization — the decision to conduct testing for such products is left to each company, since they are not subject to the current domestic laws, the “Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances” and the “Occupational Safety and Health Law.” In order to ensure worker safety, we believe it is necessary to know the sensitizing potential of these intermediates prior to handling them. The major problem of employing the GPMT for such purpose is that it takes approximately one month to complete while there are numerous numbers of the intermediates to be checked to assure the safety of our workers, thus making it very difficult obtaining timely data.

## 2. Local Lymph Node Assay (LLNA)

The LLNA test system was developed in Europe, mainly as an alternative to animal testing, to both reduce the amount of pain to which animals would otherwise experience and to reduce the number of subject animals required.<sup>15)–17)</sup> While the GPMT has “induction” and “challenge” as its testing steps, the substance is evaluated during the induction step in the LLNA. The advantage of this test method is its relatively short test period, which is approximately one week (Fig. 6).

### Test property

- ① Detection of lymphocyte cell proliferation
- ② Low sensitivity (compare with GPMT)
- ③ Short test period (1 week)



**Fig. 6** Local Lymph Node Assay (LLNA)

Table 5 depicts the advantages and shortcomings of the LLNA and GPMT test methods.

Compared to the GPMT, the LLNA has the following shortcomings: it cannot detect substances having weak sensitizing potential; and its operation is slightly more complicated since it uses RI-labeled com-

**Table 5** Comparison of LLNA and GPMT

	LLNA	GPMT
Duration	1 week	4 weeks
Cost performance	Low	High
Sample scale	1g	20g
Sensitivity	Low	High
Cross-reaction Test	No	Yes
Registrability	Yes	Yes

pounds. Nevertheless, the LLNA also has the several advantages: it can detect substances having strong sensitizing potential that may induce human skin rash; it allows for the comparison of relative skin-sensitizing potential intensity among several chemicals by comparing their threshold concentration of giving the positive signals of each chemical; it is more inexpensive and less time-consuming. Furthermore, because LLNA test methodology has been approved recently in the guidelines of the EC (2004) and OECD (2002)<sup>18)</sup> LLNA testing is expected to soon become the most popularly used test for skin-sensitization potential.

In 1998, the LLNA was introduced in our laboratory and we have used it to evaluate chemical skin-sensitizing potential ever since. As a result, we have become able to ensure the safety of our workers much faster than before. Moreover, by understanding the sensitizing potential of chemicals based on EC3 values, we can provide more appropriate facilities and better protection for workers.

However, some of the chemicals that cause human skin rashes can be detected only by the GPMT, not the LLNA.<sup>16)</sup> Moreover, since the LLNA requires a dermal route of exposure, there is the problem of relatively low detection capacity when testing water-soluble chemicals having short skin retention times.<sup>18)</sup> Therefore, we expect that many aspects of the testing system will need improvement for the future, including the selection of vehicle that can better detect such chemicals.

## 3. *In Vitro* Test Methods (Peptide-binding assay) and their Correlation with *In Vivo* Test Methods

Although the LLNA has been recognized as an effective alternative (refinement and reduction), it cannot be completely replaced with the conventional methods since it uses animals. Furthermore, in EU countries, by 2009 there will be prohibitions in place

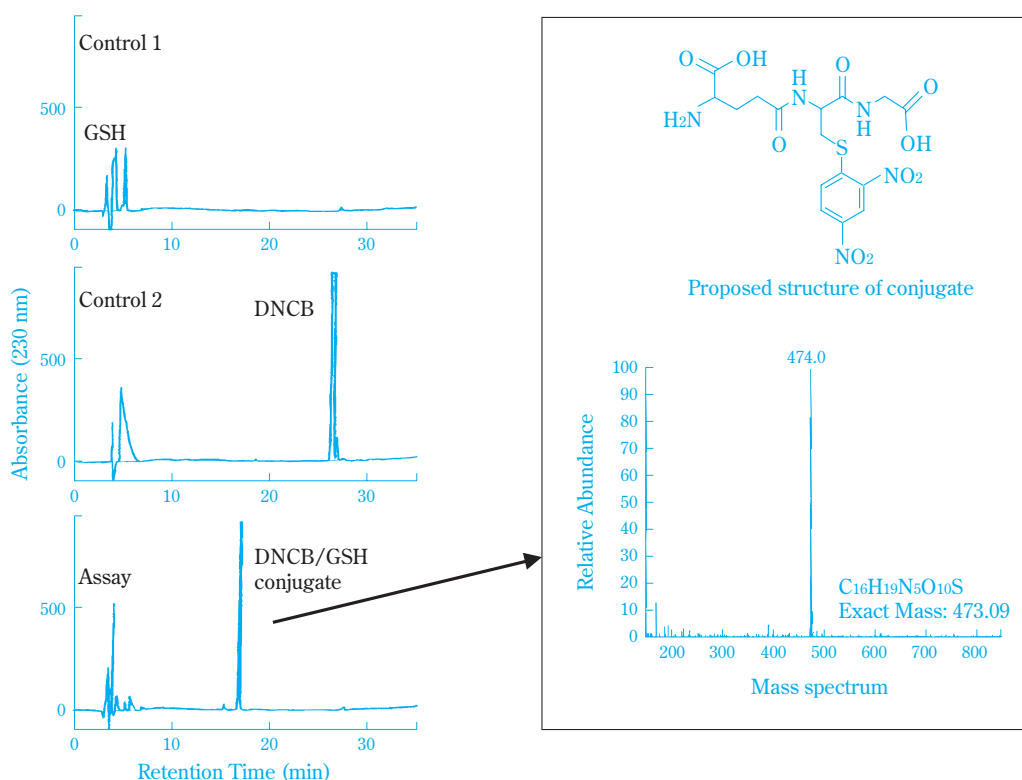
against the sale of any cosmetics or their raw materials for which animal testing has been conducted. Under these circumstances there is a pressing need to develop alternative methods that do not use animals in the evaluation of skin-sensitizing potential. Our company also has a policy to obtain data in the earliest possible stage regarding the sensitizing potential for raw materials and intermediate products. While the speed of product development is rapidly increasing, even the LLNA requires an approximately one week to test a chemical and it is the great difficulty of obtaining such data during early stages for all of the chemicals handled by our company. Therefore, our company hopes to obtain a skin-sensitizing potential screening method that has a shorter test period.

As described above, in the process whereby a chemical has the potential to cause skin-sensitization, the chemical must first penetrate the skin and then react with the proteins by covalent bond in the organism. With conventional methods, in regard to this first step, the skin-sensitizing potential of a target chemical is estimated by analyzing the reactivity of a similar chemical and by calculating the logP or logKo/w values as an index for skin penetrability.<sup>19)–21)</sup> Under such circumstances, our company has paid particular attention to the reactivity of chemicals to proteins with

in the organism and together with the Organic Synthesis Research Laboratory, has jointly developed a method of evaluating the skin-sensitizing potential within a day, using LC mass spectrometry.<sup>22)</sup>

It is generally known that chemicals having sensitizing potential react to amino-acid residues comprised of proteins (particularly cystein or lysine). Based on this knowledge and by paying attention to the reactivity of such chemicals to proteins, we have developed the methods to evaluate the sensitization potential by assessing the formation of chemical-peptide conjugates through the following test: the subject chemical was mixed with glutathione (a tripeptide composed of glutamic acid, cystein and glycine) under controlled conditions and the reaction mixture was analyzed with the LC mass spectrometer.

2,4-Dinitrochlorobenzene (DNCB), which possesses sensitizing potential, was mixed with glutathione, as shown in **Fig. 7**. The reaction mixture was then analyzed with the LC mass spectrometer. As a result, a peak was detected, which indicated the conjugate of the DNCB and glutathione. **Table 6** depicts the results of analyses for reactivity to glutathione, conducted using the 82 samples for which skin-sensitizing potentials had already been tested and clarified (61 sensitizer, 21 non-sensitizer).



**Fig. 7** Analysis of the formation of conjugates (LC-MS)



**Table 6** Relativity of *in vitro* (peptide-binding assay) and *in vivo* test

	<i>in vitro</i> Positive	<i>in vivo</i> Negative	total
<i>in vitro</i> Positive	30	2	32
<i>in vitro</i> Negative	31	19	50
total	61	21	82

Concordance = 60%

*in vitro* Positive Predictability = 94%

Of the 61 samples that were found to be positive through *in vivo* tests, such as GPMT and LLNA, 30 chemicals (49%) were found to be positive in the binding assay and 31 chemicals (51%) were found to be negative in the same test. Of the 21 samples that were found to be negative through *in vivo* tests, two chemicals (approx. 10%) were found to be positive in the binding assay and 19 chemicals (approx. 90%) were found to be negative in the same test. Therefore, the rate of concordance reached 60% (49/82). However, it was discovered that the positive predictivity for chemicals that were judged to be positive in the binding assay, was 94% (30/32).

#### 4. Future Challenges and the Utility of Alternative Methods for Determining Skin-Sensitizing Potential

Based on the examinations conducted to date, our company has been efficiently performing LLNA and GPMT tests in order to obtain skin-sensitizing potential data at an early stage. In this testing process the primary evaluation is first conducted using various documents, the test results for existing chemicals and peptide-binding assays (*in vitro*) according to the importance of the product and the applicable regulations. However, major challenges, such as (1) quantitativity; and (2) predictability, must still be addressed in the future.

The skin-sensitizing potential of chemicals can be quantitatively estimated by using the GPMT and LLNA tests. Our company workers can also compare the relative degrees of potential among the intermediate products they are handling, thus enabling them to select appropriate forms of protection. However, peptide-binding assays can provide only qualitative results. To address this issue, research with particular focus upon chemical reactivity, similar to that performed by our company, is being conducted by another research group led by Gerberick (P&G). This

research group quantitatively expresses chemical reactivity by measuring residual-SH groups in an attempt to compare the potential for skin sensitization. Therefore, in the future it will be desirable to establish a quantitative method for comparing reactivity, which utilizes the concentration of peptide-conjugates and, as an index, uses the time required to generate such peptide-conjugates.

Moreover, with respect to predictivity, another challenge is that alternative methods produce many false-negative samples and a slight number of false-positive samples, as described previously. Two samples that produced false positives in our company's experiments also produced false positives in tests conducted by Gerberick. The researchers have concluded that skin penetrability is the cause of this phenomenon. Thus, further analysis is needed for some chemicals that show reactivity through *in vitro* testing systems, due to the following reasons: there is a possibility that skin-sensitizing potential has not been recognized due to other factors, such as the subject chemical not easily penetrating actual human skin; even though a chemical may penetrate the skin and react with protein, the conjugate is not recognized by LCs or T cells; and an *in vivo* test method providing a negative result may not have been appropriate. On the other hand, since many false-negative chemicals are very likely to demonstrate sensitizing potential after being metabolized within an organism, future testing systems need to be improved by adding metabolic activation system.

We shall ensure greater safety for our workers by evaluating the skin-sensitizing potential of chemicals at an early stage and allowing our workers to take appropriate precautions. To achieve these goals, we shall continue to improve alternative screening methods for skin-sensitizing potential and shall develop methods having greater accuracy.

#### Conclusion

As described above, each alternative method has its own sensitivities. These alternative methods detect only confined endpoints of the toxicity, so to speak, the detection systems restricted to the evaluation of a specific reaction. Therefore, it is not surprising that alternative methods can accurately detect toxicity for some chemicals but not for other chemicals.

Nonetheless, alternative methods do possess many

advantages, such as: results can be obtained in a shorter time period; only a small amount of sample is required; more samples can be tested simultaneously; and testing is inexpensive. When considering innovations in the alternative methods used for chemical safety evaluation, it is necessary to use these alternative methods properly, based on an accurate understanding of the detection principles and the sensitivity of each method. Therefore, we believe it is better to use alternative methods as part of the testing in a tiered evaluation scheme (a step-wise evaluation) in order to detect toxicity. Some examples follow: using the umu test to rule out chemicals that have been found positive in the test, from candidate chemicals for development, because it is highly likely that these chemicals will also be found positive in the Ames test conducted after the umu test; or when there is no capacity to conduct animal experiments in a timely manner for a large number of chemicals, chemicals that have been found positive in peptide-binding assays should be treated for the time being as chemicals that do possess sensitizing potential. These alternative methods can be strong tools that can reduce the cost and time required to obtain results, on the condition that we understand the special features of each method and use them properly.

The alternative methods used for chemical safety evaluation described in this paper are still under development. We will continue our efforts to improve these methods, with the challenge of solving many problems and expanding applications, in order to establish the best possible evaluation method.

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