Review

CURRENT STATUS OF CONDUCTING FUNCTION TESTS
IN REPEATED DOSE TOXICITY STUDIES IN JAPAN

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(Received March 3, 1997; Accepted July 5, 1997)

ABSTRACT — It seems desirable to conduct as many function tests as possible in repeated dose toxicity studies, but is it practicable? The current status of conducting function tests in repeated dose toxicity studies in Japan was investigated by a literature survey of more than one thousand papers published in seven Japanese toxicology journals during the past 10 years and by a questionnaire survey directed to toxicologists among the Japan Pharmaceutical Manufacturers Association (JPMA) member companies.

The function tests often carried out in repeated dose toxicity studies were, for example: 1) electro-retinography (ERG) and visually evoked potential (VEP) for visual test and tonometer for intraocular pressure; 2) auricular reflex, evoked response audiometry (ERA) and auditory brainstem response (ABR) to sound stimuli; 3) respiration and heart rate, electrocardiogram (ECG) and blood pressure by noninvasive cuff methods or using electronic devices such as telemetry; 4) body temperature, spontaneous motility and some adaptation tests (using rotarod and sloped plate in rats); 5) indocyanin (ICG) or bromosulfophthalein (BSP) for hepatic test; 6) phenolsulfonphthalein (PSP) and creatinine clearance for renal test; and 7) immunoglobulin, leukocyte phagocytosis, lymphocyte blastogenesis and natural killer cell (NK) for immuno-reaction test.

Limitations to conducting function tests in repeated dose toxicity studies and issues to be resolved were discussed, based on questions and suggestions given by the respondents to the questionnaire. Although it certainly seemed desirable to conduct as many function tests as possible in repeated dose toxicity studies, most of the function tests so far introduced into toxicity studies were not satisfactory, because those tests could not be carried out under the restricted conditions of repeated dose toxicity studies, and not much reliable data from function tests were obtainable.

A variety of function tests should firstly be incorporated into single dose toxicity studies together with development of a new concept for methodology in safety pharmacology.

KEY WORDS : Function test, Repeated dose toxicity study, Rat, Dog, Monkey

INTRODUCTION

Repeated dose toxicity studies are mainly comprised of three technological components, i.e. a) daily or weekly monitoring of health conditions of test animals, including determination of food intake and change in body weight throughout the course of a study, b) examinations based on clinical pathology and hematology at the interim or end of a repeated dose study, and c) anatomical and histopathological examinations of the tissues and organs removed at the end of a repeated dose treatment. Thus, repeated dose toxicity studies focus mainly on toxic effects of test compounds for morphology such as cell proliferation and degradation. However, the studies can provide only poor information about effects of test compounds on physiological functions such as visual and auditory sensation, cardiac output and peripheral resistance, and hepatic and renal metabolism. Therefore, it certainly seems desirable to conduct as many function tests as possible in repeated dose toxicity studies. However, the question is what kinds of function tests can be achieved in toxicity studies without disturbing the studies themselves, or how can useful and reliable data be obtained by function tests under the restricted conditions of repeated dose studies.

A working group of the Preclinical Research Subcommittee, Japan Pharmaceutical Manufacturers Association (JPMA), investigated the current status of conducting function tests in repeated dose toxicity studies by a literature survey of

seven Japanese journals published during the past 10 years and by a questionnaire survey directed to toxicologists of JPMA member companies. The present paper will introduce the results of these investigations.

The function tests may be generally defined as tests of physiological functions such as visual and auditory sensors, heart rate and blood pressure, liver metabolic capacity and kidney absorption and excretion. In addition to those, the function tests in these investigations involved some additional examinations which were to be conducted using special instruments during or before sacrificing the test animals. They involved morphological examinations on the anterior chamber and fundus of the eyes, fecal occult blood test, determinations of body temperature, enzyme activity in urine and the plasma levels of antibodies and a variety of hormones.

LITERATURE SURVEY

What kinds of function tests and how often they were carried out in repeated dose toxicity studies were investigated by a literature survey of repeated dose toxicity studies reported during 1984-1993 in the following seven Japanese journals: *Oyo-yakuri* (Pharmacometrics), *Kiso to Rinsho* (Basic and Clinical Report), *Yakuri to Chiryo* (Jpn Pharmacol Ther), *Iyaku hin Kenkyu*, *Nihon Kagakuryoho Gakkaishi* (Jpn. J. Chemother.), *Zen Rinsho* (Preclin Rep Cent Inst Exp Anim) and *J. Toxicol. Sci.*

Table 1. Number of repeated dose toxicity studies in which some function tests were conducted with rats, dogs and monkeys. JPMA investigated the current status of conducting function tests in repeated dose toxicity studies by a literature survey of Japanese journals published during the past 10 years.

Category of tests	Rat studies	Dog studies	Monkey studies
Total number of studies reviewed	742	381	73
Ophthalmic	538	348	62
Cardiovascular-respiratory	26	300	36
Auditory	103	79	0
Neuro-behavioral	22	90	14
Hepatic	16	119	3
Renal	22	95	2
Gastro-intestinal	22	46	10
Immunological	4	5	4
Endocrinological	9	15	not investigated
Reproductive	18	7	not investigated

Repeated dose toxicity studies reviewed

A total of 1,196 repeated dose toxicity studies reported (742 with rats, 381 with dogs, 73 with monkeys) were investigated. Test compounds were administered in most studies orally (57%) and intravenously (24%), but in some studies by subcutaneous, intraperitoneal, transdermal and intramuscular injection or by inhalation. The length of repeated dose treatment was 4 weeks in about 400 studies, 13 weeks in 400 studies, 26 weeks in 200 studies and over 26 weeks in 200 studies.

Number of function tests conducted in repeated dose toxicity studies

Function tests as reported were classified into the following 10 categories: ophthalmic, cardiovascular-respiratory, auditory, neuro-behavioral, hepatic, renal, digestive, immunological, endocrinological, reproductive, and others. Table 1 summarizes a variety of function tests reported for each animal species. The popular function tests conducted with rats (742 studies in total) were mainly ophthalmic (538 studies) and auditory examinations (103 studies). On the other hand, a variety of function tests were conducted with dogs (381 studies in total): ophthalmic (348 studies), cardiovascular-respiratory (300 studies), hepatic (119 studies), renal (95 studies), neuro-behavioral (90 studies), and auditory (79 studies). Most function tests with monkeys (73 studies in total) were ophthalmic (62 studies), cardiovascular-respiratory (36 studies), and neuro-behavioral (14 studies).

Details of ophthalmic examinations

Some kinds of ophthalmic examinations were reported in 538 rat studies. The anterior chamber was examined visually in 238 studies or by using a slitlamp or other ophthalmoscopes in 198 studies, and the fundus was examined in 433 studies. Electroretinography (ERG) was reported in 20 studies; 7 of these studies concerned antibiotics. These examinations were repeated twice or triply, i.e. before, during or at the end of repeated dosing. These ophthalmic examinations were conducted on all test animals in some studies and on a limited number of subgroup animals in other studies.

Out of 348 dog studies with ophthalmic examinations, the anterior chamber was exam-

ined visually in 154 studies and by slitlamp or other ophthalmoscopes in 182 studies. Although pupillary reflex (17 studies) and wink reflex (4 studies) were rarely reported, it was not clear whether or not these tests were actually conducted or were involved as parts of the visual examinations. Some ocular fundus examinations were reported in 309 studies. ERG was tested in 26 studies and tonometer for intraocular pressure in 14 studies. There were some reports of visually evoked potential (VEP) tests, and determination of pupillary diameter or tear volume. Out of 62 monkey studies, the anterior chamber was examined in 57 cases and the fundus in 39 cases.

The examinations revealed some abnormalities or toxic effects on the ophthalmic function in 10 to 20 % of the studies.

Details of function tests on the cardiovascular-respiratory system

Function tests on the cardiovascular-respiratory system were reported in only 26 out of 742 rat studies, ECG examination in 18 studies, heart rate count in 12 studies and blood pressure measurement in 5 studies. On the other hand, a number of cardiovascular tests (300 out of 381) were reported in dog studies. Respiration rate was counted in 39 studies. Heart rate was counted in 113 studies by palpation or auscultation. Blood pressure was measured in 55 studies, mainly by noninvasive cuff methods. Respiration rate and heart rate were counted on all test animals at least twice in a study or weekly in most studies, but blood pressure was measured only once or twice in a study. Some abnormalities or toxic effects on respiration rate, heart rate and blood pressure were detected in 20 to 30% of the studies.

ECG recordings on dogs were reported in 282 studies. However, the positioning of animals for ECG recording was clearly specified in only 52 studies. Animals were restrained for ECG recording in 45 studies in standing, dorsal, sling or recumbent positions.

Out of 73 toxicity studies with monkeys, heart rate was counted in 15 studies, blood pressure was measured in 7 studies and ECG was examined in 26 studies. Some abnormalities caused by test drugs were reported: four cases on heart rate and one case each on ECG and blood pressure.

Details of auditory examinations

Auditory function was tested by auricular reflex in all 103 studies with rats. This test was conducted twice or triply during a study on all test animals in most studies. Abnormalities were reported in only three studies.

There were 79 dog studies in which the auditory function was tested by auricular reflex, escape responses or startle responses. Devices generally used for the tests were Galton whistles, pitchpipes, start guns or buzzers and audio meters. An evoked response audiometry (ERA) test and an auditory brainstem response (ABR) test were each conducted in one study. These tests were conducted in most cases twice or triply in a study on all test animals. There was no case reporting any abnormality in dog studies.

Details of other miscellaneous function tests

Other functions tested with rats were the phenolsulfonphthalein (PSP) test for renal function in 18 studies, skin sensitivity to pain stimulation in 18 studies, forced balance (rotarod) test in 18 studies and determination of estrus cycle in 15 studies. Those with dogs included determination

of body temperature and fecal blood occult tests in 90 studies, PSP tests for renal function in 91 studies, bromosulfophthalein (BSP) tests for hepatic function in 60, indocyanin green (ICG) tests for liver function in 52 studies and determination of adrenal and gonadal hormones. Those with monkeys included determination of body temperature and fecal blood occult tests in most studies.

QUESTIONNAIRE SURVEY

The current status of function tests in repeated dose toxicity studies was also investigated by a questionnaire survey in order to get more detailed information. Eighty-seven out of 99 JPMA member companies responded to the survey. The questionnaire also asked for the purpose of conducting function tests in toxicity studies and significance / trouble in conducting those tests.

Current status of conducting functional tests in toxicity studies

Table 2 shows the number of pharmaceutical companies conducting some function tests in repeated dose toxicity, usually as routine work or

Table 2. Number of pharmaceutical companies currently conducting some function tests in repeated dose toxicity studies with rats, dogs and monkeys. JPMA investigated the current status of conducting function tests in repeated dose toxicity studies by questionnaire survey directed to toxicologists of member companies.

Category of tests	Rat studies			Dog studies			Monkey studies		
	Total number of answers	Usually	Sometimes	Total number of answers	Usually	Sometimes	Total number of answers	Usually	Sometimes
Ophthalmic	90	78	1	88	74	5	53	46	2
Cardiovascular-respiratory	75	1	9	86	63	15	47	25	7
Auditory	82	21	10	77	28	7	45	5	1
Neuro-behavioral	76	4	8	78	30	18	44	12	7
Hepatic	79	2	4	79	19	16	46	5	4
Renal	83	6	23	81	20	22	45	7	7
Gastro-intestinal	79	5	22	78	11	25	45	4	11
Immunological	78	0	18	74	0	17	45	0	19
Endocrinological	87	1	35	76	2	29	44	0	12
Reproductive	87	2	20	78	3	10	42	1	2

Total number of answers: Number of companies that answered the question in total.

Usually: Number of companies usually conducting some tests as routine activity.

Sometimes: Number of companies conducting some tests on a case-by-case basis.

sometimes case by case (depending on the situation).

As the above-mentioned literature survey indicated, some ophthalmic examinations were conducted in toxicity studies with rats, dogs and monkeys by the majority of companies that answered. In addition to the some ophthalmic examinations, a variety of other function tests such as cardiovascular-respiratory, neuro-behavioral, auditory, hepatic and renal examinations were conducted in dog toxicity studies.

Tests for each category of function

Ophthalmic

Visual and fundus examinations were routine activities in most companies that answered, and slitlamp tests with rats, dogs and monkeys were done by 50-70 % of the respondents. Function tests by ERG or tonometry were done case by case depending on the specificities of drugs, *e.g.* antibiotics.

Cardiovascular

Almost all respondents were conducting ECG tests with dogs and monkeys as a routine activity. Determination of blood pressure was routine for some companies, but case by case (mainly with cardiovascular drugs) for others.

Auditory

Almost all respondents were testing auditory function by auricular reflex. Case-by-case decisions were made based on the side effects of some categories of drugs, *e.g.* antibiotics, chemotherapeutics and some cardiovascular drugs. Electro-physiological studies such as ERA and ABR with rats or guinea pigs were performed mostly for antibiotics, because of the side effects of this class of drugs.

Neuro-behavioral

Determination of body temperature in dogs and monkeys as a routine activity was reported by many respondents. Case-by-case tests involved determination of spontaneous motility and some adaptation testing (using rotarod and sloped plate) with rats.

Hepatic and renal

Frequent ICG or BSP tests were conducted as routine or case by case in studies with dogs, but also sometimes with rats and monkeys. PSP and creatinine clearance tests were conducted as routine or case by case. Determination of

enzyme activity in urine was frequently carried out in relation to some potential pathological changes in the kidneys.

Digestive

A fecal occult test for gastrointestinal bleeding was conducted with rats routinely by four companies and case by case by 17 companies, with dogs routinely by 11 companies and case by case by 25 companies, and with monkeys by seven companies. The drugs tested were mainly CNS drugs, NSAID and antitumor drugs. Measurement of spleen enzyme activity in blood or urine was not routine. This test was added to studies with rats by seven companies, with dogs by 10 companies, and with monkeys by four companies. Some companies determined gastric juice volume, localization of bleeding sites using a stomach camera or by injecting dye, and identification of gastric flora.

Immunological

For immuno-reaction tests, some companies measured immunoglobulin, leukocyte phagocytosis, lymphocyte blastogenesis or natural killer cell (NK) activity in toxicity studies with rats, dogs or monkeys. A variety of antibodies was measured in toxicity studies on bio-products with rats by 18 companies, with dogs by 14 companies, and with monkeys by 17 companies.

Endocrine

Because of some pharmacological specificities of test drugs or because some abnormalities were found in previous studies, plasma levels of a variety of hormones were measured in toxicity studies. For example, thyroid hormones in rats were measured by 24 companies, in dogs by 17 companies and in monkeys by six companies; adrenal hormone in rats by 17 companies and in dogs by 18 companies; pituitary hormone in rats by 19 companies, in dogs by 15 companies and in monkeys by seven companies; gonad hormones in rats by 15 companies, in dogs by 10 companies and in monkeys by four companies; and pancreatic hormone in rats by six companies and in dogs by five companies.

Reproductive

Estrous cycle checks on rats were conducted by three companies routinely in conjunction with fertility and teratology studies. This test was conducted case by case in studies by 16 companies. Sperm count and activity tests were conducted in

many companies, mainly because some abnormalities in reproductive organs were found in previous studies.

Difficulties and limitations of conducting function tests in repeated dose toxicity studies

There is no need to stress the significance (or expectation) of conducting function tests in order to improve the quality of repeated dose toxicity studies. However, it is important to clarify the problems that need to be resolved and the limitations of conducting function tests in toxicity studies. A number of questions were raised by respondents to the above-mentioned questionnaire. The following were major comments and questions.

For example, function tests such as determination of cardiovascular parameters should be carried out under physiologically normal and stable conditions. An ECG is one of the most popular function tests in dog toxicity studies. However, as far as a sufficient database from ECG under standardized conditions of the posture of test animals, positioning of electrodes and lead methods can be developed, ECG analysis cannot be very helpful for histopathological examination. On the other hand, a careful analysis of ECG pattern may be useful for detecting abnormal myocardial excitation and conduction, because there is little or no difference in the placing of ECG electrodes with either recumbent or standing positions of dogs (Eckenfels 1980). Determination of blood pressure by noninvasive methods in dogs and rats cannot be highly accurate (Hassler *et al.*, 1979; Petersen *et al.*, 1988). On the other hand, modern techniques with implanted electrode-sensors and telemetric cardiovascular monitor (Kutz *et al.*, 1985; Sato *et al.*, 1994; Gillam *et al.*, 1985 and Coyle *et al.*, 1994) make cardiovascular function tests in conscious animals practical in some situations. However, it may be not be practical to treat test animals in repeated dose toxicity studies with such surgical invasion.

Some methods for function tests in toxicity studies are applications of principles and devices developed for humans. However, the reliability or accuracy of those methods when used with animals are not established. For example, the audiometry test by auricular reflex in animals is non-

invasive, simple and easy, but this method cannot be accurate due to, for example, development of adaptation to the stimuli. The threshold measurement in the auricular reflex will be affected by conditioning to sound stimuli (Laurell and Borg *et al.*, 1986). Ototoxicities of canamycine and trimethyltin were detectable in rats, but there was a variation in sensitivity in different strains (Matsuzawa and Suzuki 1985; Fechter *et al.*, 1986). Appropriate methods for detecting ototoxicity have not been well established with dogs or monkeys (Tokuriki *et al.*, 1990; Pickrell *et al.*, 1993). ERG and/or VEP recording in toxicity studies has become common these days. However, there are still problems with the precision and reproducibility of these methods, *e.g.* restraint and use of anesthetics and/or noise and trouble due to physical contact with attachments. Determination of intraocular pressure in animals by tonometers and ABR for auditory test are other methods that need to be verified.

BSP and ICG tests for hepatic function and PSP test for renal function do not seem too useful because conditioning for the tests in toxicity studies is difficult and troublesome to make the tests sufficiently accurate. For example, it is difficult to obtain urine samples from dogs in accordance with a planned time table. Dogs are species-specific in having extra-hepatic excretion.

Because it is extremely difficult to detect CNS and neuro-toxicity by post-mortem histopathological examinations, neuro-behavioral observation and analysis in the course of toxicity studies are important (Gerber and O'shaughnessy 1986; Mattsson *et al.*, 1989; Gad 1989). The Environmental Protection Agency (EPA) has issued a guideline for CNS toxicity studies (1993), but there are no such guidelines available for pharmaceutical drugs. For example, quantitative determination of spontaneous locomotion activity and learning in rats can be measured with an automated open field system with infrared beams. However, the fact is that rats are nocturnal. An automatic, remote observation system is necessary, because dogs and monkeys behave differently in the absence or presence of observers. Magnetic Resonance Imaging (MRI) may be useful to examine the morphology of central nervous tissues in a noninvasive way (Wolf *et al.*, 1992; Whelan *et al.*, 1988; Carvlin *et al.*,

1992; Fujimoto *et al.*, 1987; Yatsushiro 1990). However, these techniques do not seem practical for repeated dose toxicity studies, and thus simpler and noninvasive technologies need to be established.

Some respondents suggested that a variety of function tests should firstly be incorporated into single dose toxicity studies, and thereby a new approach (concept and methodology) to safety pharmacology should be developed.

CONCLUSIONS

The literature survey as well the questionnaire investigation indicated that a number of function tests were conducted in repeated dose studies as a routine activity or on a case-by-case principle. Although it certainly seems desirable to conduct as many function tests as possible in repeated dose toxicity studies, most of the function tests so far introduced into toxicity studies are still not satisfactory, because those tests could not be carried out under the restricted conditions of repeated dose toxicity studies, so not much reliable data from function tests were obtainable. It was suggested that a variety of function tests should firstly be incorporated into single dose toxicity studies.

ACKNOWLEDGMENTS

The authors express their sincere appreciation to the JPMA member companies that contributed to this survey. Special thanks are extended to Mr. Takashi Kobayashi (Roussel Morishita), Mr. Osamu Sadanaga (Kyowa), Mr. Nobuyuki Tanaka (Otsuka Pharmaceutical), Mr. Yuji Nakano (Asahi Chemical Industry), Dr. Kuninobu Fujii (Nikken Chemicals), and Dr. Nobuo Matsuoka (Dainippon Pharmaceutical).

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