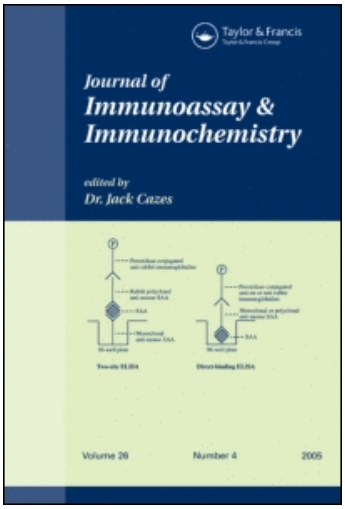

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A Radioimmunoassay for the Anticonvulsant and Neuroprotective Agent, MK-801%

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A RADIOIMMUNOASSAY FOR THE ANTICONVULSANT AND NEUROPROTECTIVE AGENT, MK-801

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ABSTRACT

A radioimmunoassay is described for MK-801, a potent anticonvulsant and neuroprotective agent. Two immunogens were prepared from N-glutaryl- and N-carboxyethyl-MK-801 by coupling through their carboxyl groups to bovine serum albumin. Radioligands were I-125-iodotyramine conjugates of the same derivatives. Both types of antisera displayed bridge recognition which could be circumvented. In the first case, specificity for N-acyl derivatives was satisfied by acetylating the analyte prior to measurement. Antisera to the N-alkyl derivative yielded a satisfactory assay for MK-801 when the heterologous radioligand was employed. The first of these strategies was adopted for the routine assay. Specificity relative to hydroxylated metabolites was a function both of antiserum selectivity and sample preparation. High plasma concentrations of drugs concomitantly administered to epileptics posed special analytical problems. Assay sensitivity is 40 pg/ml in plasma and the interassay CV is about 5%.

(KEY WORDS: MK-801, radioimmunoassay, neuroprotective, anticonvulsant, NEUROGARD^{R}, dizocilipine maleate).

INTRODUCTION

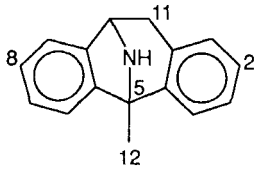
MK-801 (dizocilipine maleate, NEUROGARD^{R}, I in Table 1) is a novel, very potent anticonvulsant (1,2) originally expected to

Table 1

CROSS-REACTIVITY OF ANALOGS

		% Reactivity
I	MK-801	100
II	(-)-Enantiomer	0.61
III	5-Ethyl	1.5
IV	N-Methyl	0.96
V	8-Methoxy	0.96
VI	2-Methoxy	6.1
VII	8-Hydroxy	1.2
VIII	2-Hydroxy	2.0
IX	N-Hydroxy	1.5
X	12-Hydroxy	2.0
XI	11-Exo-hydroxy	1.2
XII	Ketamine	0.003

All compounds were subjected to the acetylation procedure and were racemic except for enantiomers of MK-801. The reactivity estimate is based on concentrations required for 50% inhibition of radioligand binding relative to MK-801 itself. Assay selectivity *in practice was considerably enhanced by the sample preparation* (extraction and back-extraction).



MK-801

Ketamine

have utility in epilepsy. Projected doses were low, and after an oral dose of 330 μg ^{14}C-labeled MK-801 in human volunteers, peak radioactivity in plasma corresponded to a concentration equivalent of 2 ng/ml, and this included metabolites (3). An immunoassay was developed to meet the sensitivity needs of clinical

trials. It was necessary to distinguish drug from the recognized metabolites, 11-exo-hydroxy-, N-hydroxy-, 8-hydroxy- and 2-hydroxy-MK-801. Furthermore, patients receiving the drug were likely to be undergoing concurrent therapy with one or more other drugs having plasma concentrations as high as 10^5-fold greater than MK-801. Even very slight cross-reactivity was a potential analytical hazard.

More recently the drug has been recognized as a potent and specific non-competitive antagonist of the neuron depolarizing action of N-methyl-D-aspartic acid, NMDA (4). Neurodegeneration consequent to an ischemic insult to the CNS has been associated with excessive releases of glutamate and aspartate, agonists at the NMDA receptor, and MK-801 has been shown to afford protection to the CNS in laboratory animals (5).

The present account is a description of the development and characterization of an assay for MK-801 in biological fluids.

MATERIALS AND METHODS

1. The following reagents were purchased from the indicated vendors:

Bovine serum albumin, cat #12660 and rabbit-γ-globulin, cat#345991 (Calbiochem). Goat or sheep anti-rabbit-γ-globulin (Arnel Products Co., NY).

All drugs and metabolites were obtainable within Merck Sharp

& Dohme Research Laboratories; remaining reagents are widely available and not dependent on the source.

2. Immunogens

a) N-glutaryl-MK-801-BSA.

MK-801 base (1 mmole) was treated in pyridine with 1.5 mmoles glutaric anhydride at room temperature. TLC (ethylacetate:toluene: methanol: water, 16:8:6:3) indicated prompt acylation (R_f: base, 0.8; product, 0.2). Pyridine was removed in vacuo and the residue was dissolved in water. After extraction of the aqueous phase with ether at pH 10, the product was precipitated as an oil at pH 5.5, or extracted into ether at pH<5. Purification by HPLC (Waters μBondapak C_{18} column, 3.9 mm x 30 cm, 10 μ particle size, eluting with 50% methanol/ water containing 0.1% trifluoroacetic acid at 1 ml/minute) yielded the product with a retention time of 7.3 minutes. Identity was confirmed by NMR. It was isolated as a semi-hard foam but could not be crystallized.

The N-hydroxysuccinimide ester (NHS active ester) was prepared in dry dioxane using 0.1 mmole N-glutaryl-MK-801, 0.1 mmole N-hydroxysuccinimide and 0.11 mmoles dicyclohexylcarbodiimide. Without isolation, one-half of the product was added to bovine serum albumin (BSA, 100 mg) in 0.1M sodium phosphate, pH 8.5. Additions were made in small aliquots with stirring at 0-4°C during a one-hour period, maintaining

the pH at 8.5 to 9.0 with NaOH. After stirring overnight at room temperature, the mixture was dialyzed exhaustively against water at 4°C. Difference spectra (vs. control BSA) indicated the incorporation of 20 moles hapten per mole BSA.

b) N-carboxyethyl-MK-801

MK-801 base (0.5 mmoles) was refluxed overnight with 0.6 mmoles methyl acrylate in dry methanol. Further addition of methylacrylate was made until TLC indicated complete reaction (ethylacetate: toluene: methanol: ammonia, 16:8:6:3. Product R_f=0.95). The solvent was evaporated, the ester hydrolysed with 0.7M NaOH, and the aqueous solution was extracted with ether before and after adjustment to pH 1. The residue after evaporation of the aqueous phase was extracted with dioxane and dried to a foam. (The structure was verified by NMR. R_f=0.2 in the above solvent, 0.45 in butanol:acetic acid:water, 8:1:1).

The NHS active ester was prepared, and reacted with BSA as in (a) above. UV absorption spectra indicated incorporation of 23-25 moles hapten per mole BSA.

3. Iodotyramine Conjugates

a) From N-glutaryl-MK-801

Iodotyramine was prepared by the method of Counsell et al (6). The principal product was di-iodotyramine, not the monoiodo derivative as claimed. A mixture of iodotyramines

(ca. 0.05 mmole) was reacted with 0.05 mmoles of the active ester described in 2a above in 0.5 ml dry pyridine. The dried crude product was subjected to HPLC (Waters μBondapak C_{18} column, 3.9 mm x 30 cm, 10 μ particle size, 50% acetonitrile in 0.1% aqueous trifluoracetic acid adjusted to pH 2.2 with triethylamine, 1 ml/min). The principal product, retention time 10 minutes was identified by mass spectrometry as the di-iodotyramine conjugate (M/Z=706).

b) From N-carboxyethyl-MK-801.

This was prepared analogously to the method described in (3a) above except that the HPLC solvent was 42% acetonitrile in 0.1% trifluoroacetic acid, pH 2.2. Fractions eluted at 5.5 and 6.5 minutes were shown by mass spectrometry to be the desired mono- and di-iodotyramine conjugates (M/Z=538 and 664 respectively).

4. Radioligands

10 μl (1 mCi) of carrier-free NaI^{125} and 5 μl(25μg) chloramine-T solution were added to tyramine hydrochloride (83 ng in 5 μl 0.01N HCl) and 10 μl 0.2M borate buffer pH 8.5. The reaction was stopped after 60 seconds by the addition of 10 μl (25μg) sodium metabisulfite.

The mixture was added to N-hydroxysuccinimide esters of N-glutaryl- and N-carboxyethyl-MK-801 (each 4 x 10^{-7} moles in 20 μl dioxane). After leaving overnight at room temperature,

purification was effected by HPLC on a Waters μBondapak C_{18} column, 3.9 mm x 30 cm, 10μ particle size, eluting with 44% methanol in 0.1% acetic acid-water at 1 ml/min. Fractions containing radioactive peaks were tested for immunoreactivity and pooled for storage at -20°C.

5. Preparation of Acylated Derivatives

N-acetyl and N-succinoyl derivatives were prepared from the respective acid anhydrides and MK-801 base in pyridine (TLC, ethyl acetate:toluene: methanol:ammonium hydroxide, 16:8:6:3, R_f values, MK-801, 0.7; N-acetyl, 0.8, N-succinoyl 0.3). The N-acetyl derivative crystallized upon solvent evaporation and was recrystallized from ethanol-water, yielding colorless needles, m.p. 169-170°C in 76% yield (elemental analysis, C, 81.7%; H, 6.07%, N, 5.28%; theoretical C, 82.1; H, 6.46, N, 5.32). NMR spectroscopy appeared to indicate the presence of two products but twin peaks coalesced at elevated temperatures and reappeared on cooling, suggesting the existence of rotamers.

6. Immunization

The conjugates were each emulsified with complete Freund's adjuvant and approximately 1 mg conjugate per rabbit was distributed between subcutaneous, intramuscular and multiple intradermal sites. Rabbits were reinjected with 0.5mg

immunogen in incomplete adjuvant (s.c., i.m), after 3 months and at intervals thereafter.

7. Sample Preparation

Serum, up to 2 ml, was made alkaline with 0.5N NaOH (0.25 ml per ml serum) and extracted with 5 ml heptane for 10 minutes on a flat bed shaker. The analyte was back-extracted from 4.5 ml of the heptane into 1 ml 0.01M acetate buffer pH 4.0.

For acetylation. 0.8 ml of the above extract was treated with 0.2 ml 0.5M K_2HPO_4 and 3 µl acetic anhydride with immediate vortexing. After 30 minutes at room temperature, 20 µl 2.5N sodium hydroxide was added. A "blank acetylation mixture" was prepared with 0.01M acetate buffer in place of a serum extract and treated similarly to serve as a diluent for the reference standard.

8. Radioimmunoassay

a) Reagents

The buffer was 0.2M borate, 0.05M EDTA, 0.1M sodium acetate, 0.1% sodium azide, 0.1% bovine serum albumin, adjusted to pH 8.5 with sodium hydroxide.

Antisera were diluted appropriately in assay buffer containing 50 µg/ml rabbit gamma globulins.

Second antibody (goat or sheep anti-rabbit-α-globulin) was diluted according to titer (generally 1:25 to 1:75) in assay buffer and the radioligand was diluted in this to a concentration of 200,000 cpm/ml.

b) Reference

The working standard consisted of 100 µl aliquots of a 1µg/ml solution of N-acetyl-MK-801 stored at -80°C. For use, this was diluted to concentrations of 15 to 5000 pg/ml in the blank acetylation mixture (see Section 7).

Recovery samples consisted of MK-801 in normal human serum (40-4000 pg/ml) or urine (0.5-50 ng/ml). Aliquots were stored at -20°C and for each assay a complete series was treated identically to the unknowns.

c) Quality Controls (N-acetyl-MK-801) were prepared in blank acetylation mixture at 0, 50, 200 and 1000 pg/ml and stored in aliquots for routine use.

d) Method

A Micromedic Automatic Pipetting Station was used to add all assay reagents simultaneously. Assay tubes received 200 µl acetylated extracts, standards or QC, 400 µl assay buffer and 100 µl each of radioligand/2nd antibody and primary antibody. Non-specific binding (NSB) tubes received buffer-gamma globulin instead of primary antibody. After incubation for >18 hours at room temperature, the tubes were centrifuged at

800 g for 45 minutes, supernatants were decanted and radioactivity in the precipitate was measured.

e) Data Processing

The standard curve was represented in terms of the customary logit-log transformation or as a second or third degree polynomial. Mean recovery was calculated for the reference samples and used to adjust the calculated values for unknowns.

RESULTS

Assay Development

For each immunogen, a corresponding radioligand was prepared bearing an identical bridging group. In both cases, assays based on the use of homologous (i.e., corresponding) radioligands exhibited strong bridge recognition and poor displacement of radioligand by the parent drug (Figure 1). This was more marked with antisera to the N-glutaryl derivative (N-GLU) than with those to the N-carboxyethyl derivative (N-CE). When radioligands were interchanged, N-CE antisera displayed enhanced sensitivity to MK-801 (Figure 2B), but N-GLU antisera remained very insensitive (Figure 2A).

The homologous N-GLU assay exhibited general specificity for N-acyl derivatives (Figure 3), leading to the assay format finally developed in which drug was extracted from the matrix and N-

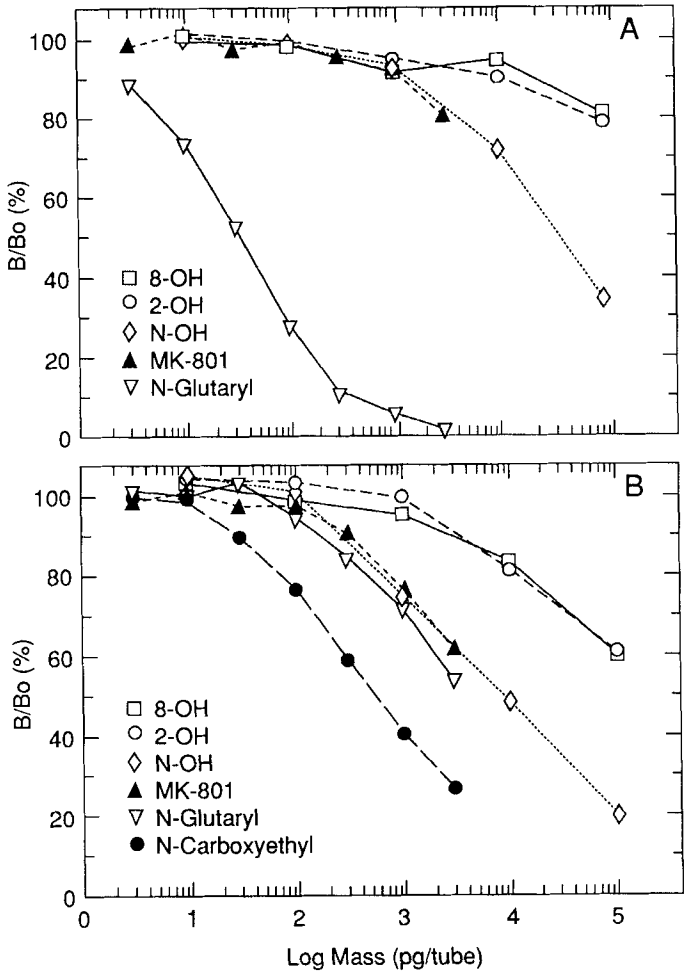


Figure 1 Relative reactivities in assays employing antisera and radioligands having common bridging groups.

A) N-glutaryl antiserum
B) N-carboxyethyl antiserum.

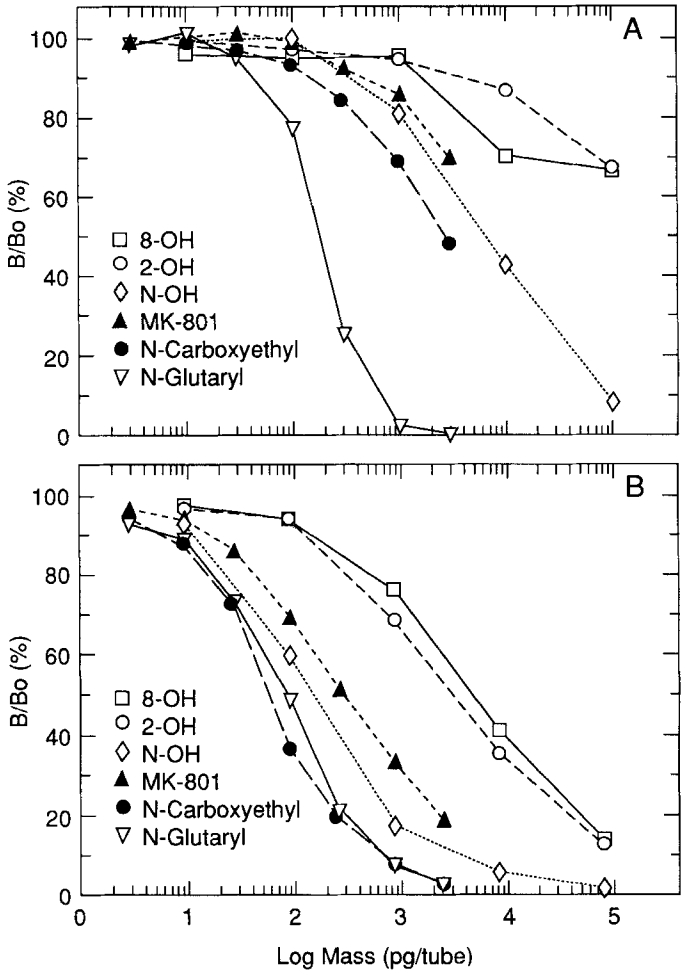


Figure 2 Relative reactivities in assays with heterologous radioligands.

A) N-glutaryl antiserum, N-carboxyethyl radioligand.
B) N-carboxyethyl antiserum, N-glutaryl radioligand.

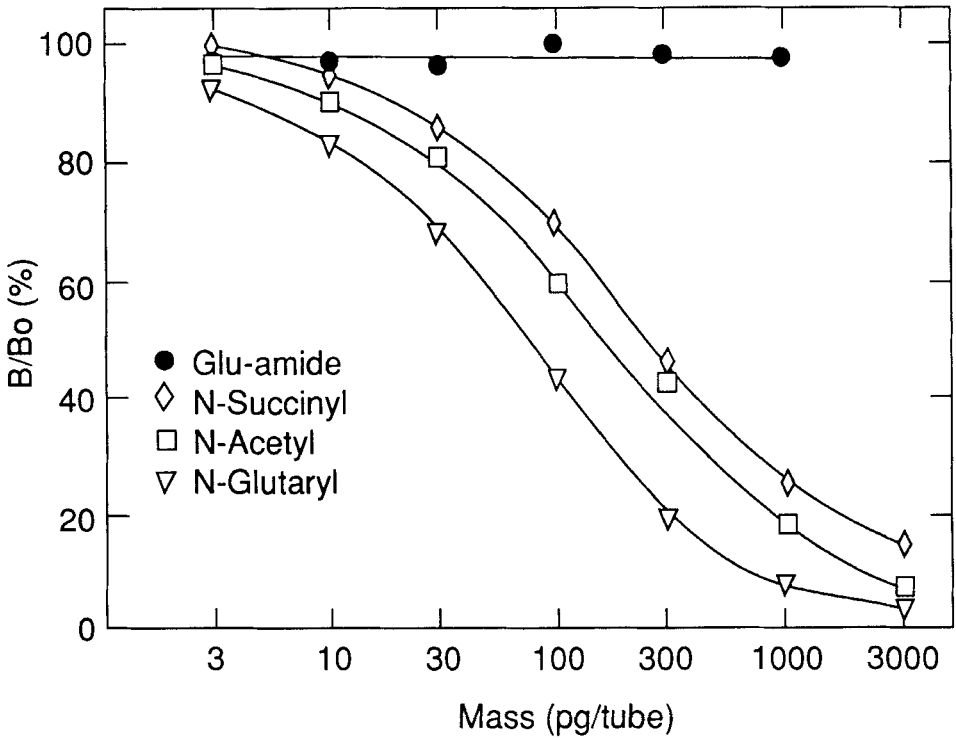


Figure 3 Relative reactivities of N-acyl-MK-801 derivatives in the N-glutaryl homologous assay. The half amide of glutaric acid was unreactive (GLU-AMIDE).

acetylated prior to RIA. Acetylation with acetic anhydride introduced considerable acetate ion into the assay matrix and this was found to enhance radioligand binding (Figure 4). Incorporation of acetate into the assay buffer at a concentration in excess of that producing a maximum effect was a means of ensuring assay stability despite some variation in the contribution of samples to the final acetate concentration.

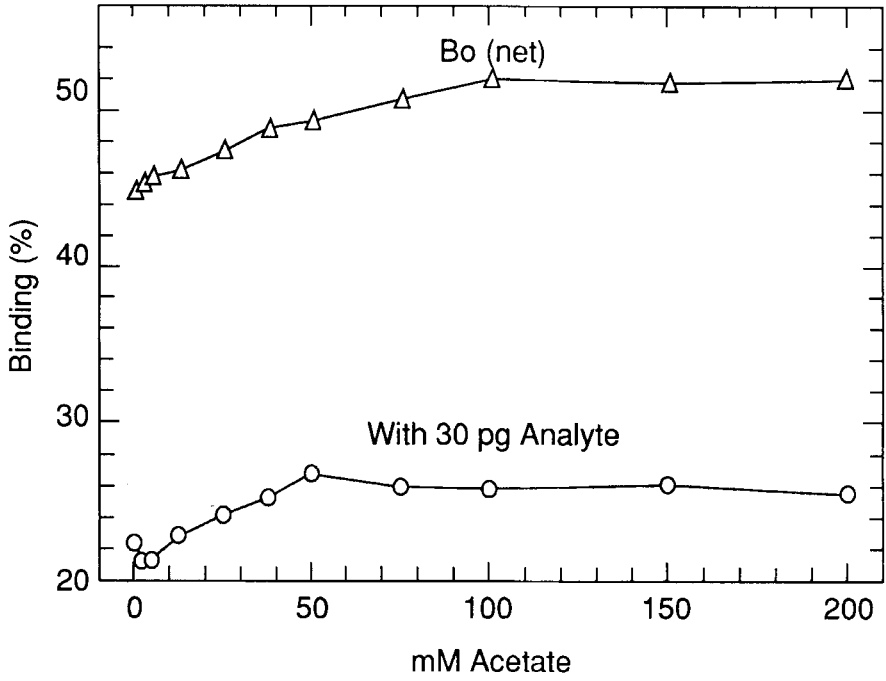


Figure 4 Effect of acetate concentration on radioligand binding.

Specificity

Unlike MK-801, phenolic metabolites (Table 1, VII, VIII) are not extractable into heptane at alkaline pH, and the N-hydroxy derivative IX is not back-extracted into pH 4 buffer. Additionally, these compounds have weak intrinsic immunoreactivity and are virtually invisible to the assay. The 11-exo-hydroxy metabolite XI is recoverable in 20% yield and (with acetylation) is one-eightieth as immunoreactive as the drug. Its effective reac-

tivity is therefore 400-fold less than MK-801 and this metabolite interferes to a negligible degree.

Primate samples obtained with ketamine anesthesia were not assayable due to extensive interference. This drug is structurally related to MK-801 and cross reacts to the extent of 0.003% at 60% of control binding (1μg ketamine per assay tube), but 10 ng/tube is equivalent to 5 pg N-acetyl-MK-801 (90% of control binding; 0.05% cross-reactive) because the response curve is shallow and not parallel to that of MK-801. N-acetyl ketamine was not tested per se but ketamine was subjected to the usual acetylation procedure without change in its activity.

Coadministration of two or more of several drugs is common in epilepsy. Since plasma concentrations of these drugs are relatively high, reactivity was assessed in this RIA. Cross-reactions were slight (Figure 5), and in many cases reduced still further when extraction steps were included (Figure 6), but potentially significant. The inclusion of phenytoin in the assay buffer at 40 ng/ml shifted most response curves but not that of N-acetyl-MK-801, substantially towards higher concentrations and this was adopted as a routine precaution. Elevation of binding by primidone was avoided by substituting gelatin for BSA in the assay buffer while the more significant problem with carbamazepine was solved by washing the initial heptane extract three times with 5 ml of 0.01N NaOH when this drug was known to be present.

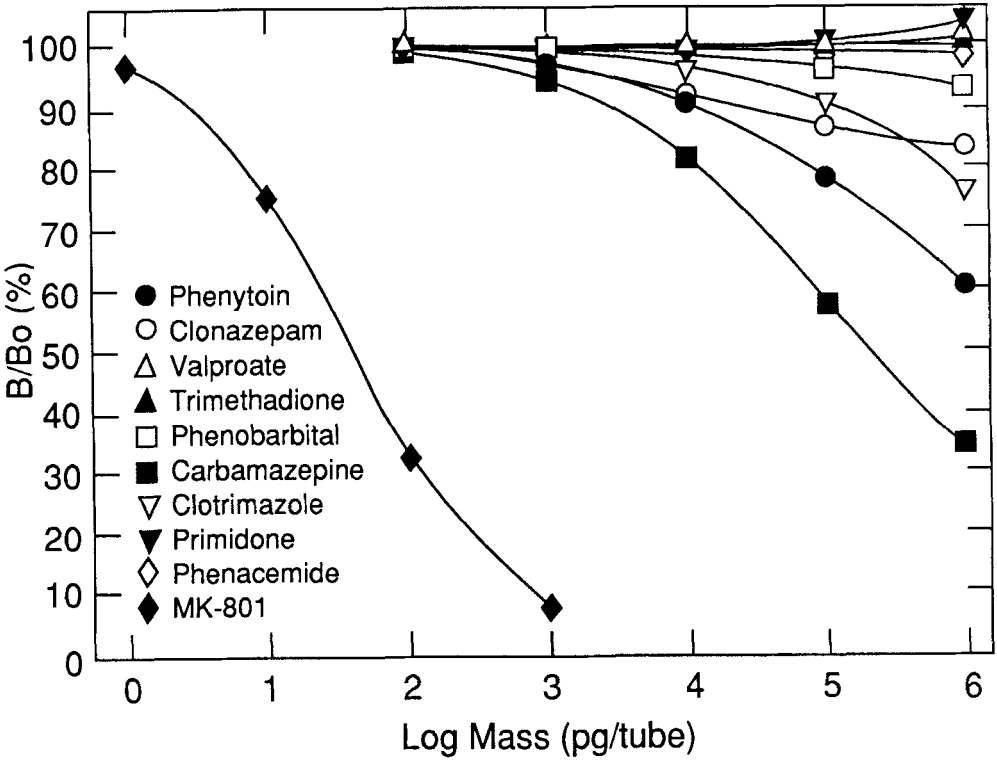


Figure 5 Intrinsic activity of anti-epileptic drugs subjected to acetylation conditions.

Sensitivity

The absolute sensitivity was 3 pg N-acetyl-MK-801 per assay tube (800 µl) corresponding to a lower limit in plasma of about 40 pg/ml MK-801 (2ml extracted).

Precision

Cumulative data for recovery samples in Table 2 display the expected type of precision profile, greater variability at low

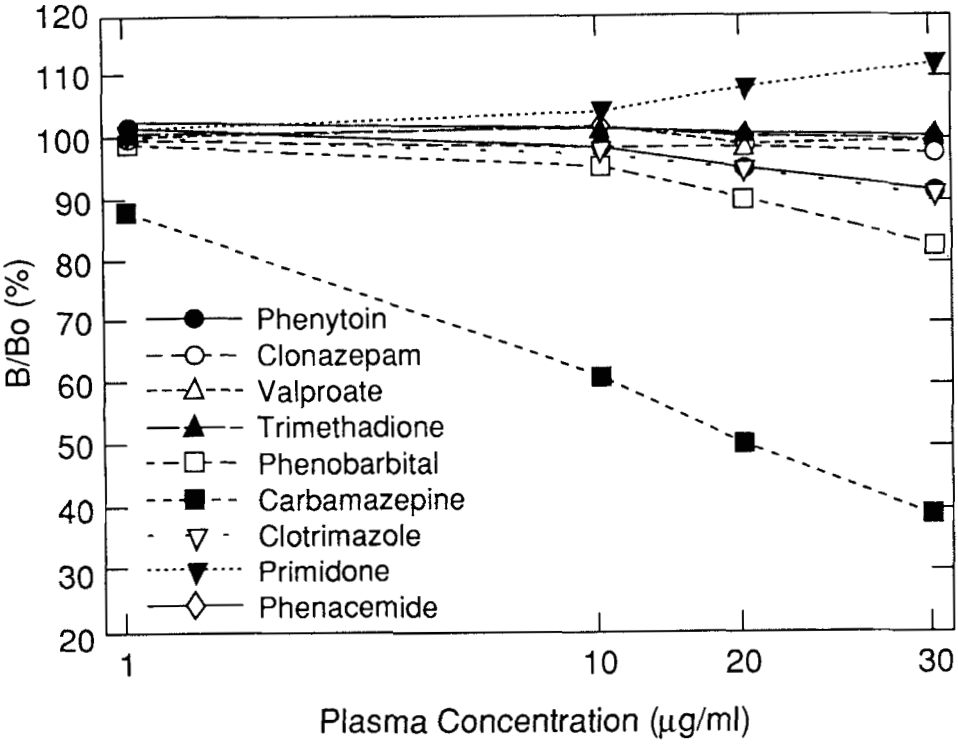


Figure 6 Reactivity of anti-epileptic drugs subjected to the total sample preparation procedure.

concentration. There is no concentration-dependent bias in recovery.

Buffer QC (N-acetyl-MK-801 in blank acetylation mixture) monitor the standard curve dilutions that are unique to each assay. Cumulative data are in Table 3. Comparison with Table 2 indicates that the sample preparation procedure per se introduced no detectable additional variability into the assay.

TABLE 2

Cumulative Statistics for Serum Recovery Samples

Conc (pg/ml)	Mean found	N	Intra-assay CV%	Inter-assay CV%
40	40.8	34	4.92	9.69
100	102	46	3.00	3.91
200	201	47	2.87	4.20
400	404	47	3.03	3.85
1000	997	47	2.49	3.92
2000	1922	44	3.16	6.24
4000	3970	44	4.36	4.74

In each assay, drug is measured in terms of a reference sample of N-acetyl-MK-801. A recovery factor is then calculated for each sample based on the nominal concentration (L.H. column). The mean value is applied as a correction factor for these samples and for unknowns. Thus, recovery samples serve as the primary standard even though calculations are made with reference to the N-acetyl-MK-801. Actual "found" values correspond to about 50% of nominal.

TABLE 3

Cumulative Statistics for Buffer QC

Conc (pg/ml)	Mean found	N	Intra-assay CV%	Inter-assay CV%
50	51.5	55	4.67	6.70
200	203	55	3.83	5.85
1000	1001	55	3.68	4.90

Accuracy

Urines from a ^{14}C study in man were assayed for parent drug by measuring radioactivity in appropriate HPLC fractions (3) as well as by the method described here. Concentrations were in the range of 0.7 to 40 ng/ml for seven samples from each of six subjects. The mean ratios of HPLC/RIA results were 1.02, 0.97,

TABLE 4

Recoveries

Clinical samples were assayed with and without addition of reference drug.

Serum	Intrinsic	plus 200 pg/ml MK-801	% of Expected
#1	169 pg/ml	387 pg/ml	104.9
#2	160	385	106.9
#3	201	422	105.2

Urine	Intrinsic	plus 4.64 ng/ml MK-801	% of Expected
#1	2.81 ng/ml	7.45 ng/ml	100.0
#2	4.67	9.42	101.2
#3	14.38	19.11	100.5

0.98, 0.98, 0.97, 0.95 for the six subjects. Concentrations of ^{14}C drug in plasma were insufficient for an analogous comparison.

Recovery and Parallelism

Data in Table 4 are representative only. Patient serum samples were assayed with and without a supplement of 200 pg/ml MK-801; for urine the supplement was 4.64 ng/ml. Recoveries were quantitative in both matrices.

The same serum samples displayed proportionality upon dilution (Figure 7A). Similar satisfactory data have been obtained for samples from several species (rat, gerbil, dog, rabbit, baboon), and for urine (Figure 7B).

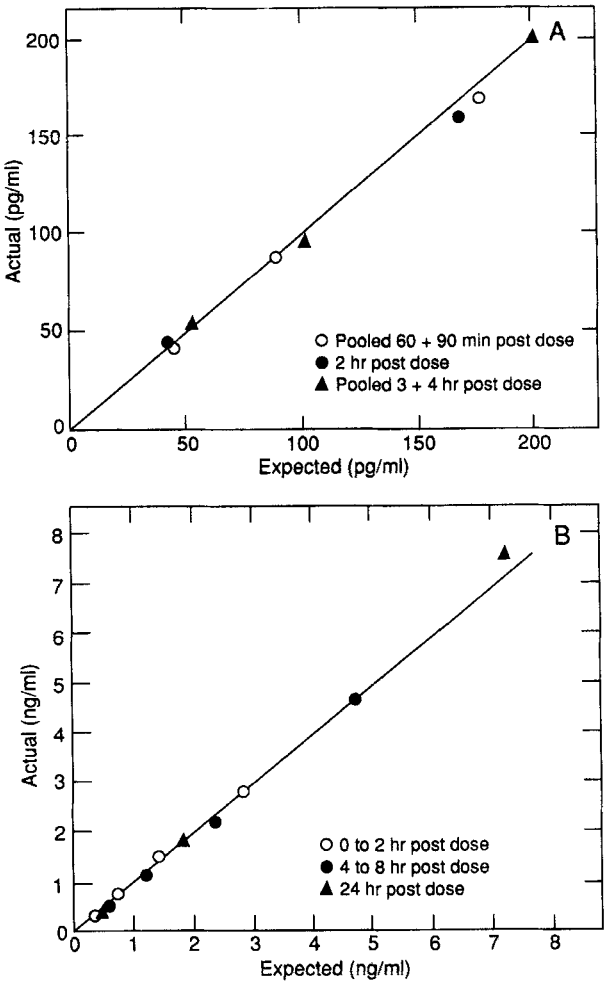


Figure 7 Proportionality to dilution ("parallelism") in (A) plasma (B) urine.

TABLE 5

Stability

Clinical samples were routinely stored at -20°C. The following were assayed on two occasions thirteen months apart to assess stability. Concentrations are in ng/ml.

		RIA #1	RIA #2
Plasma	A	0.282	0.262
	B	0.265	0.261
Urine	A	13.9	14.3
	B	25.7	24.8
	C	21.0	22.5

Stability

Clinical samples stored at -20°C were reassayed at intervals to assess stability. Table 5 shows results for plasma and urine samples assayed on two occasions thirteen months apart. There is clearly no stability problem.

DISCUSSION

Two immunogens were prepared. In one, the basic character of the nitrogen was retained with the expectation that antisera would not discriminate strongly between the tertiary and secondary amines. For the second immunogen, the expectation was that both the amine and the amide would be immunoreactive. Other possibilities for immunogen preparation are limited since the amine is hindered and there are no other functional groups.

Both antisera displayed a strong degree of bridge recognition, and the homologous radioligands were poorly displaced by the drug. However, two solutions were possible. Firstly, the N-carboxyethyl-MK-801 antisera bound the heterologous radioligand, albeit at a lower titer, and displacement occurred with MK-801. As anticipated, all specificity was lost for substituents on the nitrogen atom, but the N-hydroxy metabolite was eliminated by the sample preparation procedures as were the less reactive phenolic metabolites. Secondly, N-glutaryl-MK-801 antisera reacted almost equally well with several N-acylated analogs and the final form of the assay included acetylation of the extracted analyte. The combination of N-glutaryl-MK-801 antisera and the carboxyethyl radioligand was only slightly responsive to MK-801, insufficiently to produce a usable assay.

The extraction procedure was designed to eliminate both matrix effects and metabolite interference. Phenolic metabolites were not solvent-extractable at alkaline pH and the N-hydroxy metabolite was not back-extracted into pH 4 buffer. Weak intrinsic activity and low recovery combined to reduce metabolite interference to a negligible level. Interference by the 11-exo hydroxy metabolite was reduced to a very low potential by a combination of recovery and reactivity factors.

It was necessary to know the overall efficiency of extraction and acetylation in each assay, for which purpose a complete series of reference concentrations was included. The recovery

for each was measured against a standard of N-acetyl-MK-801 and mean recovery was used to correct observed values for unknowns. Use of an absolute reference rather than a calibration curve derived from extracted samples furnished a more certain calibration and permitted routine assurance that sample processing was reproducible. Although extraction recovery was virtually complete, acetylation efficiency was only about 50% and independent of analyte concentration. The inclusion of 0.1M acetate in the assay buffer was designed to eliminate the effects of any small variations in acetate concentrations between samples, and the reference standard, N-acetyl-MK-801, was prepared in a blank-acetylation mixture to maintain a constant matrix.

The agreement between HPLC analysis and RIA of urine samples served to validate the assay for this matrix. For plasma, no alternate method presently exists having the necessary sensitivity and the usual criteria of parallelism and recovery were applied to selected clinical samples to satisfy these minimum requirements.

The assay has been applied to many clinical samples. Patients with epilepsy are liable to receive several drugs concommitantly and many analyzed samples contained one or more such drugs at concentrations in excess of the analyte by several orders of magnitude. Some of these possessed very slight immunoreactivity and were extractable only in low yield but had the potential, due to very high concentration, to influence the

assay. In particular, phenytoin displayed slight but real inhibition of radioligand binding over a large concentration range with a very shallow slope, and therapeutic concentrations were potentially capable of mimicking low concentrations of MK-801. Addition of this drug to the assay buffer reduced B-zero slightly without significant effect on the standard curve but reduced sensitivity to additional phenytoin as well as shifting responsiveness to several other drugs toward higher concentrations. Potential interference by carbamazepine could only be positively avoided by reducing extraction recovery and extra alkaline washes of the heptane extract were specifically included when this drug was co-administered.

The effects of other drugs at concentrations several orders of magnitude greater than those of MK-801 are illustrative of the hazards of immunoassay when the matrix is not totally defined and constant. Specificity may be exquisite with respect to metabolites or endogenous materials but it is still only relative. Weak, but presumably specific, cross-reactivity in the case of ketamine can be attributed to recognizable structural similarity to MK-801. From a practical point of view, ketamine is very immunoreactive by virtue of relatively high plasma concentrations when used as a primate anesthetic, and samples obtained in this way could not be assayed for MK-801.

The amine function in MK-801 is known to be sterically hindered and derivatization with bulky substituents is difficult.

The heterogeneity suggested by NMR spectroscopy disappeared and reappeared on heating and cooling, behavior consistent with the existence of a pair of rotamers whose interconversion is inhibited by hindered rotation. It would be speculation to suggest that this relates to one curious feature of the assay, the reproducible approximately 50% recovery. This limit is associated solely with the acetylation step and is not consequent to incomplete extraction or losses by adsorption. The impact of acetate concentration upon the assay imposed a practical limit on the quantity of acetic anhydride that could be used but the excess is very considerable and there seemed to be no prospect of a substantial increase in yield.

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Address request for reprints to Dr. M. Hichens, Department of Drug Metabolism, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

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