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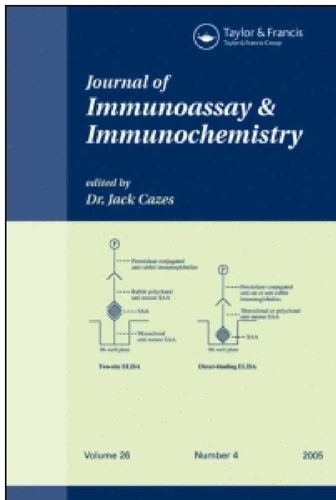

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Radioimmunoassay of Tricyclic Antidepressant and Some Phenothiazine Drugs in Forensic Toxicology

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RADIOIMMUNOASSAY OF TRICYCLIC ANTIDEPRESSANT AND SOME PHENOTHIAZINE DRUGS IN FORENSIC TOXICOLOGY

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ABSTRACT

A radioimmunoassay suitable for screening blood and urine samples for tricyclic antidepressant and some phenothiazine drugs in forensic toxicology is described. The assay displays a remarkably broad spectrum of cross-reactivity that enables therapeutic or sub-therapeutic levels of most tricyclic antidepressants and a number of phenothiazines in blood to be detected. The antiserum is commercially available and the radioligand, a radioiodinated conjugate of N-acetyl-L-histidine and nortriptyline, is easily prepared. Blood samples require prior extraction to reduce the background but urine may be assayed directly. 75 µl of blood or 100 µl of urine are required. Haemolysis or decomposition of the samples, common anticoagulants and sodium fluoride do not affect the results. Data for 57 compounds are presented.

INTRODUCTION

A number of radioimmunoassays for the analysis of tricyclic antidepressant drugs in body fluids have been described (1 - 19). A double-antibody enzyme immunoassay (20) and two radioreceptor assays (21, 22) have also been reported. The radioimmunoassays, with the exception of those described by Kamel et al (14), and Mason et al (19), use tritium-labelled tricyclic antidepressants as the radioligands. All

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these assays display cross-reactivity towards a number of tricyclic antidepressants, but most of them have been used to monitor levels of individual drugs, sometimes in conjunction with a selective extraction. In forensic toxicology however, unlike the clinical situation where specificity for a single drug is advantageous, an assay with a broad range of cross reactivity is preferable. The detection limits of such an assay and thus the levels of individual drugs excluded by a negative result can be established to a reasonable degree of certainty, and the usefulness of the assay can be evaluated by comparing the detection limits with the therapeutic ranges of the drugs where these are known. Mason et al (19) have recently described a broadly specific tricyclic antidepressant assay for forensic use, but the detection limits are such that overdose rather than therapeutic blood levels are likely to be detected. For the last five years in this laboratory, we have used a variant (23) of the assay developed by Aherne et al (3) to screen toxicological samples for therapeutic levels of a range of tricyclic antidepressants, but we have now devised an assay with a radioiodinated ligand that is simpler to use since it avoids liquid scintillation counting.

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MATERIALS AND METHODS

Buffer

0.067M phosphate of pH 7.4 containing 0.2% bovine γ-globulin (Cohn Fraction II, Sigma Chemical Company, Fancy Road, Poole, Dorset, U.K.) and 0.1% sodium azide.

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PEG Solution

23% w/v polyethylene glycol (PEG) of molecular weight 6000 in buffer containing no bovine γ-globulin.

Antiserum

Sheep anti-nortriptyline serum (2), Batch HP/S/1-VIIIA, was purchased from Guildhay Antisera, Department of Biochemistry, University of Surrey, Guildford, Surrey, U.K. It is used at an initial dilution of 1:80 in buffer which binds about 50% of the radioligand added to each assay tube.

Radioiodinated N-Acetyl-L-Histidine-Nortriptyline Conjugate

This is prepared as described by Kamel et al (14) by carbodiimide condensation of N-acetyl-L-histidine and nortriptyline followed by Chloramine-T iodination of the conjugate. One synthesis yields sufficient conjugate (approx. 5 mg) to last for many years. Radioiodination of the conjugate (2 μg) and isolation of the product takes about 15 minutes and is carried out 3 - 4 times per year. Instead of Sephadex column chromatography (14), we isolate the radioiodinated product using a silica Sep-Pak cartridge (Waters Associates, Chester Road, Hartford, Northwich, Cheshire, U.K.). The iodination mixture is transferred to a dry cartridge and unreacted ^{125}I is eluted with 3 ml water followed by 1 ml methanol. The product, which contains about 45% of the initial activity, is eluted with a further 1 ml methanol and stored in a silanised glass vial at $4^{o}C$. About 80% of the product can be

bound by excess antiserum and it is usable for at least two half-lives of the isotope. For use in an assay, the methanolic radioligand solution is diluted about 1:1500 in buffer to give 10^4 cpm per 50 µl aliquot.

Standards

Solutions of 0, 5, 10, 20, 30, 40 and 50 ng/ml amitriptyline in buffer are stored in silanised glass vials at 4^oC. Other standards may be used if required.

Sample Preparation

Blood (75 µl) is buffered to pH 9.2 and shaken for 10 min with 500 µl ethyl acetate. After centrifugation (1 min, 12000 g), 400 µl of the organic extract are evaporated to dryness in silanised glass tubes under compressed air at room temperature. The residue is dissolved in 10 µl methanol followed by 140 µl buffer to give a 1:2.5 dilution of the original sample. Further dilution may be necessary to ensure that the result lies on the dose-response curve. Urine is assayed with no pretreatment other than dilution with buffer if necessary.

Assay Protocol

50 µl each of standard or sample, radioligand and antiserum are added to duplicate sets of polypropylene microcentrifuge tubes which are then vortexed and incubated 1 h at room temperature. 500 µl of PEG solution are added, the tubes are vortexed, centrifuged (2 min, 12000 g) and the supernatants are aspirated. The precipitates are

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counted in a γ-counter. Two extra tubes containing only 50 μl radioligand are also counted to measure the total activity per tube. The results are plotted on any convenient coordinates.

RESULTS AND DISCUSSION

Our aim was to devise an assay with an ^{125}I-labelled radioligand and a broad spectrum of cross-reactivity towards tricyclic antidepressants. When we used the radioligand of Kamel et al (14) with the antiserum developed by Aherne et al (2), the resulting assay fulfilled our requirements and proved convenient and reliable. A positive result requires confirmation by an alternative method, a standard practice in forensic toxicology, while a negative result indicates the absence of all the drugs in Tables 1 and 2 at concentrations greater than the detection limits of the assay. Approximate therapeutic ranges, where known, of the various compounds in blood are also included in the tables. Obviously, significant levels of drugs that cross-react weakly in the assay may not be detected, and so a negative result may or may not merit further investigation depending on the circumstances of a particular case, the available background information and the volume of the sample.

Amitriptyline is a convenient standard to use in the assay if the presence of a particular drug in the samples is not suspected. Cross-reactivity data and detection limits for blood and urine are given in Tables 1 and 2. The detection limits were determined by assaying blank

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TABLE 1

Cross-Reactivities, Detection Limits and Therapeutic Ranges of Tricyclic Antidepressants and Related Compounds.

	Cross-Reactivity*	Detection Limit, ng/ml		Therapeutic Range in Blood, ng/ml (Ref.)	
		Blood	Urine		
Amitriptyline	43.5	41	28	35 - 202	(24)
				16 - 500	(25)
Butriptyline	55.6	31	22	60 - 280	(24)
Carbamazepine	0.1	6500	22000	3500 - 9400	(24)
Clomipramine	27.8	45	51	20 - 143	(24)
				16 - 282	(25)
Cyproheptadine	52.6	17.5	34		
Desipramine	100.0	15	12.5	11 - 110	(24)
				8 - 280	(25)
Dibenzepin	66.7	25	27	180	(24)
Dothiepin	47.6	36	25.5	17 - 64	(24)
				25 - 420	(25)
Doxepin	66.7	25	21	28 - 171	(24)
				5 - 115	(25)
Imipramine	71.4	23	17	9 - 126	(24)
Iprindole	18.9	52.5	84		
Maprotiline	40.0	20	42	26 - 196	(24)
				45 - 1558	(25)
Mianserin	1.2	500	1500	30 - 120	(24)
Nortriptyline	52.6	36	23	46 - 253	(24)
				10 - 275	(25)
Noxiptyline	52.6	19	29		
Opipramol	66.7	29	17.5		
Protriptyline	58.8	50	21	95 - 288	(24)
				10 - 376	(25)
Trimipramine	27.0	60	47	310, 600	(24)

* Weight of desipramine required to displace 50% of radioligand x 100/weight of compound required to displace 50% of radioligand.

blood extracts (n = 50) and blank urines (n = 33), averaging the responses (expressed as percentage binding of the total label) and adding three standard deviations. Detection limits for individual drugs were then extrapolated from dose-response curves. In the case of blood, the quoted detection limits are corrected to allow for the initial dilution factor of 2.5. The blank blood and urine samples were from healthy subjects who had not ingested drugs. The blood samples were all haemolysed and varied in condition from fresh to putrid. The urine

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TABLE 2

Cross Reactivities, Detection Limits and Therapeutic Ranges of Phenothiazines and Related Compounds.

	Cross-Reactivity*	Detection Limit, ng/ml		Therapeutic Range in Blood, ng/ml (Ref.)
		Blood	Urine	
Acetophenazine	3.9	212	405	
Acetylpromazine	2.4	375	910	
Butaperazine	1.0	925	1750	0 - 430 (24)
				20 - 3000 (25)
Carphenazine	2.1	375	800	
Chlorpromazine	10.5	150	135	100 - 2000 (24)
				20 - 300 (25)
Chlorprothixene	14.5	115	91	40 - 300 (24)
				4 - 100 (25)
Clopenthixol	13.1	50	135	
Diethazine	18.2	52	80	
Dimethothiazine	No cross-reaction			
Dimethoxanate	5.8	125	310	
Ethopropazine	7.5	100	270	
Fluphenazine	0.5	1875	3200	1 - 22 (25)
Flupenthixol	0.6	1125	2950	2 - 10 (26)
Isothipendyl	3.3	237	550	
Mesoridazine	0.2	4375	>10000	10 - 1050 (24)
Methdilazine	71.4	21	17	
Methotrimeprazine	14.5	50	135	50 - 140 (24)
				36 - 389 (27)
Methoxypromazine	17.5	50	100	
Metopimazine	No cross-reaction			
Pecazine	23.3	50	67	
Perazine	16.9	67	85	
Pericyazine	3.1	237	585	
Perphenazine	16.4	67	90	0.4 - 30 (24)
Pipazethate	0.2	1750	>10000	
Piperacetazine	8.3	80	235	
Pipamazine	23.8	311	54	
Prochlorperazine	6.2	62	360	1000 - 2000 (24)
				1000(toxic) (28)
Promazine	55.6	25	24	1000 - 2000 (24)
				1000 (toxic) (28)
Promethazine	15.9	37	115	3 - 23 (25)
Propiomazine	0.3	2750	6500	
Proquamezine	0.9	750	1750	
Prothipendyl	20.0	60	70	
Thiethylperazine	1.1	625	1650	
Thiopropazate	5.8	125	300	
Thioproperazine	No cross-reaction			
Thioridazine	1.8	625	850	50 - 5000 (24)
				140 - 2600 (25)
Thiothixene	No cross-reaction			
Trifluoperazine	1.1	375	1750	1000 - 2000 (24)
				1 - 30 (25)
Triflupromazine	0.6	1625	2500	
Trimeprazine	50.0	30	28	160 - 250 (24)

* See footnote, Table 1.

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samples were up to 18 months old. Heparin, EDTA, potassium oxalate or sodium fluoride present in some of the samples did not affect the results. Whole blood gave an unacceptably high background and so an extraction step is necessary. Urine can be assayed without pre-treatment.

The mean recovery of amitriptyline from blood spiked at 75 ng/ml was 83% and the intra-assay coefficient of variation was 9% (n = 12). The extraction efficiencies for chlorpromazine, chlorprothixene, doxepin and imipramine were found to be 103%, 80%, 69% and 98% respectively.

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Non-specific binding (approximately 5%) is low enough to be ignored.

Therapeutic or sub-therapeutic levels of 12 tricyclic antidepressants are detectable, as are low levels of the four tricyclic antidepressants whose therapeutic levels are not known (Table 1). Mianserin, which is a tetracyclic compound, cross-reacts poorly and cannot be detected in therapeutic amounts. The cross-reactivity pattern differs considerably from that obtained using ^{3}H-labelled radioligands with the same antiserum (2, 10), and so only speculative conclusions are possible concerning the determinants involved in the antigen-antibody reaction. The side-chain, as well as the ring system or part thereof, is certainly involved since iprindole and maprotiline,

which have the same side-chains as imipramine and protriptyline respectively but atypical ring systems, are both recognised by the antiserum. The anticonvulsant carbamazepine has the same ring system as opipramol and protriptyline but an atypical side-chain. It cross-reacts poorly, however, which suggests that the ring system alone is a weak determinant.

The degree of cross-reaction with phenothiazines and related compounds was unexpected since the phenothiazine ring systems differ from those of the tricyclic antidepressants. Fifteen of the 40 phenothiazines studied are detectable at blood levels of 100 ng/ml or less (Table 2). Few therapeutic levels of phenothiazines are given in the literature and so the assay cannot be evaluated as rigorously for phenothiazines as for tricyclic antidepressants. When screening samples, however, the possibility that a positive result may be due to a phenothiazine should not be overlooked. The side-chains of the phenothiazines undoubtedly influence antibody binding and it is possible that the ring system in toto rather than in part is involved since, in general, substitution at the 2-position of the phenothiazine nucleus markedly decreases cross-reactivity.

The cross-reactivities of tricyclic antidepressant and pheno thiazine metabolites have not been investigated, but it is likely that metabolites contribute to the assay response and thus lower the detection limits.

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The assay has been used routinely in forensic casework for 9 months and no problems have arisen.

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