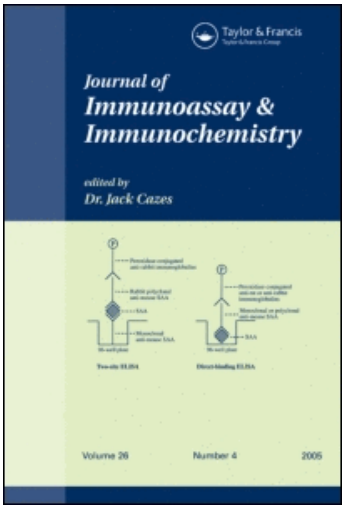

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WATER IMMISCIBLE SOLVENT BASED IMMUNOASSAY

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ABSTRACT

A competitive monoclonal antibody-based immunoassay which quantifies a hydrophobic hapten (R_X) in water immiscible solvents, obviating the need of a pre-extraction step, has been developed. Approximately linear dose response profiles of analyte, over the range 1-20 $ugml^{-1}$ in the hydrophobic solvents, hexane, toluene and xylene were obtained. UV spectrophotometric analyses of R_X dosed hexane confirm the phenomenon of antibody-mediated transfer of analyte from the organic to the aqueous milieu. Preliminary data on the effect of water immiscible solvents on the immunoreactivity of a monoclonal antibody in free solution are presented. The potential industrial applications of water immiscible solvent based immunoassays are discussed.

(**KEY WORDS**: Monoclonal antibody, organic solvent, hapten immunoassay.)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; PBS; phosphate-buffered saline; OD, optical density.

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INTRODUCTION

A novel and rapid immunochemical approach for the quantitative determination of analyte in water immiscible solvents has been developed. In this report we describe a competitive enzyme-linked immunoassay (ELISA) which generates a linear dose-response with a hydrophobic hapten in the micromolar concentration range. Essentially, a limiting concentration of a monoclonal antibody in aqueous buffer is mixed carefully for a few minutes, with an analyte-containing water immiscible solvent, for example, hexane, toluene and xylene. After phase separation, aliquots of the lower aqueous layer are incubated in the wells of a microtitre plate, coated with a derivatised form of the analyte, enabling quantification of analyte in a standard ELISA format (1).

The concept of a physiologically derived molecule, ie., an antibody retaining function in organic solvents is both unexpected and unpredictable. It has been shown, however, that immobilised anti-hapten antibodies will bind hapten, present in organic solvents, with high affinity and specificity (2). It has also been demonstrated that anti-peptide antibodies coupled to paramagnetic particles retain immunoreactivity (3). Matsuura and workers have observed that antibodies in free solution exhibit the capacity to bind a hydrophobic shell fish toxin in 90-100% methanol (4). However, these researchers did not exploit this observation to develop a quantitative immunoassay

Here, we report, for the first time, a quantitative immunoassay for a hydrophobic hapten in the water immiscible solvents, hexane, toluene and xylene. The performance and potential industrial applications of this approach are discussed.

MATERIALS AND METHODS

Analyte selection

The hapten used in this study as a model analyte, (R_X) is a hydrophobic derivative of 2,4 dinitrophenol (octanol : water partition coefficient, (estimated) = log K_{OW} 6.14), having a MW of approximately 350 daltons. This hapten was selected on the basis of its property of remaining with the organic phase and not partitioning into aqueous buffer.

Monoclonal antibody culture and purification.

A monoclonal antibody, 1E8, directed towards R_X, was generated using conventional hybridoma technology (5, 6). 1E8 was cultured in RPMI1640 glutamax medium supplemented with foetal calf serum (5%) (GibcoBRL, Life Technologies Ltd., PO Box 35, Trident House, Renfrew Road, Paisley). Antibody purification was carried out by affinity chromatography (7, 8) using protein-G sepharose (Pharmacia Biotech Ltd., 23 Grosvenor Road, St. Albans, Herts.) according to the manufacturer's instructions. Briefly, cell supernatant (500ml) was applied to a protein-G sepharose column (bed volume, 6ml) equilibrated with binding buffer (20mM sodium phosphate, pH 7). The column was washed with 5 bed volumes of binding buffer before elution of 1E8 in 0.1M glycine buffer, pH3. After neutralisation, peak fractions (OD_{280}) were pooled and dialysed extensively against phosphate buffered saline (0.2mM, pH7.4). Affinity purified 1E8 (1mgml^{-1} protein) containing sodium azide (0.1%) was stored at 4^{o}C until use.

Antibody Specificity

The antibody, 1E8, used in this study was selected on the basis of specificity for the 2,4 dinitrophenyl group. Analogues of the hapten, R_x , in which

TABLE 1

Reactivity of 1E8 with structurally related analogues of R_x

Hapten	I_{50}
R_x	2 uM
Dinitrophenol	> 1 mM
4-(4-nitrophenyl)butyric acid	24 uM
2,4 dinitrophenylmethylester	40 uM
2,4 dinitrophenylglycine	4 nM

I_{50} defines the molar dose of analogue required to displace the binding of 1E8 by 50% in an ELISA.

the substituent at C-1 of the aromatic ring was varied and the linkage to C-1 was via an oxygen atom, or a nitrogen atom, or a methylene carbon atom, were tested for binding affinity with 1E8 in a typical ELISA. The assay was carried out essentially as described in the solvent based immunoassay protocol (see below), but in which the solvent extraction step was omitted (Table 1).

Solvent based immunoassay protocol.

Concentrates of R_X ($2mgml^{-1}$, w/v) were prepared carefully in each of the solvents hexane, toluene and xylene (GPR grade, BDH, Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leics.). Concentrates were diluted in the appropriate solvent to give 20, 10, 5 and 1 $ugml^{-1}$ solutions for assay.

R_X dosed solvent (4ml) and R_X free solvent were dispensed into screw cap glass bottles (capacity, 8ml) to which were added solutions of 1E8 (1ml at 1/100, 1/200, 1/500 and 1/1000 dilutions) in 0.2mM phosphate buffered saline(PBS), pH7.4. The glass bottles were carefully mixed by inversion for 2 min, either manually or, on an end to end rotary mixer. After phase separation (approximately 10-20 seconds), volumes (100ul) of the lower aqueous phase were carefully removed and dispensed into the wells of a microtitre plate coated with an R_X - human serum albumin (HSA) conjugate. This step was carried out ensuring minimal carry over of solvent. After incubation for 1 hour and washing with PBS solution containing Tween-20 (0.05%), second antibody solution (rabbit anti -mouse horseradish peroxidase conjugate , 1/1000 in PBS, Dakopatts, Dako Ltd., 16 Manor Courtyard, Hughenden Avenue, High Wycombe Bucks.) was added to each well (100ul) and the plate incubated for a further hour. Finally, after washing, substrate solution (3,3',5,5'-tetramethylbenzidine, Sigma chemical Co.Ltd., Fancy Road, Poole, Dorset) was added (100ul /well) and colour development monitored at 650 nm on an ELISA plate reader (V_{max} , Molecular Devices, Unit 6, Raleigh Court, Rutherford Way, Crawley, West Sussex).

The effect on immunoreactivity of exposing 1E8 to water immiscible solvent was examined by mixing a solution of the antibody with hexane (1:4, v/v), on a rotary mixer over a time course (0-20 min). Aliquots of the aqueous phase were removed and monitored for binding to R_X -HSA as described above.

Analyte UV absorbance profiles.

In order to confirm that antibody-analyte binding occurred during the mixing

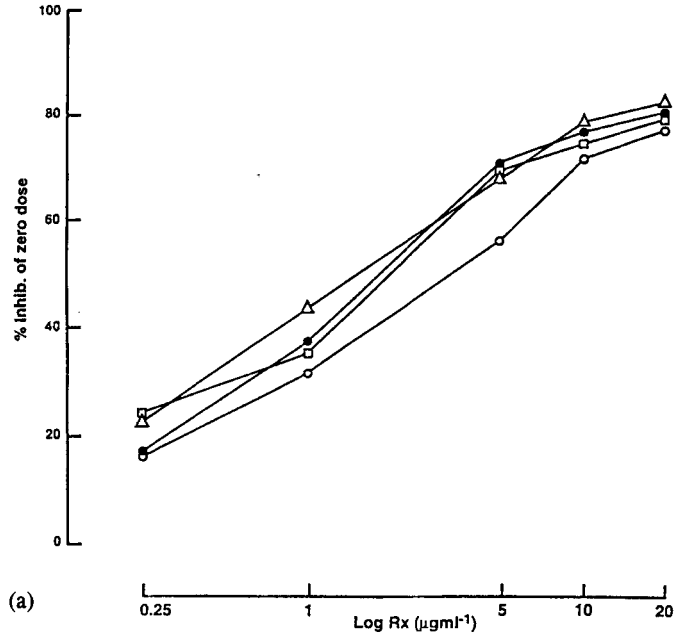
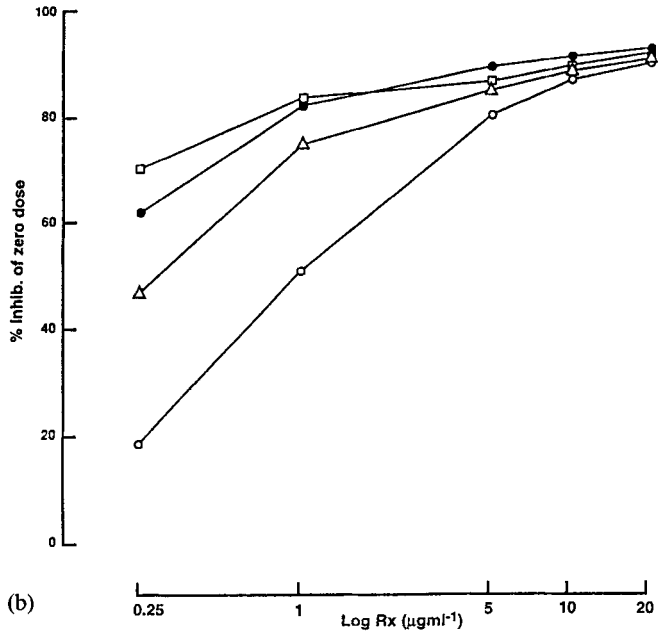


FIGURE1. Dose response of R_X in organic solvents at various antibody dilutions. (a) Hexane, (b) Toluene and (c) Xylene. R_X dosed solvents were mixed for 2 min with diluted aqueous 1E8 (o--o, 1/100; ∇--∇, 1/250; ●--●, 1/500 and □--□, 1/1000) at a ratio of 1:4 (1E8:solvent, v/v). Aliquots of the aqueous layer were assayed as described in Materials and Methods. Percent inhibition of zero dose is plotted as a function of the logarithm of dose concentration. Data points represent the means of triplicate estimations.

stage, a UV absorbance profile of Rx dosed hexane (20ugml^{-1}) was obtained using a diode array spectrophotometer (8452A, Hewlett Packard Ltd., Eskdale Road, Winnersh, Wokingham, Berks.). The solvent was then mixed for 15 min, as described above, with a relatively high concentration of 1E8 (approximately 1mgml^{-1} protein). After mixing, a UV absorbance scan of the organic phase was repeated. Control experiments were carried out using antibody diluent and mouse immunoglobulin (IgG,1mgml^{-1}, Sigma).



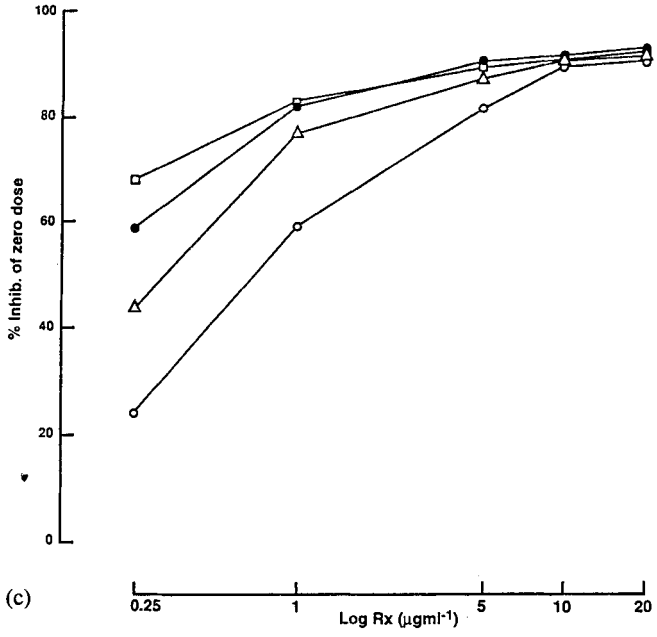


FIGURE 1. Continued

TABLE 2

Effect of water immiscible solvent on 1E8 immunoreactivity.

Time (min)	0	1	2	5	10	20
Reduction of 1E8 R_X -HSA Binding (%)	-	7	4	4	5	6

A limiting dilution of 1E8 was mixed for up to 20 min with hexane (1:4, 1E8:hexane, v/v). Antibody immunoreactivity was monitored using R_X coated microtitre plates, as described in Materials and Methods, and is expressed as the percentage reduction in total 1E8 binding compared with unmixed antibody.

RESULTS

R_X dose-response data.

Dose-response data of 1E8 and R_X (0-20ugml^{-1}) in hexane, toluene and xylene are presented in Fig.1. A quantitative estimation of R_X , over the dose concentration range tested, was obtained using each solvent and at each dilution of 1E8. Closely parallel responses were observed at various antibody dilutions where hexane comprised the vehicle for analyte dose. Assay performance appeared to be more sensitive to 1E8 dilution in the presence of toluene and xylene.

Solvent effect on immunoreactivity.

Exposing 1E8 to hexane, by rotary mixing over the course of 20 min, had little effect on immunoreactivity as determined by a measure of the capacity of 1E8 to bind R_X -HSA adsorbed to the wells of a microtitre plate (Table 2). A

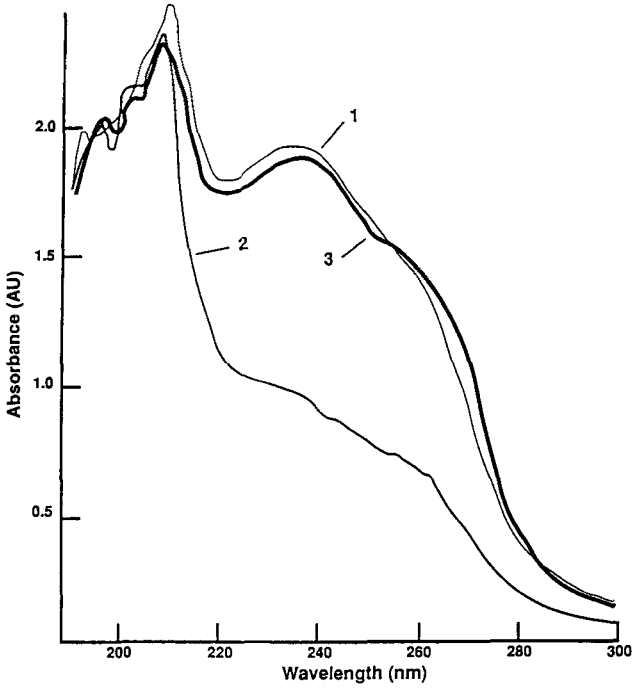


FIGURE 2. UV Spectrophotometric analyses of R_X dosed hexane. R_X hexane (20 $ugml^{-1}$) was analysed using a diode array spectrophotometer before (1) and after mixing with an aqueous solution of 1E8 (2) and antibody diluent containing murine IgG (3) as described in Materials and Methods.

maximum loss of 7% binding was observed compared with the binding of the unmixed control.

R_X UV absorbance profiles.

The UV absorbance profile of R_X hexane (20$ugml^{-1}$) over the wavelength range 200-300 nm is shown in Fig.2.1. Peak absorbance values were recorded at 236 nm. After mixing with 1E8 for 15 min, a reduction of approximately 1.0 absorbance unit in the organic phase was obtained (Fig.2.2). No UV

absorbance differences were observed after mixing R_X hexane with antibody diluent and mouse IgG aqueous control solutions (Fig.2.3).

DISCUSSION

The results of this study demonstrate that a monoclonal antibody may be used effectively to quantify analyte in water immiscible solvents obviating the requirement of a pre-extraction step.

The assay procedure described here requires a total time of about 2.5 hours. However, modifications of this format using, for example, analyte coated tubes and labelled 1E8 permit a reduction in assay time to less than 30 min per sample. A ratio of 4:1 (solvent:aqueous antibody, v/v) was found to be optimal (data not shown). Because of the large molar excess of analyte relative to antibody in this assay system, care is required to avoid the carry over of solvent when transferring aliquots of the aqueous phase to the coated microtitre plate. Moreover, variable traces of solvent in this stage of the assay may lead to imprecision. Prior removal of the organic phase to waste, using, for example, a pipette, may facilitate the transfer step.

1E8 was not purified to homogeneity thus rendering an elucidation of the kinetics of antibody binding in solvent difficult in this case. However, on the basis of OD_{280} readings, a limiting concentration of antibody was utilised. Our findings suggest that the optimisation of a water immiscible solvent based immunoassay must take into account the phenomenon of antibody mediated transfer of analyte from the organic to the aqueous milieu and the conditions necessary for competitive inhibition.

The effect of mixing 1E8 with hexane for up to 20 min indicates that a monoclonal antibody has a relatively high tolerance for this solvent (Table 2).

This experiment was not carried out in the presence of toluene and xylene. However, it may be conjectured that subtle differences in antibody toleration of solvent may account for the greater sensitivity to antibody dilution observed in the dose-response data illustrated in Fig.1. Kilbanov (1986) has documented the observation that enzymes work in organic solvents and has postulated that water molecules bound tightly to an enzyme are not removed in the presence of hydrophobic organic solvents (9). It has been demonstrated that the enzyme horseradish peroxidase has vigorous catalytic activity in several water immiscible solvents (10). By analogy, it is presumed that the hydrophobicity of the solvents investigated in this study will not displace the film of water which surrounds the molecules of 1E8 which, therefore, retain immunoreactivity. The UV absorbance profiles of R_X containing hexane, before and after mixing with 1E8, confirm that antibody mediated transfer of hydrophobic hapten from the organic to the aqueous phase occurs (Fig. 2).

The measurement of hydrophobic compounds invariably is complicated by extraction and purification steps which often are time consuming and protracted. This study highlights the potential of an immunoanalytical approach to facilitate the monitoring of hydrophobic analytes in areas of industry hitherto unexplored. The application of water immiscible solvent based immunoassay for monitoring hydrophobic analytes in, for example, petrochemicals, agrochemicals, oils , fats, environmental pollutants and organic waste etc., opens up exciting prospects and new challenges.

REFERENCES

1. Voller, A., Bidwell, D.E. and Bartlett, A.. The Enzyme Linked Immunosorbent Assay (ELISA), Dynatech Europe, 1979.

2. Russell, A.J., Trudel, L.J., Skipper, P.L. Groopman, J.D., Tannenbaum, S.R. and Kilbanov, A.M. Antibody-antigen binding in organic solvents. Biochem. & Biophys. Res.Com.1989; **158**: 80-5.

3. Weetall, H.H. Antibodies in water immiscible solvents. J Immunol Methods 1991;**136**:139-42.

4. Matsuura, S., Hamono, Y., Kita, H. and Takagaki, Y. Preparation of mouse monoclonal antibodies to okadoic acid and their binding activity in organic solvents. J Biochem. 1993; **114**: 273-8.

5. Galfre, G. and Milstein, C. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol 1981; **73**: 3-46.

6. Goding, J.W. In: Monoclonal Antibodies: Principles and Practice. 2nd. edition, London: Academic press. 1986: 59-93.

7. Akerstrom, B., Brodin, T., Reis, K. and Bjork, L. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. Immunol. 1985; **135**: 2589-92.

8. Wilchek, M., Miron,T. and Kohn, J. Affinity chromatography. Methods in Enzymol. 1984; **104**: 3-56.

9. Kilbanov, A.M. Enzymes that work in organic solvents. Chemtech 1986; **16**: 354-9.

10. Kazandjian, R.Z., Dordick, J.S. and Klibanov, A.M. Enzymatic analyses in organic solvents. Biotechnol Bioengin.1986; **28**: 417-21.