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Propofol-imprinted membranes with potential applications in biosensors

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Abstract

Herein we describe a new thin film imprinted polymer for propofol (active ingredient of a commonly used general intravenous anaesthetic) developed on a membrane support. Propofol binds to this polymer in 2 min and can be removed and tested in 1 min, giving a total assay time of 3 min. The non-specific binding to the polymer is below the detection limit (0.1 μg/ml) and the response rate is below the rate of metabolism of the anaesthetic. Tests performed with this polymer against propofol-spiked blood samples showed good linearity and specificity at clinically relevant concentrations of 1–10 μg/ml and the working range for the system is 0.1–50 μg/ml. The polymer is easily regenerated and can be re-used for subsequent testing (in blood up to 5000 cycles). These results suggest suitability for use in an on-line propofol detection system.

Keywords: Propofol; Biosensor; Molecular imprinting; Anaesthetic

1. Introduction

Propofol is a short-acting intravenous (i.v.) anaesthetic with a sleep induction period of 30–50 s, one bolus injection giving sedation for 4–6 min [1]. The rapid metabolisation of propofol in the body makes it necessary to have a continuous infusion. However, metabolic rates vary between individuals, making continuous monitoring of propofol desirable. The most suitable monitoring system for an anaesthetic would be an on-line biosensor using an antibody- or a molecularly imprinted polymer (MIP)-based detection system.

There are cases when antibodies for a substance cannot be developed or when the antigen binding is poor. Such was the case for 2,6-diisopropylphenol (propofol). Propofol is a small molecule in which the phenolic hydroxyl is sterically shielded by the adjacent isopropyl groups. Attempts at making propofol antibodies [2] have led to unreliable results, giving little selectivity and recognition in the desired clinical concentration range (1–10 μg/ml).

As the antibody approach was not met with success, a propofol-imprinted polymer was synthesised and characterised [3]. Molecular imprinting is a technique used to synthesize polymers (MIPs) capable of recognising a given target molecule (template). The presence of a molecular template in the polymerisation mixture induces the formation of binding sites. After template removal these binding sites have cavities with shape and functional group recognition complementary to the target compound.

The general properties of MIPs suggest they can be successfully used as antibody analogues [4]. MIPs have several advantages over classical antibodies. For example they can be tailor-made for specific toxic substances, the imprint molecule can be recovered after polymerisation, and they are resistant to pH changes and organic solvents.

The initially synthesised MIP re-bound propofol over approximately 15 min and the supernatant required subsequent testing by HPLC. As part of a biosensor-type system, the response time has to be below the metabolisation rate. In previous work the propofol MIP demonstrated a response time considered too slow, and the free polymer particles floating in the test solution required separation prior to HPLC determination of propofol.

Based on previous results [3], the response time of the polymer was found to decrease with increased surface

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area and decreased particle size, as a reduced number of non-specific binding sites improved the selectivity. The short binding time can also be achieved by allowing easy access to the active sites of the polymer. All these characteristics can be replicated when the polymer is synthesised on a support, such as a thin film polymer. The support provides controllable surface and shape, and also eliminates the particle removal step necessary with powdered polymer.

Both membrane filters and glass (microscope slides) were tested as supports in conjunction with this type of polymer. Previous reports of polymers successfully being synthesised on membranes [5,6] and on glass supports [7] for a wide range of applications, offer a basis for preparation of thin polymeric layers with large surface areas.

Our aim therefore was to create a system of MIP-based measurement of propofol featuring the above design advantages.

2. Experimental

2.1. Materials

4-Acetoxystytrene, 2,6-dicholorimidequinone hydrochloride (Gibbs reagent), 4-nitrophenol, 2,4-dinitrophenol, 2,6-diisopropylphenol, diisopropylethylamine, 2,6-di(tertbutyl)-4-methylphenol, ethyleneglycoldimethacrylate (EDMA), 1,1′-azo-bis(cyclohexanecarbonitrile) (ABCHC), and silica gel (Davisil, grade 645, 60–100 mesh, 99+%) were obtained from Sigma-Aldrich. Toluene, methanol, hexane, all of them AnalaR, were obtained from BDH.

ABCHC was recrystallised from methanol before use. EDMA was washed with sodium hydroxide/sodium chloride and dried over $MgSO_4$ and silica before use to remove the inhibitor. The Teflon membranes, Fluoropore laminated and non-laminated (Fluoropore flat sheet filter membranes, PTFE, 0.45 μm cut-off, 47 mm diameter, Fluoropore filter type FH), were obtained from Millipore, USA, the cellulose membrane (cellulose ester dialysis tubing, MCO 6000-8000, 23 mm flat diameter), from Spectrapor, USA, and the Nylon membrane (Nylon flat sheet filter membranes, 0.45 μm cut-off, 47 mm diameter), from Phenomenex, New Zealand. Untreated microscope slides were used as glass supports (25 mm × 75 mm).

3. Methods

3.1. Polymer preparation

3.1.1. Monomer synthesis

The monomer was prepared as described previously [3]. After silica column purification, the carbonate (>99% purity) was obtained as a pale yellow oil. Purity was checked by NMR (Bruker, 400 MHz). The monomer was stored at 4 °C under nitrogen.

3.1.2. Polymer synthesis

Covalent polymers were prepared generically according to the procedure of Whitcombe et al. [8]. The template was propofol-(4-vinylphenyl) carbonate. We used EDMA as a cross-linker, in different ratios to the monomer (discussed below).

The untreated support was placed on a glass sheet and a known amount of polymerisation mixture was spread evenly on the support. The polymerisation took 24 h to complete at 70 ± 2 °C, in a glassware oven. Based on observations when making the polymer as small particles, we investigated different solvents as porogens (both polar and non-polar). Toluene (0.5 ml/g monomers) was used for all experiments reported in this paper.

The hydrolysis of the template involved soaking the membranes or the glass slides in 1 M NaOH in methanol for 1 h, then rinsing with dilute HCl and water. Cleanup was performed by stirring in methanol for 24 h, followed by air-drying.

For each imprinted polymer the corresponding non-imprinted polymer was made. The polymerisation mixture had the same composition as the respective imprinted one, but without the template.

3.1.3. Batch binding

The batch binding experiments were performed by dipping the polymer on support into 2 ml propofol solution (0.2 mm in methanol) and allowing 2 min contact time. The solution was subsequently analysed by HPLC (Shimadzu system, $\lambda = 220$ nm, column Prodigy 3 μm ODS (3) 100 Å, 100 mm × 2.00 mm, 70% acetonitrile–water, oven 35 °C, RT = 3.5 min) to determine the propofol concentration. The membranes and glass slides were rinsed with 2 ml methanol/water 1:1 (v/v) mixture to avoid carryover. The propofol bound on the membrane was released in a methanol/bicarbonate buffer, pH 9.6, 1:1 (v/v) mixture. This solution was allowed to come in contact with the polymer for 1 min with strong stirring. The released propofol concentration was assessed by a colorimetric test (see below) and also by HPLC. When assessing the latter solution by HPLC, the pH of the sample was first lowered to 7 by treatment with dilute HCl.

After propofol release, the membranes were stirred strongly in water for 5 min to avoid carryover from the last step. For the Teflon membranes, this step was also used for drying the membrane and preparing it for the next testing cycle.

For all experiments discussed below, a minimum of two replicates were performed and the mean value presented.

3.1.4. Binding from blood solutions

The blood samples were prepared by spiking blood with known amounts of Diprivan to reach concentrations of clinical relevance. Fifteen minutes were allowed between spiking with Diprivan and testing the blood to allow propofol to bind to the blood components and thus mimic the real-life

situation. A blood aliquot thus prepared was mixed with 4 parts methanol. After 30 s (for settling of precipitated proteins), 2 ml of the supernatant were removed and used as the test solution. This was then subjected to batch binding experiments.

3.1.5. Colorimetric test

The reaction was developed based on the experiment described by Gibbs [9] with specific modifications that enabled us to differentiate between sub-microgram concentrations. One part Gibbs reagent 0.45 mm solution in methanol was added to 2 parts propofol extract from the membrane (in methanol/bicarbonate buffer). Upon mixing, the colour develops within seconds and can be read at 594 nm (Spectrophotometer UV-1601, UV-Vis, Shimadzu). The blue colour of the complex intensifies due to propofol concentration, while at low concentrations the yellow colour of Gibbs prevails.

4. Results and discussion

The covalent imprinting technique offers fewer degrees of freedom for the template in the polymerisation mixture, thus giving better specificity and selectivity for the target molecule in the propofol-imprinted polymer. The re-binding of the template involves a chemical reaction in the active site. We aimed to develop a fast rebinding test for the propofol. The classical covalent approach was inappropriate as the recognition step would have been too slow. On the other hand, non-covalent imprinting offered more non-specific binding due to the physical, rather than chemical, constraints applied to the template during polymerisation, but with faster recognition time. Consequently, a strategy involving covalent imprinting with a sacrificial spacer approach was used [8]. In this approach, a polymerisable template is used for imprinting, but the recognition and re-binding are carried out in a non-covalent manner.

Propofol ‘sticks’ to most materials [10], consequently it is readily adsorbed by acrylic polymers after moderate or long contact times. The dynamic binding to the imprinted sites is however much faster than the non-specific adsorption (occurring at equilibrium binding), as has been established in our previous study on MIP particles [3]. The problem of non-specific adsorption is largely circumvented by using short contact times, in a dynamic binding protocol. The optimum binding time in the polymer thin films was found to be 2 min, at which time very little non-specific adsorption has occurred and the binding in the imprinted sites is almost complete.

4.1. Choosing the porogen

The polymerisation mixtures (containing 1:19 monomer: EDMA and 5%, w/w ABCHC) needed to be made in porogens capable of wetting most of the substrates and solubilising the template. The solvents displaying these characteristics were found to be methanol, hexane, and toluene. These solvents were used as porogens. In addition to pure solvents, hexane/toluene, 9:1, v/v, previously used in bulk polymerisation studies [3], was also investigated as a porogen.

Methanol and hexane are not viable porogens in the preferred polymerisation system. They both have low boiling points and are likely to evaporate before the polymerisation occurs, yielding non-porous polymers. The hexane–toluene mix gave high specific (in the MIP) and non-specific (in the non-imprinted polymer) binding: 51±2 and 44±2%, respectively. The polymer has to have non-specific binding below the detection limit in order to be useful in a biosensor-type system. Toluene was chosen as polymerisation solvent as it decreased the non-specific binding dramatically. All subsequent tests were carried out using toluene as the porogen.

4.2. Choosing the support

Glass slides and membranes of varying composition were tested as supports. Membranes were employed only as supports for the polymer, and not as filtering units. High cross-linking, and resultant high surface area, made them suitable candidates for these experiments. The thickness of the membrane was not investigated as a limiting factor, as the membranes were immersed in solution and no care was taken in keeping the surfaces flat, but only in keeping all of the polymer-coated support in contact with the test solution. The binding characteristics of the resulting polymers are listed in Table 1.

Propofol is a highly hydrophobic molecule, so it migrates only slowly towards a hydrophilic substrate. Our results (see Table 1) suggest that hydrophilic surfaces are not suitable for this experiment. On the other hand, propofol tends to bind to most of the materials it comes in contact with. The only two substrates reported so far that do not have this problem are glass and Teflon. This may explain the results, especially the high non-specific binding observed with cellulose and with the Teflon laminated (polyethylene backing) membrane (see Table 1).

When subjected to mechanical pressure, the thin film on the glass surface started to flake off, whereas the polymer on the Teflon membrane was not affected. Scanning electron microscopy (SEM) (Fig. 1) showed that the polymer partially coated the Teflon membrane fibres. A stable thin film of polymer was thus achieved by using Teflon as the polymer support. The binding is easily quantified by having a set amount of polymerisation mixture on each support (see below). As observed in Fig. 1, the film does not uniformly cover the Teflon membrane. Teflon is highly hydrophobic and the non-laminated PTFE membranes (100% Teflon) repel water. When the partially coated membrane is immersed in water after a test, the hydrophobic–hydrophilic interactions

Table 1
Effect of substrate and monomer: cross-linker ratio on specific and non-specific binding

Support	Monomer:EDMA ratio	Specific binding (%)[a]	Non-specific binding (%)[b]
Nylon (polyamide)	1:19	5	7
Cellulose ester	1:19	35	35
Glass, untreated	1:1	19	0
Laminated PTFE (polyethylene backing)	1:1	35	16
Non-laminated PTFE	1:19	43	37
Non-laminated PTFE	1:10	32	26
Non-laminated PTFE	1:1	24	0

Binding was calculated as the percentage bound out of a 0.2 mm propofol solution in methanol over 2 min and the results were assessed by HPLC.
[a] Binding to the imprinted polymer.
[b] Binding to the blank.

are so strong that the methanolic solution trapped in the membrane is repelled towards the water and the membrane dries out. After propofol removal, the membrane thus dried is ready for subsequent testing and carryover of propofol is avoided.

As a consequence of these results, the support of choice was the PTFE non-laminated membrane. All subsequent experiments involved polymers made on this support.

4.3. Cross-linking degree

The polymer on the membrane has different characteristics than the polymer developed in monolithic form. The composition of the polymerisation mixture was further modified to find the minimum amount of EDMA required to achieve molecular imprinting. Three different monomer:EDMA ratios were tested, 1:1, 1:10, and 1:19,

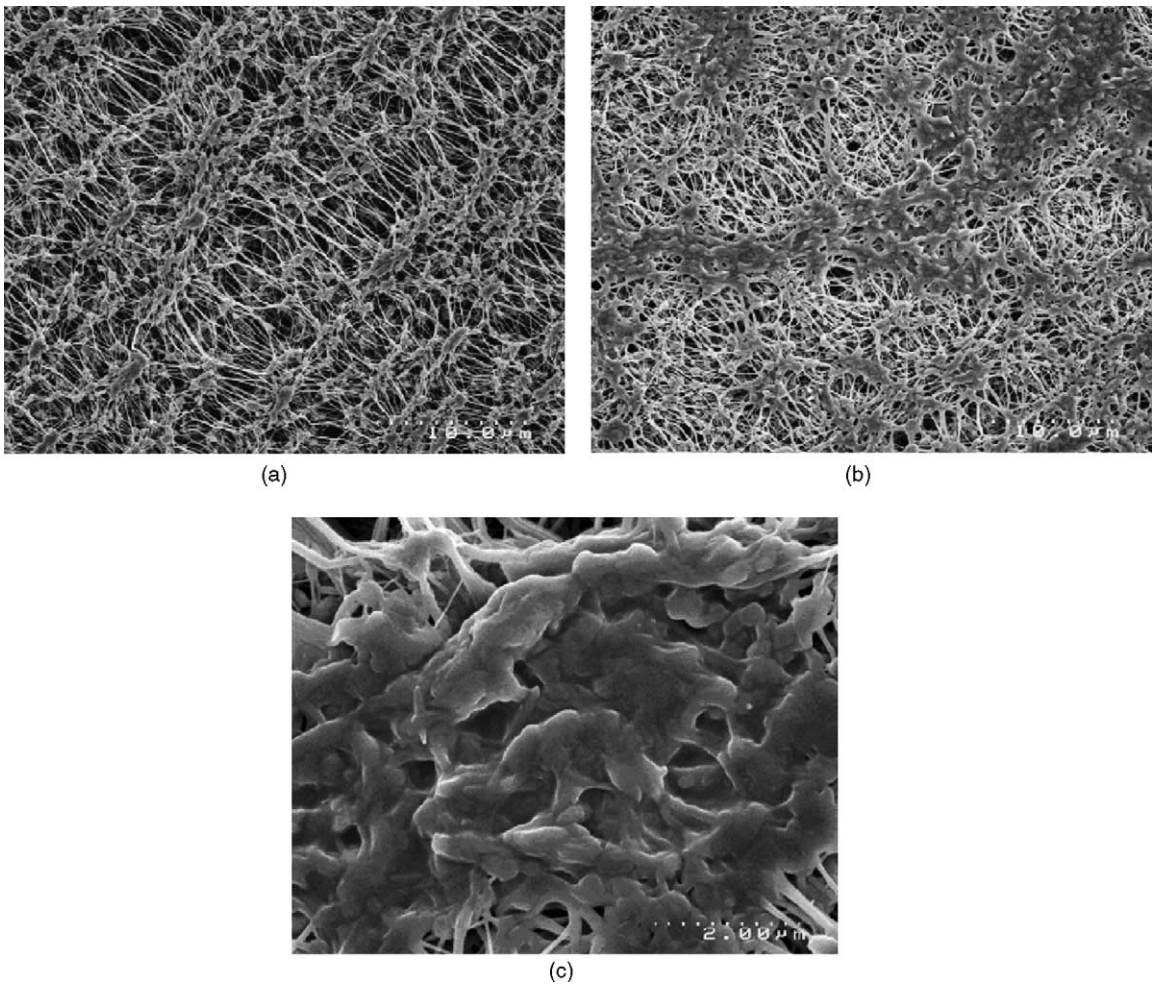

Fig. 1. SEM of polymer on PTFE membranes: (a) commercial membrane, (b) polymer on membrane, and (c) close-up on polymer.

as shown in Table 1. Using a low amount of cross-linker decreased both the specific and the non-specific binding. Based on the results obtained for this lot of experiments, all subsequent experiments were performed with a 1:1 monomer:cross-linker ratio in the polymerisation mixture.

4.4. Membrane loading

Different amounts of polymer mixture were loaded onto the supports in order to create a film of desired thickness, to generate enough coating on the membrane so as to have recognition of the template in the clinical concentration range and maintain the hydrophobicity of the support.

Three different loadings were tested: 0.1, 0.2 and 0.4 ml polymerisation mixture on each membrane support. The latter proved to be too much as it overloaded the membrane, and covered it completely. On the contrary, 0.1 ml did not produce enough polymer to provide linearity in the desired concentration range. As a consequence for this particular application 0.2 ml polymerisation mixture/membrane was chosen and this amount of polymer was used for all subsequent tests. The propofol-MIP on support obtained proved to give reliable replicates upon synthesis and testing of different batches. The S.D. for 60 replicates upon testing against a 2 μg/ml solution was ±0.1 μg/ml.

4.5. Linearity tests

Less than 1% of the total propofol is found in the blood as free molecules, the rest being strongly bound to proteins [11]. This means that the polymer would have to compete for propofol alongside proteins and this can result in high response variation due to minor blood composition differences between individuals.

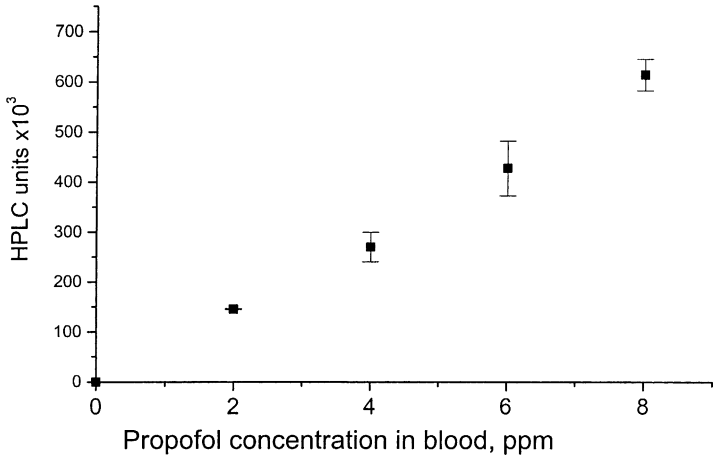

Fig. 2. Propofol recovery from blood by protein precipitation; all samples were done in triplicate and the supernatant was assessed by HPLC analysis (blood mixed with methanol 1:1, v/v) ($r^2 = 0.9891$).

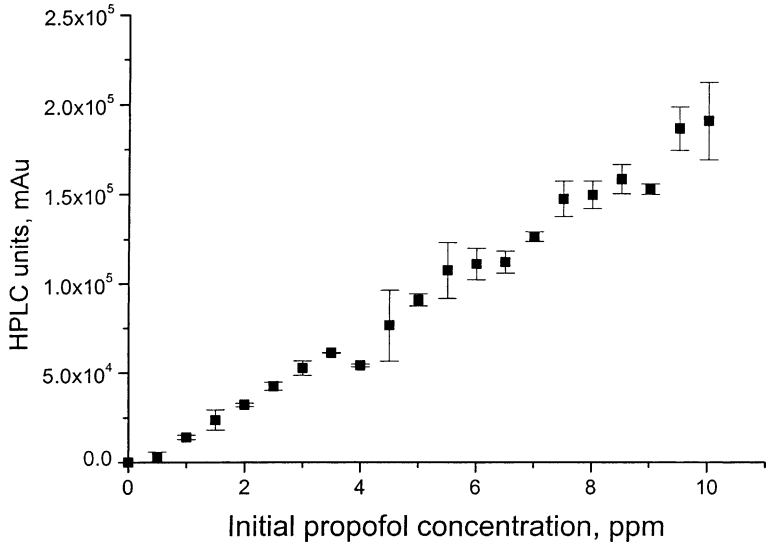

Fig. 3. HPLC determination of the propofol-imprinted polymer response upon testing against propofol standards (propofol was bound in the polymer, then released in the extraction solvent).

To avoid this, propofol needs to be separated quantitatively from blood proteins prior to testing it. Extraction of propofol with cyclohexane [12] gives reliable results, but it takes a long time (15 min) and releases the propofol into a non-polar solvent.

Treating blood with methanol leads to total protein precipitation and release of propofol into the methanol [13]. Literature [14] suggests that the precipitation method is not free from interference, as other compounds are extracted alongside the target molecule, which results in a coloured extract that includes propofol.

A precipitation experiment was performed to see whether propofol was fully recovered from the test solutions. The propofol (see Fig. 2) was released effectively alongside various other blood components, so precipitation of blood in methanol was chosen as a means to get the propofol in contact with the MIP membranes. The off-colour of the methanol supernatant, due to various interfering compounds, was an impediment in assessing the propofol concentration at this stage as the interfering substances would affect the Gibbs test. The solution cleanup step is offered by the MIP binding and subsequent release in a solvent of choice.

The linearity of release of propofol from the membrane was then assessed. Methanol was chosen as the extraction solvent, combined with bicarbonate buffer, pH 9.6, 1:1 mix (v/v), using 1 min exposure with strong stirring to remove propofol from the polymer. The increase in pH is enough to partially ionise the propofol. This disrupts the hydrogen bonds in the polymer and releases the propofol into solution.

Fig. 3 shows the relationship between propofol recovered from the membrane and initial concentration in the test solution. A pilot experiment was performed with the propofol in methanol, at different concentrations, to assess the linearity of extraction. Both the depleted propofol solution (in which the MIP was incubated) and the recovered solution (generated by extraction from the MIP) were analysed by HPLC.

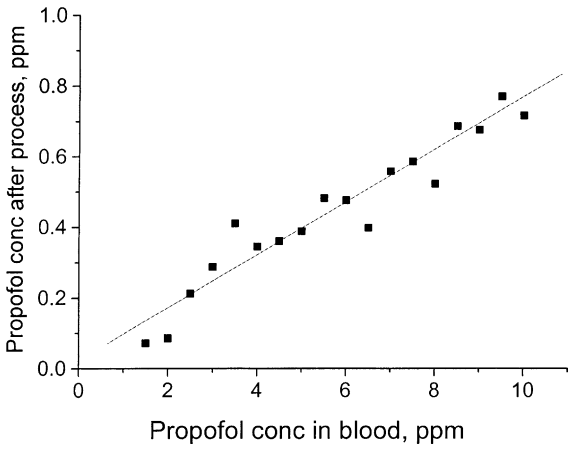

Fig. 4. HPLC determination of the propofol-imprinted response upon testing against spiked blood. Note the different values for the *y* axis—due to the dilution by precipitation from blood ($r^2 = 0.9519$).

The same experiment was then performed with propofol in blood. Blood (heparinised sheep blood, 125 units/ml) was spiked with known amounts of the commercial formulation containing propofol in order to get concentrations between 0 and 10 μg/ml (clinical range) in steps of 0.5 μg/ml. The results in blood (all samples assessed by HPLC) are shown in Fig. 4 and an estimation of the propofol concentration in blood can be given based on the amount present in the final sample.

4.6. Colorimetric test

The HPLC method for propofol determination takes minimum 5 min/sample. As one bolus injection of 10 μg/ml propofol gives sedation for 4–6 min, the propofol concentration has to be measured rapidly (preferably less than a minute) and the expense and complexity of the HPLC method has to be avoided.

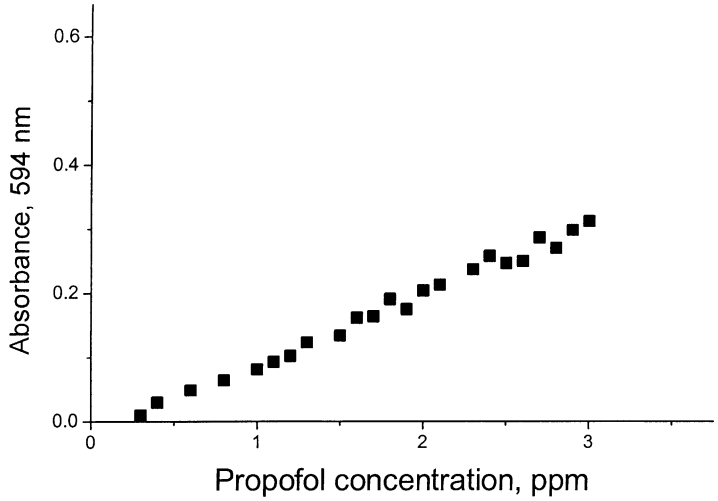

Fig. 5. Calibration curve for propofol at low concentrations using a modified Gibbs test. The calibration shows linearity in clinical range concentrations.

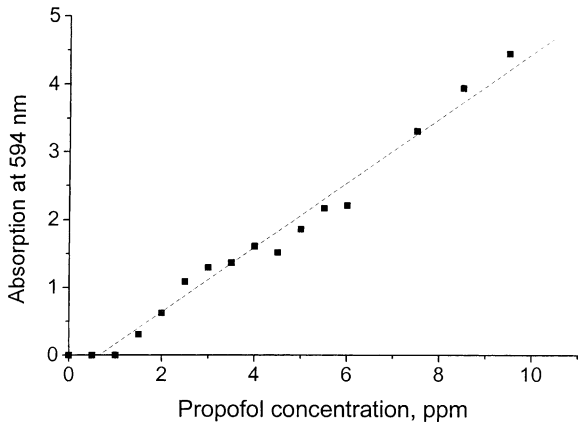

Fig. 6. Spectrophotometric determination of the propofol-imprinted polymer response when using propofol standards.

Colorimetric tests are well documented [15,16], but they are not specific for propofol. Three different colour reagents, 4-nitrophenol, 2,4-dinitrophenol, and 2,6-dichloroimidoquinone (Gibbs reagent) were evaluated. The concentration of the complex formed was assessed by spectrophotometry.

The first two compounds formed a complex with propofol, but were not sensitive enough at low concentrations. The Gibbs complex concentration was determined by spectrophotometric reading at 594 nm and the resulting calibration curve is depicted in Fig. 5. The calibration gives reliable results down to less than 0.5 μg/ml propofol present in the sample. In real samples, propofol concentrations between 0–2 μg/ml are monitored, due to the 1:5 dilution of blood in methanol. The linear response provided by Gibbs reaction over this range (Fig. 5) suggests the usage of this new detection system in a biosensor-type system.

The propofol released from the membranes was tested with the Gibbs colorimetric method (Fig. 6). Comparison of HPLC and Gibbs tests proved that we can use the newly developed system to give an accurate estimation of the propofol concentration initially present in the sample.

5. Conclusions

These results show that we have developed a new fast and reliable test for propofol in blood. The MIP has been developed as a thin film on a membrane support. The test involves rapid, non-equilibrium binding in a propofol-imprinted polymer, then release in an appropriate solvent and subsequent concentration assessment by colorimetric determination. The total cycle time is 3–4 min (below the metabolisation rate). In the non-equilibrium system, the non-specific binding is below the detection limit. Further tests need to be performed against other anaesthetics that could be present in the blood. The results obtained so far with propofol extracted from blood compared to those with propofol in methanol suggest that the test is free of interferences from blood. The selectivity of the polymer is coupled with a colorimetric test to achieve a fast detection method. Regeneration of the polymer on the support takes 5 min to complete and the polymers can be subjected repeatedly to the test in blood (up to 5000 cycles) without loss of sensitivity or selectivity.

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