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# The preparation of a molecular imprinted polymer to 7-hydroxycoumarin and its use as a solid-phase extraction material

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**Abstract**

A molecular imprinted polymer (MIP) was prepared to 7-hydroxycoumarin (7-OHC). A number of preparation parameters were examined by ultraviolet (UV) spectroscopy, including the amount of solvent used for reaction, equilibration time, selectivity and capacity of material. The polymer which showed the most selectivity for 7-OHC was then packed into cartridges and used as a solid-phase extraction sorbent. An extraction procedure was then developed from first principles. The cartridges were examined for selectivity of 7-OHC over some other members of the coumarin family. 7-OHC was then extracted from urine using this solid-phase extraction (SPE) method, and its concentration determined using capillary zone electrophoresis (CZE). The method was found to be linear over the range 10–50 μg $ml^{-1}$. Inter- and Intra- assay precision studies were performed to validate the method. © 1997 Elsevier Science B.V.

*Keywords:* Molecularly imprinted polymer (MIP); 7-hydroxycoumarin (7-OHC); Capillary zone electrophoresis (CZE); Solid-phase extraction (SPE)

## 1. Introduction

Coumarin occurs naturally in a number of plants and has found uses in the treatment of cancer, brucellosis, rheumatic disease and burns [1]. Moran et al. [2] showed that approximately 63% of the total dose of coumarin administered to patients was recovered as the 7-hydroxycoumarin (7-OHC) metabolite within 24 h. It has been determined in biological samples by a number of techniques including spectrofluorimetry [3,4], HPLC [5] and CZE [6,7].

In most biopharmaceutical analyses, it is first necessary to extract or remove the drug/metabolites of interest from interfering components in the sample matrix. Solid-phase extraction (SPE) is a technique which has found wide application in the

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area of sample preparation [8], the analyte of interest being sorbed onto the solid phase, while the interferents are washed to waste. This area of sample preparation is ever growing with a variety of sorbents available including ionic, $C_{18}$ and mixed functionality materials [9].

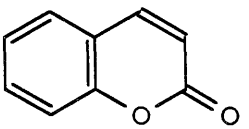
To date, a number of molecularly imprinted polymers (MIPs) have been prepared to a number of compounds including amino acids, amino acid derivatives and drug [10] species. These materials have also found use as stationary phases in HPLC. The preparation of these materials is in three stages: (1) copolymerization of the monomers and crosslinker monomers in the presence of the target molecule; (2) grinding and sieving of the particles to give a material with an appropriate range of particle size; and then (3) the removal of the target molecule from the polymer network. For instance, Sellergren [11] prepared a MIP to pentamidine (PAM) and used this as a SPE material to selectively retain PAM. This involved the preparation of the polymer in glass tubes, which were fitted with column end fittings, and then connected to a HPLC system.

In this work a molecularly imprinted polymer was prepared to 7-OHC and a number of parameters were evaluated by UV spectroscopic studies. A number of spectroscopic studies were then carried out on the polymer, which included: (a) the amount of solvent used in the polymerization reaction; (b) the selectivity of 7-OHC over some other coumarins, including coumarin and 7-diethylamino-4-methylcoumarin (Fig. 1); (c) the amount of time necessary for equilibration of the polymer with the test solution; and (d) the capacity (uptake of 7-OHC) of the material. The recovery and selectivity of this polymer was determined by packing the MIP into cartridges and using it as a SPE material. These columns were then used for the determination of 7-OHC in urine, and the method validated for the extraction of 7-OHC from urine samples in the pharmacokinetically important range of 10–50 μg $ml^{-1}$.

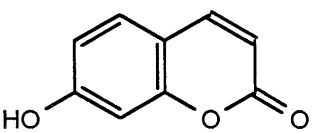
## 2. Experimental

### 2.1. Reagents and solvents

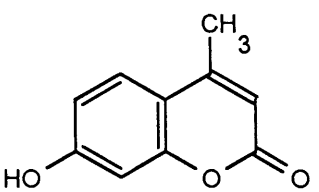
HPLC grade methanol was obtained from Labscan Analytical Sciences (Dublin, Ireland). Analar grade sodium hydroxide and phosphoric acid



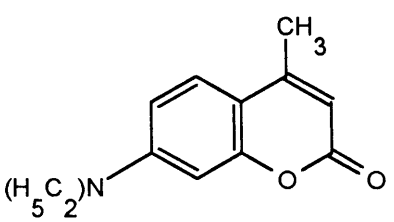

coumarin




7-hydroxycoumarin




7-hydroxy-4-methylcoumarin




7-diethylamino-4-methylcoumarin

Fig. 1. Chemical structures of coumarin, 7-hydroxycoumarin and 7-diethylamino-4-methylcoumarin.

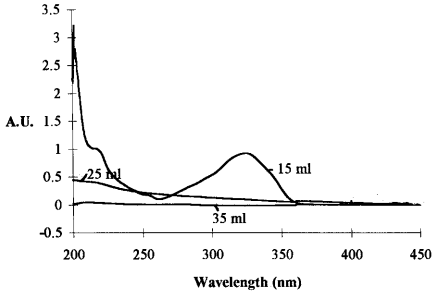



Fig. 2. Investigation of the optimal volume of solvent required for the polymerization reaction.

were supplied by BDH (Poole, UK). Analytical grade dipotassium hydrogen phosphate and potassium dihydrogen phosphate were used as buffer salts and were obtained from Merck (Darmstadt, Germany). The electrolyte solution used was 0.025 M phosphate buffer, pH 7.5, which was prepared daily by preparing a solution which was 0.02 M $K_2HPO_4$ and 0.005 M $KH_2PO_4$ in deionized water. 7-Hydroxycoumarin (7-OHC) and the other coumarin compounds were obtained from Sigma (St. Louis, MO). 7-Hydroxycoumarin standards were prepared from a 1 mg $ml^{-1}$ stock solution in methanol.

### 2.2. Instrumentation and operating conditions

The capillary used for CZE analysis was a 27 cm by 50 μm (I.D.) fused-silica column (Beckman Instruments), with a capillary-to-detector distance of 20 cm. The capillary was prepared by rinsing with 0.1 M sodium hydroxide for 1 min and then with buffer solution for 1.2 min; this procedure

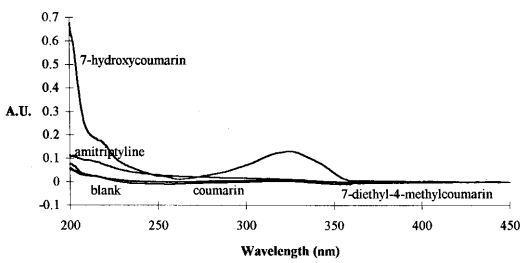



Fig. 3. The selectivity of the polymer prepared to 7-hydroxycoumarin, in comparison to some other coumarins and amitryptyline.

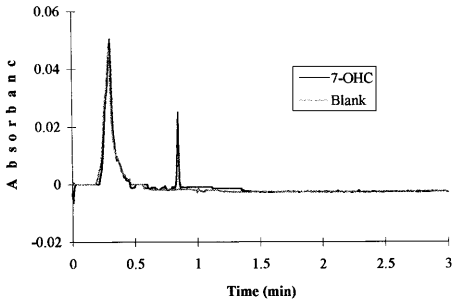



Fig. 4. CZE analysis of blank urine and spiked urine sample containing 50 μg $ml^{-1}$ 7-hydroxycoumarin. Background electrolyte consists of 0.05 M phosphate buffer, pH 7.5, temperature 25°C, voltage 20 kV, detection 210 nm.

was based on a method previously reported by Bogan et al. [7] The sample was then applied to the capillary by a 3-s pressurized injection (0.5 p.s.i.) and separation was achieved with an applied voltage of 20 kV (rise time 0.2 min) at 25°C. Typical running current was 100 μA. The resultant electropherogram was monitored at 210 nm with a photodiode array detector using Beckman System Gold software. The migration time for 7-OHC was 0.92 min.

### 2.3. Preparation of polymer

The polymerization was carried out based on a method by Vlatakis et al. [12] by mixing methacrylic acid (0.45 g, 5.2 mmol), 7-hydroxycoumarin (0.21 g, 1.4 mmol), ethylene glycol dimethacrylate (EDMA) (4.68 g, 23.5 mmol) and azo-isobutyronitrile (AIBN) (0.06 g, 0.37 mmol). Chloroform (15 ml) was added and the mixture was degassed under vacuum and then sonicated

Table 1
Inter-assay precision and linearity

| Amount added (μg $ml^{-1}$) | Mean amount found ($\pm$SD) | % CV (R.S.D.) |
|---|---|---|
| 10 | $11.30 \pm 0.71$ | 6.28 |
| 20 | $18.95 \pm 0.85$ | 4.49 |
| 30 | $29.07 \pm 0.60$ | 2.06 |
| 40 | $39.95 \pm 0.76$ | 1.90 |
| 50 | $50.71 \pm 1.11$ | 2.18 |

Mean CV = 3.38%

Table 2
Intra-assay precision and linearity

| Amount added (μg ml$^{-1}$) | Mean amount found (± S.D.) | % CV (R.S.D.) |
|---|---|---|
| 10 | 10.92 ± 0.26 | 2.38 |
| 20 | 18.91 ± 1.19 | 6.29 |
| 30 | 29.03 ± 1.62 | 5.58 |
| 40 | 39.93 ± 0.78 | 1.95 |
| 50 | 50.17 ± 2.21 | 4.41 |

Mean CV = 4.12%.

for 5 min. It was then sparged with nitrogen for 5 min. The polymerization was carried out by heating the mixture to 60°C for 12 h under nitrogen. As the polymer was formed it precipitated out of solution. This was ground in a pestle and mortar to give particles in the range 45–65 mm. The material was then placed in a Soxhlet apparatus and the 7-hydroxycoumarin was extracted twice using 300 ml methanol: acetic acid (90:10% v/v). Repeated sedimentation of the polymer removed any fines present. The particles were then dried under vacuum and stored at ambient temperature until use.

### 2.4. Column preparation and SPE procedure

The columns were prepared by a method described by Boyd [13] which involved the use of syringe barrels. Two filter paper discs were placed in a 2 ml syringe barrel and 0.40 g of polymer was added to the barrel and two more filter discs were placed on top. The columns were preconditioned by flushing with 3 ml of methanol, 2 ml of water and 1 ml of buffer solution. The column was then compressed with a syringe plunger. A sample (250 μl) of undiluted urine was applied to the top of the column. The column was then washed with 2 ml of deionized water and allowed to dry for 30 min. The drug component was then eluted with 3 ml of methanol. This eluate was evaporated to dryness under a stream of nitrogen at 60°C, and reconstituted in 250 μl of phosphate buffer (0.025 M, pH 7.4) prior to analysis using CZE.

### 2.5. Calibration and calculation

Evaluation of the extraction procedure was carried out using a five-point calibration graph covering the concentration range 10–50 μg ml$^{-1}$. The slope and the intercept of the calibration graph were determined through linear regression of the drug peak heights. Individual peak-heights were then interpolated on the calibration graph to determine values of concentration found as compared to concentration added.

## 3. Results and discussion

### 3.1. Spectroscopic evaluation of MIPs

#### 3.1.1. Selection of amount of solvent for polymerization

The volume of chloroform (15, 25 and 35 ml) required in the polymerization reaction was examined initially. The polymers were prepared in the usual manner using methacrylic acid, EDMA, 7-OH coumarin and varying amounts of chloroform. Known amounts (0.10 g) of polymer were stirred in 10 μg ml$^{-1}$ solutions of 7- OHC for 60 min. The polymers were then filtered, rinsed with methanol and dried, and suspensions (1 mg ml$^{-1}$ of polymer) prepared in acetonitrile. These were then scanned in the region 200–450 nm. From the results obtained it would seem that the polymer prepared in 15 ml of chloroform was more selective for 7-OHC than the other two polymers (Fig. 2).

#### 3.1.2. Selectivity

To determine the selectivity of the polymer prepared using 15 ml chloroform, known amounts (0.10 g) of polymer were stirred in solutions of 7-OHC, coumarin, 7-diethylamino-4-methylcoumarin, amitriptyline and methanol (blank). The polymers were filtered, rinsed and dried, and suspensions prepared in acetonitrile. These were then scanned in the region 200–450 nm. From the results obtained in Fig. 3 it can be seen that the polymer prepared against 7-OHC was reasonably selective for this compound over some other coumarins and drug compounds.

Table 3
Results for recovery of 7-hydroxycoumarin

| Concentration (μg ml$^{-1}$) | 7-Hydroxycoumarin peak height | | |
|---|---|---|---|
| | Authentic standards | Extracted standards | Recovery (%) |
| 10 | 0.00357 | 0.00329 | 92.16 |
| 20 | 0.00703 | 0.00589 | 77.38 |
| 30 | 0.00972 | 0.00910 | 93.62 |
| 40 | 0.01288 | 0.01198 | 93.01 |
| 50 | 0.01528 | 0.01461 | 95.62 |

Mean recovery ( ± S.D.) = 90.36 ± 7.37%.

*3.1.3. Effect of stir time*

The next parameter examined was the effect of 'stir' or (equilibration) time. This is defined as the amount of time necessary for the polymer to interact with the analyte and as such allow the 7-OHC to be taken up by the polymer. Small amounts of the polymer were equilibrated with solutions of 7-OHC for 30, 60, 120 and 180 min. The polymers were then filtered, rinsed and dried as before, and then scanned in the region 240–450 nm. From the results obtained it was shown that the uptake of 7-OHC increased up to 60 min, but then decreased on further increasing the time, and hence it was decided to use equilibration times of 60 min for further studies.

*3.1.4. Determination of capacity of polymer*

To determine the capacity of the selected polymer, solutions of various concentrations of 7-hydroxycoumarin (1 and 100 mg ml$^{-1}$; 50, 10 and 1 μg ml$^{-1}$) were mixed with known amounts of the MIP. After mixing the polymer with the sample solutions for 1 h, the polymer was filtered, rinsed and dried. Solutions were prepared from the dry polymers in acetonitrile and these were then examined by UV spectroscopy. From the UV scans obtained, it was seen that the polymer was unable to absorb 7-OHC in large quantities from the highly concentrated solutions. Hence, for the amount of material used (0.10 g polymer), the maximum concentration of 7-OHC which could be extracted with good efficiency (95–100%) was 10 μg ml$^{-1}$ 7-OHC.

*3.2. Development of solid-phase extraction procedure*

*3.2.1. Development of extraction procedure*

Since the main aim of this work was to incorporate the MIP prepared to 7-OHC into a SPE cartridge, the MIP was packed into cartridges as described in the Experimental Section. To optimize the extraction, a number of parameters were varied, which included the pH of the buffer used prior to the application of the sample to the SPE column, the wash step and the elution step. Each of the parameters was examined in triplicate. The columns were preconditioned with methanol and then water.

The pH of the buffer was the first parameter varied. This was carried out by applying 1 ml of buffer of appropriate pH to the pre-conditioned cartridges. The pH was varied using Britton–Robinson buffers in the pH range 3–10. From the data obtained, the recoveries were found to be about 40% at pH values of 3–4, 80–96% between pH 5–7, and 0–5% at higher values. A pH of about 6.0 therefore proved optimum for application of samples to the SPE cartridge containing the MIP to 7–OHC.

The next parameter studied was the wash step. This is an important stage, so as to remove any interfering compounds without eluting the target analyte. A number of wash procedures were examined including 100% $H_2O$ and $H_2O$/methanol (50:50 v/v). The washings were dried down to determine if any 7-OHC had been eluted at this

stage. Based on the results obtained, it was decided to use water, as it gave rise to cleaner extracts (when dealing with urine), and no 7-OHC eluted, whereas some 7-OHC eluted when $H_2O$/methanol mixtures were employed. The volume of wash solution was also varied in the region 1–5 ml, with 2 ml being found to be the most suitable.

The final step was to select a suitable elution solvent, and a number of solvents were examined, including methanol, diethyl ether, hexane:diethyl ether (50:50 v/v) and ethyl acetate. Methanol gave rise to the best recoveries, with the amount of solvent used being varied in the region 1–5 ml, with 3 ml being found optimum for the elution of 7-OHC. Poor recoveries were found with the other solvents: hexane and hexane:diethyl ether (50:50 v/v) gave rise to recoveries in the region 0–5%, whereas diethyl ether gave rise to values of 30–40%; in addition, ethyl acetate gave rise to a large number of interfering peaks for urine analysis. An increase in the volumes of these solvents did not improve matters.

*3.2.2. Recovery*

The recovery of 7-hydroxycoumarin from urine using the MIP-SPE procedure was measured by calculating the percentage difference between the peak heights of extracted standards and those of authentic standards in the relevant concentration range. The mean recovery was found to be $90.36 \pm 7.37\%$ (Table 3). Fig. 4 depicts a typical electropherogram of a spiked and a blank urine sample following SPE extraction using the MIP column.

*3.2.3. Assay validation*

The method was then validated over the concentration range 10–50 μg $ml^{-1}$. The data presented in Table 1 and Table 2 demonstrate the inter- and intra- assay variations for the method. Inter-assay variation was assessed singly in four replicate runs. Intra-assay was determined in quadruplicate over the same range.

Linearity is defined by the correlation coefficient of the regression line and accuracy is defined by the percentage difference between 'added' and 'found' concentration for inter-assay values presented in Tables 1 and 2, and a correlation coefficient of 0.994 or better obtained.

## 4. Conclusions

The area of solid-phase extraction as a method of sample preparation is ever increasing in popularity, and in many cases replacing liquid/liquid extraction protocols. The number of phases available for this technique is also increasing, and these include ion-exchange materials, polymers and mixed functional phases, and this in turn expands the range of components which can be extracted by this technique.

Although polymers have already found use in this area, the use of an MIP allows a polymer material to be prepared to a specific compound. This would be particularly useful if the compound is unsuitable for solid-phase extraction by any of the materials previously mentioned. This could also be particularly useful when a component has to be removed from its metabolites or compounds with very similar structures.

This study shows that a MIP prepared to a 7-hydroxycoumarin allowed it to be selectively retained, while allowing endogenous components to be removed. It was capable of extracting 7-OHC in the range 10–50 μg $ml^{-1}$, which is the range reported for pharmacokinetic studies using HPLC and CZE methods of analysis. The presented analytical scheme can thus in favourable cases enrich and clean-up a sample to a level that allows direct analyte determination upon desorption. It should prove to be a suitable alternative to conventional liquid/liquid extraction techniques.

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