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Application of Molecularly Imprinted Polymer for Solid Phase Extraction and Preconcentration of Hydrochlorothiazide in Pharmaceutical and Serum Sample Analysis

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A new polymeric sorbent prepared by utilizing molecular imprinting technology was used for the selective extraction of hydrochlorothiazide (HCT) from pharmaceutical and human serum sample. The molecularly imprinted polymer (MIP) was prepared using HCT as the template, methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EDMA) as the cross-linker monomer, and dimethylformamide (DMF) as a solvent. The optimized conditions of MIPs as a selective sorbent for the preconcentration of the HCT were studied. The results showed that the drug could be quantitatively and selectively maintained in the column to be then eluted from the sorbent by using methanol-acetic acid mixture (9:1). HCT could be determined spectrophotometrically at λ_{max} = 270 nm. This method made it possible to quantitize HCT in the range of 0.1-21.0 μg ml^{-1}, by less than 0.55% of RSD%, with a detection limit (S/N = 3) of 0.073 ng ml^{-1}. The preconcentration factor of 1000, recoveries of up to 96% and retention capacity of 75.0 mg g^{-1} were achieved using this technique.

Keywords: Hydrochlorothiazide, Molecularly imprinted polymer, Solid-phase extraction, Preconcentration

INTRODUCTION

Hydrochlorothiazide, 6-chloro-3,4-dihydro-2h-1,2,4-benzothiazine-7-sulfon-amide-1,1-dioxide (Fig. 1), is a diuretic agent used for hypertension treatment that reduces plasma volume by increasing the excretion of sodium, chloride, water and to a lesser extent of potassium ion [1-3]. It is often used in mixture with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme inhibitors, or more recently, as angiotensin receptor blockers [4-10]. A range of analytical techniques, such as high performance liquid chromatography (HPLC) [11-16], capillary electrophoresis [17,18], spectrophotometry [19,20] and chemometric techniques [21-23] are used for its determination in pharmaceutical samples. Most of the reported methods suffer from drawbacks such as complicated procedure, requirement of costly instruments and mostly low detection ability.

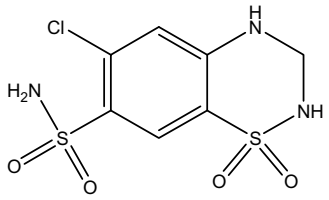

Fig. 1. HCT structure.

Even though several important advances continue to be made in analytical instrumentation, sample preparation is still the rate-limiting step and most prone to errors in many analytical techniques. Traditionally, liquid-liquid extraction used to be the preferred technique for clean-up samples [24]. These extractions resulted in relatively clean extracts with good recoveries, but were, at the same time, time-consuming

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and large quantities of solvents were used which often exerted environmental and health hazards. On the other hand, these methods can not be used in low concentration levels of analytes and complex matrices. The solid phase extraction (SPE) is one of the powerful and important preconcentration methods that has been generally employed to solve these problems. Furthermore, SPE technique is cost effective, quite fast with satisfactory recoveries. In the last decade, one of the most promising technical applications based on the use of MIPs has been molecularly imprinted solid-phase extraction (MISPE). Many studies have focused on extracting compounds from biological samples [25-30]. The use of MIPs in SPE has obvious advantages mainly when a selective extraction must be performed and the commonly used sorbents lack selectivity. Then, MISPE allows not only the analyte to be preconcentrated but also the other compounds present in the sample matrix to be removed. MIPs are cross-linked synthetic polymers which are obtained by copolymerization of a monomer with a cross-linker in the presence of a template molecule. After polymerization, the template is removed from the porous network by washing, leaving cavities in the polymeric matrix that are complementary in size, shape, chemical functionality and conformational to the template. Thus, when a sample is loaded onto an MIP in a typical SPE procedure, the polymer is able to selectively rebind the analyte (*e.g.* the template). Consequently, the analyte is eluted from the cartridge ideally free of co-extracted compounds. The selectivity of MIP arises from the synthetic procedure followed to prepare the MIP, in which a template molecule is linked, by covalent bonds or noncovalent forces, to suitable monomer (s) containing functional groups. This link is responsible for the subsequent specific binding sites imparted to the MIP. In this study, we wish to demonstrate a selective SPE and preconcentration method using MIP for the effective clean-up and enrichment of HCT in pharmaceutical and human serum sample. Chen et al. merely reported the synthesis of MIP-HCT but did not offer any practical application of the system [31].

EXPERIMENTAL

Apparatus

A UV-Vis NIR spectrophotometer (Jasco, double beam and model V-750, Japan) was used for the recording of the absorption spectra. An oil bath thermostat was used for the control of the reaction temperature. A peristaltic pump (Ismatec model, MCP standard V7.00, Glattbrugg-Zürich, Switzerland) was used for adjusting the flow rate and driving force. Tygon rubber tube with i.d 0.76 mm was used for delivery of the solutions. A Metrohm pH meter (model 827, Herisau, Switzerland) was used to adjust pH.

Chemicals

HCT, dibucaine, desipramine, isoxicam were obtained from Sigma-aldrich (Steinheim, Germany). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), 2,2′-azobis (2-isobutyronitrile) (AIBN), dimethylformamide (DMF), acetone, HPLC-grade methanol and acetic acid were purchased from Merck (Darmstadt, Germany). The universal buffer (0.04 M) solutions in the pH range of 2.0-7.0 were prepared using phosphoric, boric and acetic acid and adjusting to the desired pH with NaOH solution (0.04 M) [32].

Preparation of MIP

Non-covalent method with HCT as a template was employed in the molecularly imprinted polymer synthesis. For the preparation of the HCT imprinted polymer, the functional monomer (MAA, 3.9 mmol), cross-linker (EDMA, 19.5 mmol), initiating agent (AIBN, 0.3 mmol) and 8.5 ml of DMF were placed in a glass bottle. The molar ratio of template/monomer/cross-linker was 1:5:25. The polymerization mixture was degassed with N_2 for 5 min, sealed under nitrogen and then left to polymerize in an oil bath at 60 °C for 24 h. Finally, the bottle was smashed and the polymer was ground into fine particles using a mortar and pestle. The product, after drying overnight, was white solid and possessed a rigid structure. In order to verify that the retention of HCT was due to molecular recognition and not due to nonspecific absorptions, a non-imprinted polymer (NIP) was prepared following the same procedure as in the absence of the template (HCT) and its selectivity was compared with the MIP performance.

Extraction of HCT from the MIP

The obtained polymers were extracted using the soxhlet apparatus in order to remove the template from the polymer

matrix. A sample of the HCT-imprinted polymer was placed inside the cellulose extraction thimble. The HCT was completely extracted from the polymer network after 57 h using a mixture of methanol-acetic acid (9:1 v/v).

Preparation of Column

A steel sieve was used to select the MIP particles with sizes between ~50-70 μm. 200 mg of the polymer was dispensed into a polypropylene column measuring 53.0 mm in length and 6.5 mm i.d. up to half way the column bed, using four or five gentle brunts at the end of the column. A 2.0 μm frit was used at the end of the column fitting to ensure no loss of polymer particles and the column was capped. The preparation of NIP column was completed in a similar manner.

General Procedure

The packed column was connected to a tygon tube with i.d. 0.76 mm for passing the solutions and a flow rate of 0.108 ml min^{-1} was adjusted. In order to optimize the extraction conditions, the concentration of HCT, either in the solution passed from the column or the solution after the elution step, was determined spectrophotometrically. This was achieved after the evaporation of the solvent and dissolving of the remaining solute in 1.0 ml of deionized water. The absorbance signal against blank solution was measured at the wavelength 270.0 nm (A_{MIP}). The aforementioned experiment was also repeated for the NIP to control the selectivity of imprinted polymer. Its absorbance signal (A_{NIP}) was measured and $\Delta A = A_{MIP} - A_{NIP}$ was followed as an analytical signal. The results showed that the NIP was not able to trap HCT in appreciable amount (lower than 1.0%). As a consequence, the NIP has only nonspecific adsorption property.

Pharmaceutical and Serum Sample Analysis

Three tablets of each formulation were dissolved in water (basic medium) in a calibrated flask, placed in an ultrasonic bath for 15 min. The mixture was filtered before further dilution for measurement. Appropriate aliquots from the working solution were taken for the determination of HCT.

A 0.5 ml human serum sample (taken from the health center of Isfahan University of Technology) was spiked with HCT (in water) to provide a working concentration of 0.46, 1.2 and 2.4 μg ml^{-1}. The serum sample was placed in a glass vial containing 4.0 ml of methanol, vortexed for 10 s, and centrifuged at 3300 rpm for 20 min. A 1.0 ml aliquot of the methanol layer was placed in another glass vial and 3.0 ml methanol was added and centrifuged again at 3300 rpm for 10 min. One milliliter of the supernatant was diluted to 10 ml and 2.0 ml of the aliquot was injected onto the column. After the solution had passed through the MIP-SPE, it was washed with 10 ml of water. Finally, the analyte was eluted with methanol-acetic acid (9:1). The eluate evaporated to dryness at ambient temperature under a stream of nitrogen, and the residue was dissolved in 2 ml of buffer solution and the concentration of HCT was determined.

RESULTS AND DISCUSSION

In order to optimize all the variables that were affected upon the determination, extraction and preconcentration of HCT steps, various effective parameters of both steps were evaluated.

Spectrophotometric Determination of HCT

The molar absorptive dependence of HCT on various pHs was studied. The relation between the absorbance and the pH is shown in Fig. 2. The maximum signal was obtained in the pH = 6.0. Therefore, for further application, this pH was used for determination step. The linear equation, in the HCT concentration range of 0.1-21.0 μg ml^{-1} with regression

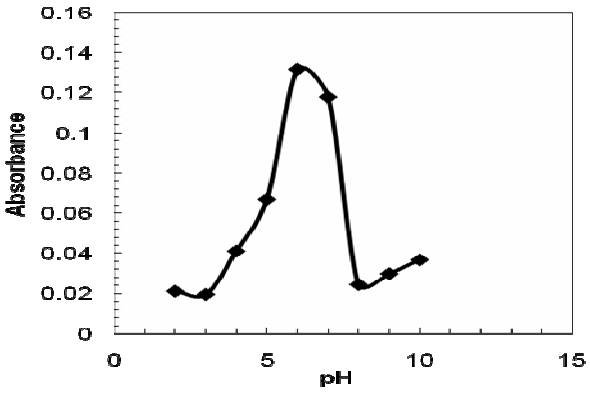


Fig. 2. Effect of pH on calibration curve. Conditions: wavelength of measurement; 270 nm, volume of buffer solution; 2.0 ml; HCT concentration, 3.0 μg ml^{-1}.

coefficient R^2 = 0.9998, was A = 0.0584 × C - 0.0024 (A is absorbance signal and C is μg ml^{-1} concentration of HCT). The detection limit ($3S_b/m$ = 3) was 0.073 μg ml^{-1}. The relative standard deviation (RSD%) for four replicate measurements of 5.0, 10.0 and 15.0 μg ml^{-1} of HCT were 0.44, 0.55 and 0.55%, respectively.

Effect of Solvent Ratio and Extraction Time

It is very important to mention that in order to obtain the best extraction time and solvent ratio (methanol-acetic acid) for the extraction of HCT from the polymer matrices, different solvent ratios (9:1, 7:3 and 5:5) and extraction times (2 to 20 h) were evaluated by spectrophotometric determination. Figure 3 shows that the solvent ratio of 9:1 and extraction time of 20 h were optimized conditions. Because of bleeding HCT from MIP, the extraction time of 57 h was selected for the complete extraction of HCT. Therefore, the solvent ratio of 9:1 and 57 h for the extraction was selected for further experiments.

Effect of pH

The effect of pH on the extraction of HCT from the MIP was studied at various pHs in the range of 2.0-7.0 (using universal buffer). In sample loading, the optimum pH should be applied for a better imposition of the analyte on the polymer network because of its chemical structure. In these circumstances, suitable hydrogen bonding with polymer was achieved. Figure 4 shows the effect of pH on the net absorbance (ΔA). The maximum net absorbance was at pH 3.0; whereas higher pH values cause a decrease in the signal. The maximum signal would represent the effect of pH on hydrogen bonding formation. Therefore, pH, 3.0 was chosen as the optimum pH.

Effect of Flow Rate

The influence of analyte flow rate was investigated by passing 10.0 ml HCT (30.0 μg ml^{-1}) in the range of 0.108-0.500 ml min^{-1}. The results illustrated that the maximum signal was obtained at the flow rate of 0.108 ml min^{-1} (Fig. 5). Therefore, the flow rate of 0.108 ml min^{-1} was selected as the optimum condition. In higher flow rates, the analyte did not have enough residence time in the column for effective interactions with the sorbent. It is notable that lower flow rate(0.108 ml min^{-1}) was not evaluated because of shortage of instruments.

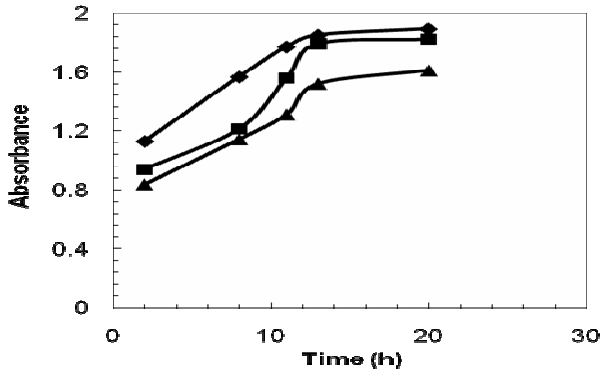


Fig. 3. Optimization of solvent ratio and time extraction. Conditions: volume of extraction solvent, 150 ml; wavelength of measurement, 270 nm: (◆) methanol-acetic acid 9:1, v/v; (■) methanol-acetic acid 7:3, v/v; (▲) methanol-acetic acid 5:5, v/v.

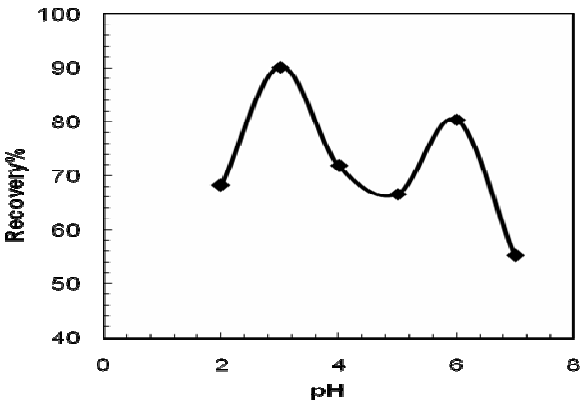


Fig. 4. Effect of pH on the adsorption efficiency. Conditions: column packing material, 200 mg; buffer solutions volume, 2.0 ml; HCT concentration, 30.0 μg ml^{-1}; flow rate, 0.554 ml min^{-1}; wavelength of measurement, 270 nm.

Retention Capacity of the MIP

Various concentrations of HCT in the range of 0.060 to 3.0 mg ml^{-1} at the same volume (10.0 ml) were passed through the column for measuring their retention capacity. The results indicated that the minimum recovery percentage of extraction

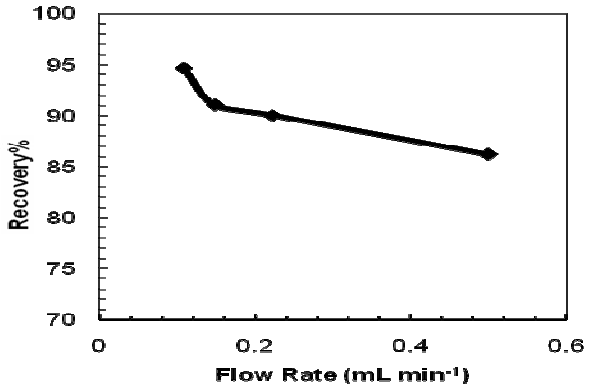


Fig. 5. Effect of flow rate on the adsorption efficiency. Conditions: column packing material, 200 mg; buffer solutions volume, 2.0 ml; HCT concentration, 30.0 μg ml^{-1}; pH, 3.0; wavelength of measurement, 270 nm.

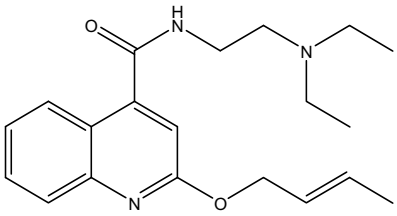
was 99.2 for the high concentration value (maximum solubility of HCT). The retention capacity (mg adsorbed HCT/g of sorbent) was found to be 75.0 mg g^{-1}.

Evaluation of Repeatability

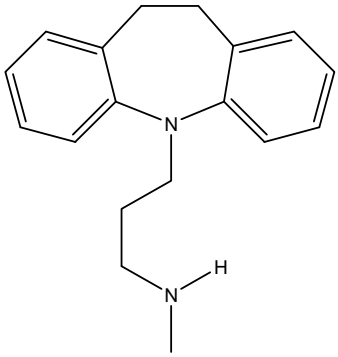
In order to evaluate the repeatability of the MIP extraction procedure, 200 mg polymer was packed onto the column and then 3.0 ml of the solution containing 500 μg ml^{-1} of HCT was passed through the column. The absorbance signal of the passed solution was measured to obtain recovery of sorbent. Then the column was eluted with the proper mixture of the solvent (2.0 ml) and 10.0 ml of distilled water was passed into the column. This column was used again, without exchange of the sorbent. The results showed that the MIP-packed column had a good repeatability with the relative standard deviation (RSD%) of 0.30% for four replicate measurements.

Evaluation of Breakthrough Volume

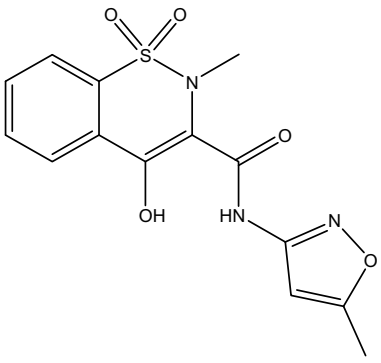
The measurement of breakthrough volume is a significant aspect of SPE because it represents the sample volume that can be preconcentrated without loss of analytes. Different volumes of 0.20 μg ml^{-1} HCT solutions in the range of 10-1000 ml were passed through the column and the signal of each eluted solution was determined. The final sample (for dissolving the trapped HCT) was 1.0 ml, and so a preconcentration factor of 1000 was obtained by this MISPE procedure. The recovery level when 1000 ml of the solution was loaded into the MIP column was 96%. It is worth mentioning that by using MIP as SPE sorbent, the limit of determination could be improved to 0.1 ng ml^{-1}.



(a)



(b)



(c)

Fig. 6. (a) Dibucaine, (b) Desipramine, (c) Isoxicam.

Evaluation of Selectivity

In order to evaluate the selectivity, three other drugs, *i.e.* dibucaine, desipramine hydrochloride and isoxicam (Fig. 6)

were chosen. The reason for the selection of these drugs was that they afford many similar functional groups which are able to bind to MIP. This experiment along with a comparison of the data with NIP results, reveal that binding to the MIP is not due to surface adsorption at all (surface adsorption <1.0%), rather, it is mainly due to the template selectively with proper cavity. The effect of these species on the determination of 0.20 μg ml^{-1} of HCT was investigated. The concentration of undesirable selected species was two hundredfold of HCT. The concentration of the added molecules, was responsible for a relative error of less than 3.0%.

Response Characteristics

In Table 1, response characteristics of the proposed method are compared with the recently reported techniques [33-38]. It is quite obvious from the table that the proposed method has a good detection limit and selectivity with excellent capacity factor. This method is simple and does not require any expensive equipments.

Analysis of Real Sample

The reliability of the MISPE method was evaluated with HCT pharmaceutical tablet samples for each concentration. Four replicates of samples were prepared and then the real pharmaceutical samples were analyzed. The analysis was performed by using the standard addition technique. The results are summarized in Table 2. Good recoveries in all samples were obtained. The application of this method was

Table1. Comparison of Some Methods for Preconcentration and Determination of HCT with Proposed Method

Method	Detection limit (ng ml^{-1})	Ref.
Time resolved chemiluminescence	160	[33]
Capillary zone electrophoresis with amperometric detection	148	[34]
Second-order derivative spectrophotometry	1570	[35]
Chemiluminescence-based liquid chromatographic	59.5	[36]
HPLC utilizing narrowbore chromatography	1000	[37]
Cerium-based chemiluminescence	44.6	[38]
Proposed method	0.1	-

Table 2. Determination of HCT in Pharmaceutical Tablets (n = 4)

Sample	Added, (μg ml^{-1})	Found, (μg ml^{-1})	Recovery (%)
1	-	4.066 ± 0.0709	-
	6.0	10.273 ± 0.0362	103.45
	12.0	15.962 ± 0.0856	99.13
2	-	4.475 ± 0.0202	-
	6.0	10.675 ± 0.0581	103.33
	12.0	16.374 ± 0.1318	99.16
3	-	3.910 ± 0.0707	-
	6.0	9.996 ± 0.0396	101.43
	12.0	15.878 ± 0.1066	99.73

Table 3. Results of HCT Determination in real Sample (n = 4)

	HCT Added ($\mu g\ ml^{-1}$)	Average of HCT found ($\mu g\ ml^{-1}$)	Recovery (%)
Serum sample	0.46	0.49 ± 0.007	106.5
	1.2	1.11 ± 0.01	92.5
	2.2	2.0 ± 0.008	90.9

estimated with human serum sample whose results are summarized in Table 3.

CONCLUSIONS

In this work, MIP was prepared as a solid phase sorbent for the selective extraction and preconcentration of HCT. The MIP showed outstanding affinity with and selectivity to HCT and was therefore suitable for the application in SPE. In order to obtain the desired conditions, various effective parameters on the procedure of extraction of MIP were carefully optimised. However, non-specific recoveries obtained from the corresponding NIP clearly indicate that the extraction of HCT from this MIP is mainly due to selective interactions with specific cavities. It is worth mentioning that the high recovery factor of 1000 ml was achieved for preconcentration.

Good recovery and precision confirm that the MISPE process is a suitable method for the sample pretreatment of HCT in pharmaceutical and human serum sample. In optimized conditions, the MISPE offered several practical advantages over other methods such as LLE and SPE with ENVI-18 materials. Finally, the developed MISPE provided superior sample clean-up.

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