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Journal of Chromatography B, 715 (1998) 379–386

JOURNAL OF CHROMATOGRAPHY B

# On-line deconjugation of glucuronides using an immobilized enzyme reactor based upon β-glucuronidase

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Received 18 December 1997; received in revised form 24 April 1998; accepted 4 May 1998

**Abstract**

An immobilized enzyme reactor based upon β-glucuronidase (BG–IMER) has been developed for the on-line deconjugation of substrates. The activity of the BG–IMER and its applicability to on-line deconjugation was investigated. The BG–IMER was coupled to a reversed-phase column ($C_8$ or $C_{18}$) and the latter column was used to separate substrates and products eluted from the β-glucuronidase reactor. The activity of the BG–IMER was followed by measurement of percent deconjugation and the parameters investigated were: substrate concentration, pH (4 to 6), temperature (r.t., 37°C), enzyme–substrate contact time using flow-rates of 0.1 to 1.0 ml/min (15–1.5 min). The glucuronides used in the evaluation of the BG–IMER were: 4-methylumbelliferyl-β-D-glucuronide, *p*-acetaminophen-β-D-glucuronide, 3′-azido-3′-deoxythymidine-β-D-glucuronide, phenyl-β-D-glucuronide, chloramphenicol-β-D-glucuronide, estradiol-17-β-D-glucuronide and morphine-β-D-glucuronide. The development of on-line HPLC deconjugation of glucuronide substrates using the BG–IMER will facilitate the identification of metabolites and quantification of aglycones in metabolic and pharmacokinetic studies.


*Keywords:* Immobilized enzyme reactor; On-line deconjugation; β-Glucuronidase; Enzymes; Glucuronides

## 1. Introduction

β-Glucuronidase enzymes are widely distributed in mammalian tissues with particularly high concentrations in the liver [1]. These enzymes catalyze the hydrolysis of β-glucuronide conjugates to yield aglycones and free glucuronic acid [2]. β-Glucuronidase plays an important role in the enterohepatic circulation of drugs [3] and the hydrolysis of β-glucuronides can contribute significantly to the overall biological activity or toxicity of a xenobiotic.

Since the glucuronidation of a parent drug or its metabolite(s) may play a role in the overall pharmacological activity of the compound, it is often necessary to measure both free (nonconjugated) and total (conjugated plus nonconjugated) drug and metabolite concentrations. Total drug concentrations are usually determined by off-line hydrolysis of the conjugates. This is a multistep process involving incubation with β-glucuronidase, extraction from the

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0378-4347/98/$19.00 
PII: S0378-4347(98)00229-1

biological matrix and analysis by a suitable chromatographic method. These procedures can be time consuming, expensive and may yield unreproducible results because of the instability of the enzymes in the assays [4,5].

One approach to the reduction of the time and expense involved in the deconjugation of glucuronides is the development of immobilized β-glucuronidase reactors for on-line conversion of these conjugates. The feasibility of this approach has been previously demonstrated by the covalent immobilization of solubilized rat liver uridine glucuronosyltransferase (UDPGT) on a cyanogen bromide-activated agarose matrix [6] and on Sepharose beads [7,8] as well as by entrapment into alginate beads in the presence of polyethyleneimine [9]. These immobilizations produced viable enzymes for in vitro studies. However, these supports could not be used in an on-line HPLC format.

An HPLC-compatible immobilized enzyme reactor (IMER) containing UDPGT has also been reported [10]. In this study, nonsolubilized rat liver microsomes were noncovalently immobilized on an immobilized artificial membrane (IAM) HPLC chromatographic support. The resulting UDPGT–IMER was coupled to a second IAM column and used on-line to study the synthesis of the glucuronides of 4-nitrophenol and 4-methylumbelliferone.

This manuscript reports the development of an IMER containing covalently immobilized β-glucuronidase {BG-IMER} and its application in an on-line HPLC system. The activity of the BG–IMER was compared to nonimmobilized β-glucuronidase in the deconjugation of a series of glucuronides. In addition, the activity and stability of the BG–IMER were investigated at various pHs and temperatures.

## 2. Experimental

### *2.1. Materials*

#### *2.1.1. Glucuronide substrates and respective alcohols*

Compounds: (**1**) 4-methylumbelliferyl-β-D-glucuronide (methylumbelliferone); (**2**) *p*-acetaminophen-β-D-glucuronide (acetaminophen); (**3**) 3′-azido-3′-deoxythymidine-β-D-glucuronide (Zidovudine); (**4**) phenyl-β-D-glucuronide (phenol); (**5**) chloramphenicol-β-D-glucuronide (chloramphenicol); (**6**) estradiol-17-β-D-glucuronide (estradiol); (**7**) morphine-3-β-D-glucuronide (morphine). Compounds **1–6** were purchased from Sigma (Oakville, Canada) while compound **7** was purchased from RBI (Natick, MA USA). The structures of these compounds are presented in Fig. 1.

#### *2.1.2. Chromatographic equipment*

A Spectra-Physics HPLC system purchased from Thermo Separation Products (San Jose, CA, USA) was used for this project. The system consisted of: an UV Spectra 100 variable wavelength detector and a Chromjet integrator linked up to a computer integration system; a single solvent (p1000) and a binary gradient (p2000) pump. A Rheodyne 7125

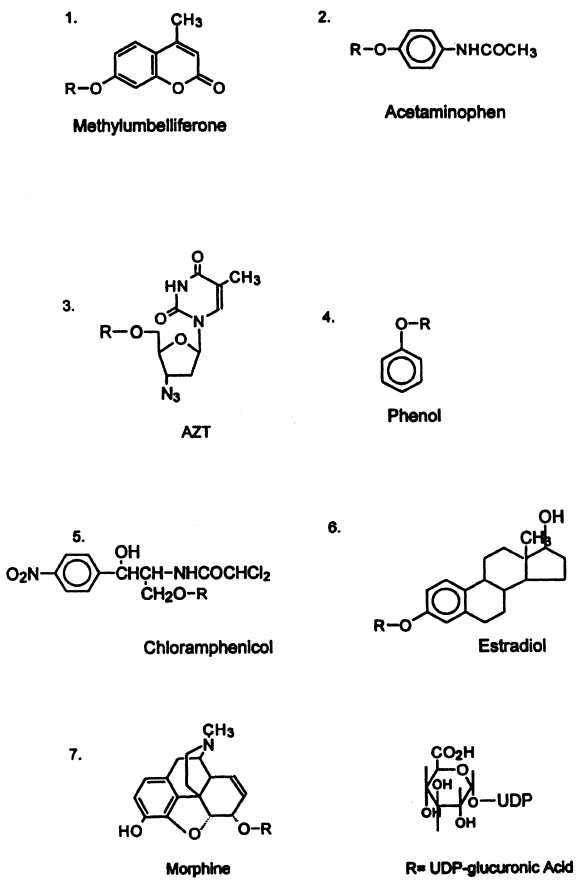



Fig. 1. Structures of the glucuronides used in this study.

injector (Rheodyne, Cotati, CA, USA) with a 100-μl loop was used for injection. A second Rheodyne injector (7125) and a Rheodyne switching valve (7010) were used for on-line connection between 2 systems to be run on-line and independently from each other. Column temperature was controlled with a Flatron Systems TC-50 column temperature controller (ASTEC, Whippany, NJ, USA.).

*2.1.3. β-Glucuronidase column*

The β-glucuronidase column was synthesized according to previously published procedures [11]. A 200 A°, 5-μm epoxide silica support packed in a 50×4.6-mm stainless steel column was used in the synthesis (Kromasil 200, Eka Chemical, Bohus, Sweden). The β-glucuronidase immobilized on the support was isolated from *Escherichia coli* K12, Rnase negative and was purchased from Boehringer Mannheim (Laval, Canada). The enzyme had a specific activity of 10 U/ml at 25°C (20 U/ml at 37°C) with 4-nitro-phenyl-β-D-glucuronide as substrate (pH 7.0) and 910 U were immobilized on the stationary phase. When not in use, the column was stored at room temperature in a solution of ammonium sulphate (1.0 *M*), sodium azide (0.01 *M*), potassium phosphate buffer (0.05 *M*, pH 7.0) (v/v/v).

*2.1.4. Reversed-phase columns*

Octylsilane ($C_8$) and an octadecylsilane ($C_{18}$) stationary phases were used for the on-line experiments. Both columns were 4.6×250-mm I.D., stainless steel columns packed with 100 A° pore size and 5-μm particle size silica purchased from Regis (Morton Grove, IL, USA). Column filters were also purchased from Regis.

*2.1.5. Mobile phases*

Mobile phase A: consisted of HPLC-grade acetonitrile (Fisher Scientific, Ottawa, Canada). The solvent was filtered and degassed under nitrogen for 15 min. Mobile phase B: consisted of ammonium acetate buffer (0.01 *M*, pH 5.0). The buffer was made by dissolving 0.8 g of HPLC-grade ammonium acetate (Fisher Scientific) per liter of Millipore water (Millipore, Bedford, MA, USA) and the pH was adjusted with acetic acid. The solution was filtered through a Millipore filtering apparatus using a 47 mm, 0.2-μm nylon filter purchased from WSC (Ottawa, Canada) and degassed under nitrogen for 30 min.

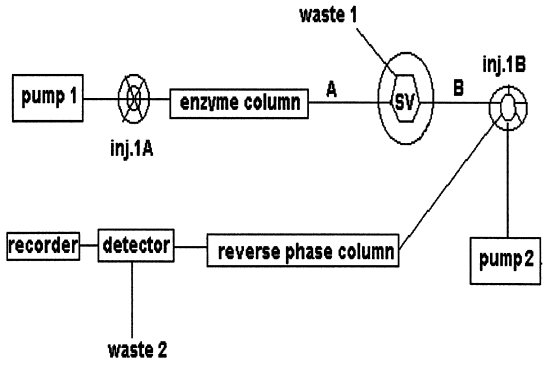
*2.1.6. Coupled HPLC system*

A schematic diagram of the coupled HPLC system is presented in Fig. 2. The following experimental steps were followed:

(1) Preparation of the system for on-line injection: Stop pump 2; place inj.IB to inject position; switch SV to B position; start pump 1 with mobile phase B through the on-line system at a flow-rate of 0.1 ml/min; place inj.IA into load position to inject an aliquot of the substrate.

(2) Injection of the substrate: 100-μl of the substrate was loaded into inj.IA and the valve switched to the inject position at a flow-rate of 0.1 ml/min for 30 min; after 30 min the SV valve was switched back to position A (flow of 0.3 ml/min), inj.IB was changed to the load position and pump 2 was turned back on at the initial flow-rate of 0.3 ml/min and mobile phase B. The analytes were concentrated on the reversed-phase column bed. The conjugated and deconjugated peaks were then separated by a HPLC gradient system.

(3) Analysis of the substrate: Substrates were analyzed using either the $C_{18}$ or $C_8$ stationary phases, at ambient temperature or 37°C. Mobile




**1A: Rheodyne injector A**

**1B: Rheodyne injector B**

**SV: Switching valve**

Fig. 2. Diagram of the on-line HPLC injection system.

phases A and B were used at different gradients for each substrate. The samples were detected at UV wavelengths of 280 nm (substrates **1**, **5**, **7**); 254 nm (**2, 4**); 270 nm (**3**); and 230 nm (**6**).

*2.1.7. β-Glucuronidase solution*

The β-glucuronidase was isolated from *Escherichia coli* K12, Rnase negative (Boehringer Mannheim) and was purchased as a solution with a specific activity of 10 U/ml at 25°C (20 U/ml at 37°C) using 4-nitrophenyl-β-D-glucuronide as substrate (pH 7.0). The strength of the solution in units of protein/ml of suspension is at least 200 units of enzyme protein/ml (suspension) at 37°C and 100 units of enzyme protein/ml at room temperature.

*2.2. Methods*

*2.2.1. Validation of the activity of the column*

Stock solutions of the glucuronide substrates were prepared by dissolving the glucuronides in ammonium acetate buffer (0.01 *M*, pH 5.0). Depending on the solubility of the glucuronide, different concentrations of the substrates were achieved (range 0.003 to 2.0 m*M*). The solutions were injected onto the BG–IMER and the mobile phase B was passed through the BG–IMER for 30 min at a flow-rate of 0.1 ml/min. The eluent from the enzyme column was passed directly on to a reversed-phase analytical column. Blanks were used as negative controls and respective alcohols of the glucuronide substrates as positive controls for retention time verification. The controls were injected on-line through the enzyme column and off-line directly onto the analytical column.

*2.2.2. Comparison of the activity of free and immobilized enzymes*

Concentrations of the substrates which were proportional to the substrate concentration injected into the BG–IMER were spiked into 250-μl of the enzyme solutions. The quantity of nonimmobilized enzyme was 910 U which was equal to the units of enzyme immobilized on the BG–IMER. Incubations were carried out for 30 min at room temperature. The reaction solutions were then centrifuged for 10 min at 12 000 *g* and 100-μl of the supernatant was immediately injected into HPLC for analysis or stored at −80°C until analyzed. HPLC gradients were modified from the HPLC gradients performed on immobilized enzyme column to separate the endogenous protein peaks extracted from the free enzyme and peaks from the substrates. Blanks and alcohols (respective to their glucuronide metabolites) were used as negative and positive controls.

*2.2.3. Effect of flow-rate on enzyme activity*

The effect of flow-rate through the BG–IMER column was undertaken using *p*-acetaminophen-β-D-glucuronide (APAP-g), and 3′-azido-3′-deoxythymidine-β-D-glucuronide (AZT-g) at concentrations of 0.17 m*M* and 0.04 m*M*, respectively. After injection onto the BG–IMER, one column volume (1.5 ml) was passed through the BG–IMER and onto the analytical column. The enzyme column was used at room temperature at flow-rates from 0.1 to 1.0 ml/min at 0.1 ml/min increments producing substrate–enzyme contact times of 15 to 1.5 min.

*2.2.4. Effect of pH on the activity of the BG–IMER*

The effect of pH on the activity of the BG–IMER was investigated using 0.01 *M* ammonium acetate buffers at pH 4.0, 5.0 and 6.0. APAP-g (1.0 m*M*) and AZT-g (0.04 m*M*) were the test substrates.

*2.2.5. Effect of temperature on the activity of the BG–IMER*

A partially pH deactivated BG–IMER was used at ambient temperature and 37°C to determine the effect of temperature on the hydrolytic activity of the BG–IMER. APAP-g was used as the substrate at a concentration of 0.27 m*M*.

## 3. Results and discussion

In the coupled LC system, the glucuronide substrates were injected onto the BG–IMER in an ammonium acetate buffer and the resulting substrate/product mixture transferred to a reversed-phase analytical column. The solutes from the BG–IMER were concentrated at the head of the analytical column and then analyzed using ammonium acetate: acetonitrile gradients. Representative chromatograms obtained from the chromatography of AZT-g on the

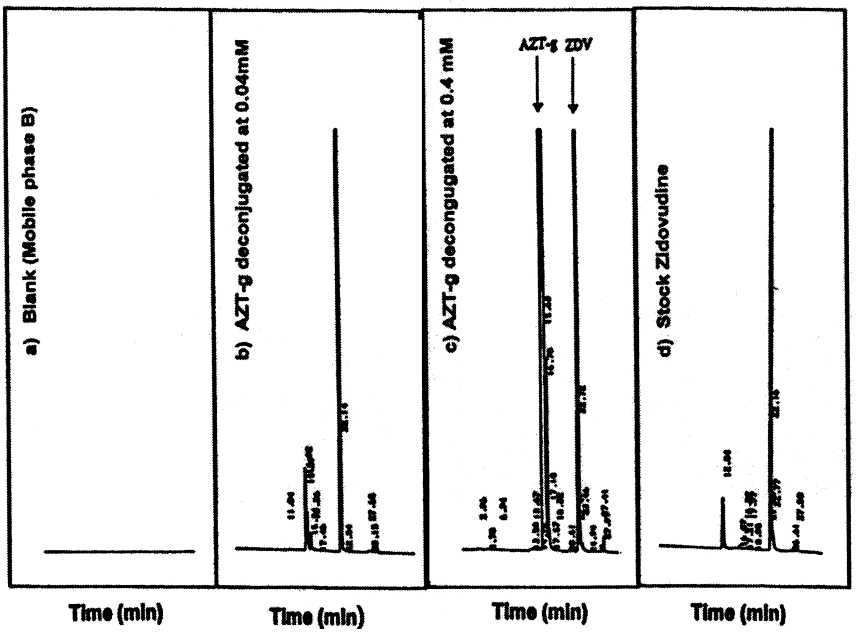



Fig. 3. Chromatograms representing the deconjugation of AZT-g in immobilized enzyme. (a) Blank (mobile phase B); (b) AZT-g deconjugated at 0.04 m*M*; (c) AZT-g deconjugated at 0.4 m*M*; (d) stock Zidovudine (AZT).

coupled BG–IMER/analytical column system are presented in Fig. 3. The BG–IMER column was used for 8 weeks (0.01 *M* ammonium acetate pH 5.0) without significant changes in the activity.

The enzymatic activity of the BG–IMER was evaluated using seven glucuronides. The results from this experiment are presented in Table 1 and demonstrate that at an average substrate concentration of 2.00 m*M*, >98% hydrolysis was achieved for five of the seven glucuronide substrates, while with AZT-g and morphine-g, the extent of hydrolysis reached only 20 and 11%, respectively.

When the concentration of AZT-g was decreased from 2.00 to 0.04 m*M* and then to 0.02 m*M*, the extent of hydrolysis increased from 20 to 76–89% and 98%, Table 1. This suggests that the low level of hydrolysis of ATZ-g observed at the higher concentration was due to saturation of the glucuronidase isoforms metabolizing AZT-g. The opposite results were obtained for morphine-g, where a decrease in substrate concentration from 2.00 to 0.04 m*M* and then to 0.02 m*M* resulted in a decrease in the extent of hydrolysis from 11 to <2% to no observable hydrolysis, Table 1. This suggests that low-affinity glucuronidase isoforms are responsible for the hydrolysis of morphine-g. This is consistent with the observation that morphine-g is relatively stable in the intestine of the dog [12].

### *3.1. Comparison of the activity of the free and the immobilized enzyme*

The enzymatic activity of the BG–IMER was compared to that of an equivalent amount of free β-glucuronidase using the seven glucuronide substrates. All of the glucuronides were hydrolyzed >98% by the nonimmobilized enzyme, Table 1. The hydrolytic activities of the immobilized and nonimmobilized enzymes were equivalent except with respect to the hydrolysis of AZT-g and morphine-g. The β-glucuronidase immobilized on the support and used in the enzyme incubations was isolated from *Escherichia coli* K12 and as such is a mixture of a number of β-glucuronidase isoforms as well as other enzymes. The results suggest that the immobilization process may be isoform selective. Thus, the glucuronidase subtypes primarily responsible for the hydrolysis of AZT-g and morphine-g are found in

Table 1
Results on validation of the activity of the enzyme: comparing deconjugation of substrates on immobilized and nonimmobilized enzyme

| Substrate | Enzyme type | Substrate concentration | | |
|---|---|---|---|---|
| | | 2.0–1.2 m*M* | 0.07–0.04 m*M* | 0.02 m*M* |
| | | % conversion | | |
| 1 | Immobilized | 98 | >98 | >98 |
| | Nonimmobilized | >98 | >98 | >98 |
| 2 | Immobilized | >98 | >98 | >98 |
| | Nonimmobilized | >98 | >98 | >98 |
| 3 | Immobilized | 20 | 76–89 | 98 |
| | Nonimmobilized | *>98* | *>98* | *>98* |
| 4 | Immobilized | >98 | >98 | >98 |
| | Nonimmobilized | >98 | >98 | >98 |
| 5 | Immobilized | >98 | >98 | >98 |
| | Nonimmobilized | >98 | >98 | >98 |
| 6 | Immobilized | N/A | >98 | >98 |
| | Nonimmobilized | >98 | >98 | >98 |
| 7 | Immobilized | 11 | <2 | 0 |
| | Nonimmobilized | >98 | >98 | >98 |

>98% refers to 100% deconjugation with <2.0% error.
N/A=Not available (substrate precipitated at 1.2–2 m*M* and therefore could only be injected at 0.5 m*M*).
Refer to Section 2, Section 2.1 and Fig. 1 for references to substrate numbers.

reduced concentrations on the BG–IMER relative to the other glucuronidase isoforms. A second possibility is that the immobilization process sterically hinders the active site of the AZT-g and morphine-g glucuronidase isoforms thereby reducing the observed activity. Both of these possibilities will be investigated through a further study of the structure–activity relationships on the BG–IMER and by the immobilization of single isoforms of UDPG–glucuronosyltransferase and β-glucuronidase, if the latter are available.

### *3.2. Effect of flow-rate on the extent of substrate hydrolysis*

The effect of the flow-rate through the BG–IMER on the extent of hydrolysis was investigated using *p*-acetaminophen-β-D-glucuronide (APAP-g) and AZT-g at concentrations of 0.18 and 0.04 m*M*, respectively. These substrates were chosen to differentiate between a substrate which appeared to be extensively hydrolyzed under all experimental conditions (APAP-g) and one whose hydrolysis was shown to be problematic (AZT-g), Table 1. The lower concentration of AZT-g was chosen because the previous experiments had demonstrated a >98% conversion at this concentration.

The flow-rates ranged from 0.1 to 1.0 ml/min and the experiment was carried out through one column volume. Thus, the substrate was in contact with the immobilized enzyme on the BG–IMER from 15 (0.1 ml/min) to 1.5 min (1.0 ml/min). The hydrolysis of APAP-g remained constant (>98%) throughout the range of flow-rates, Fig. 4. The deconjugation of AZT-g remained >98% up to a flow-rate of 0.4 ml/ml and then began to decrease to 70% at a

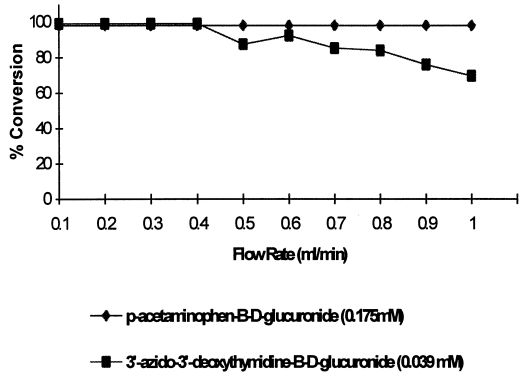



Fig. 4. A graph representing the results from flow-rate change on substrate (AZT-g)/enzyme contact (0.1–1.0 ml/min).

flow-rate of 1.0 ml/min. The results indicate that for glucuronides which are easily hydrolyzed on the BG–IMER, flow-rates of 1.0 ml/min or more can be used in the development of optimized analytical methods. For more difficult substrates such as AZT–g, lower flow-rates may be necessary to achieve quantitative conversions.

### 3.3. Effect of pH on BG–IMER activity

In this experiment, we investigated the activity of the column in relation to pH. The optimum enzymatic activity of the β-glucuronidase enzymes occurs between pH 5.0 to 7.5 [13]. Thus, the activity of the BG–IMER relative to the hydrolysis of APAP-g was determined at pH 4.0, 5.0 and 6.0.

The previous experiments were performed by passing glucuronide substrates through the BG–IMER column with 0.01 *M* ammonium acetate at pH 5.0. Under these conditions the BG–IMER column was stable for at least 8 weeks. At the completion of the experiment using 0.01 *M* ammonium acetate at pH 5.0 and 6.0, a >98% and 92% conversion was observed when APAP-g was used as the substrate. When the pH of the buffer was lowered to 4.0, circulated through the BG–IMER column until equilibration (30 min), the conversion of APAP-g was reduced to 46%. We then returned the BG–IMER column to 0.01 *M* ammonium acetate at the optimum pH of 5.0 and injected APAP-g through the column. We no longer achieved a >98% conversion but only a 20% conversion. We then preceded to test another substrate AZT-g (0.04 m*M*), to check if the reduction of activity by the BG–IMER column using APAP-g was substrate related. Similarly, at pH 5.0, a 90% hydrolysis of AZT-g was observed before exposure to pH 4.0 and only 12% conversion after. A graph representing these results are presented in Fig. 5. The effect of exposing the BG–IMER column to pH 4.0 was irreversible.

### 3.4. Effect of temperature on BG–IMER activity

APAP-g was passed through a BG–IMER which had been previously exposed to a pH 4.0 buffer. The results of this experiment are presented in a graph in Fig. 6. The experiment was carried out at ambient temperature and with a pH 5.0 buffer. Under similar

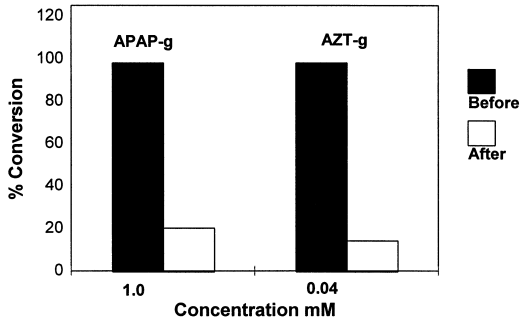



Fig. 5. A graph representing intrinsic activity of the column (assessed each time at pH 5.0) before (solid bar) and after (clear bar) exposing the column to a pH 4.0 buffer.

chromatographic conditions, >98% of the APAP-g had been hydrolyzed. However, after exposure to the pH 4.0 buffer, the extent of conversion had dropped to 30%. When the temperature of the BG–IMER was raised to 37°C, a >98% hydrolysis of the APAP-g was again obtained. The results indicate that temperature can be used to increase the enzymatic activity of the BG-IMER.

### 3.5. Deconjugation of chloramphenicol-β-D-glucuronide in urine

The application of the BG–IMER column in the deconjugation of glucuronides in urine has been investigated [14]. Fig. 7 is a chromatographic representation of the deconjugation of spiked chloramphenicol-β-D-glucuronide in human urine. The HPLC system was rearranged for an off-line direct injection of the urine samples and then on-line through the BG–IMER column for deconjugation and into a $C_8$ column for analysis of the products.

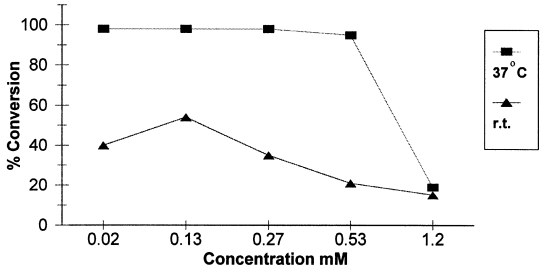



Fig. 6. A graph representing the effect of temperature on the deconjugation of APAP-g using a partially deactivated column.

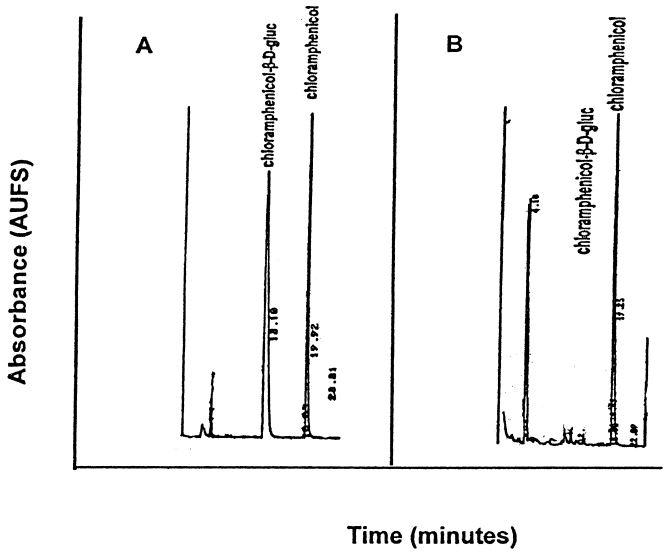



Fig. 7. Chromatograms representing (A) control standards chloramphenicol-β-D-glucuronide and chloramphenicol, without BG–IMER column and (B) spiked urine sample with hydrolyzed chloramphenicol-β-D-glucuronide (0.2879 m*M*), with BG–IMER column.

## 4. Conclusion

An on-line HPLC method has been developed for the deconjugation of glucuronides. The activity and stability of the BG-IMER was tested through various assays using different substrates. The results of these tests demonstrates that the BG–IMER has equivalent enzymatic activity for most substrates when compared to free β-glucuronidase solution. Its use is however much easier in the deconjugation of glucuronides. We are currently studying the application of this β-glucuronidase column for biological samples.

## Acknowledgements

This project was supported in part by a grant from BioChem Therapeutics. We wish to thank Dr.Terry Bowlin of BioChem Therapeutics for his general support.

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