Column packed immobilized β-glucuronidase and arylsulfatase for the cleavage of conjugates - Studies on the stability during storage and during repeated use, and on the avoidance of analyte carry-over

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Abstract

Purified β-glucuronidase (*GRD*) and arylsulfatase (*ARS*) were co-immobilized on an agarose gel matrix. The cleavage of conjugates using such immobilized enzymes was faster than using soluble enzymes (nitrophenyl conjugates were almost completely hydrolyzed within 15 min and morphine conjugates within 1h). Packing the immobilizates in columns allowed repeated use of the enzymes. Analyte carry over could be avoided by washing the column with methanol-acetate buffer (0.1 M containing 0.5 M NaCl, pH 5.2) (20/80). These columns allowed repeated use for at least 70 times. Therefore, we can conclude that cleavage of conjugates by recyclable immobilized *GRD/ARS* may be an effective and convenient alternative for the cleavage of conjugates and may be useful in the automation of sample preparation.

Keywords: immobilization, β-glucuronidase, arylsulfatase, cleavage of conjugates, stability, carry over of analyte

1. Introduction

In clinical and forensic toxicology or doping control drugs and poisons are usually identified in urine. In systematic toxicological analysis cleavage of conjugates is an important step. This is usually done by acid or enzymatic hydrolysis [1-2]. Acid hydrolysis is fast (15 min), complete and cheap, but it may lead to decomposition of analytes and formation of artifacts [2] and the extracts are rather dirty. The enzymatic hydrolysis avoids these disadvantages. β-Glucuronidase (*GRD*) and arylsulfatase (*ARS*) specifically hydrolyze glucuronide and sulfate conjugates and are used under mild pH and temperature

conditions. The disadvantages of the enzymatic procedure are that it needs long incubation times (several hours) and that matrix from the enzyme solution may contaminate the urine thus increasing the background. Furthermore, the enzyme preparations are relatively expensive. To avoid these disadvantages, we tried to increase the enzyme to substrate ratio by immobilizing an excess of purified enzymes and thereby to make the enzymes recyclable. For a repeated use the immobilized enzymes in the column should be sufficiently stable under storage and usage conditions and analyte carry over must be excluded.

2. Experimental

The determination of activities of GRD and ARS and the immobilization of purified GRD/ARS on activated agarose (Affi-Gel) and the column packing are described in [3]. 4-Nitrophenol (*NP*), 4-nitrophenyl glucuronide (*NPG*) and 4-nitrophenylsulfate (*NPS*) were simultaneously determined in urine by RP-HPLC, free morphine was determined in urine by GC-MS. Both methods as well as the different hydrolysis procedures are described in [3].

2.1 Determination of usage stability

One immobilizate column was continuously used in a heating block at 35°C for 70 hours. During this time 100 incubations were made (40 *NPG/NPS* spiked urine samples, 30 authentic urine samples containing morphine conjugates and 30 drug free urine samples). The hydrolysis efficiency was tested using *NPG* and *NPS* spiked urine samples. The columns were incubated for 5 min with 1 mL portions of a standard urine spiked with 10 mM *NPG* and *NPS*. The eluate was analyzed by RP-HPLC. The hydrolysis yields were calculated as part of the initial hydrolysis yield. The efficiency of the cleavage of the relatively stable morphine conjugates was studied by GC-MS determination of the free morphine after 1h of incubation of the authentic urine sample. The total morphine concentration determined after acid hydrolysis (15 min) was defined as 100 %.

2.2 Determination of analyte carry over

Blank urine was spiked with dihydrocodeine, paracetamol, phenobarbital (250,000 ng/mL), ibuprofen (25,000 ng/mL) and perazine (125,000 ng/mL). 1 mL portions of this urine were incubated in an immobilizate column and eluted with 10 mL of 0.1 M acetate buffer containing 0.5 M NaCl, pH 5.2 (standard acetate buffer). The column was then washed with either 10, 20 or 30 mL of 20 % methanol in standard acetate buffer and then twice with 10 mL of standard acetate buffer. In the last eluate the presence of the test substances was checked by GC-MS as described in the following:

10 mL of eluate (pH 5.2) were extracted with 10 mL of a mixture of dichloromethane-isopropanol-ethylacetate (1:1:3, v:v:v). The pH of the aqueous residue was adjusted to 8.6 - 8.9 with 1 M sodium hydroxide solution and then extracted with the same solvent mixture. The combined organic extracts were evaporated and the residue was dissolved in 100 µL of methanol and derivatized with 100 µL of an ethereal diazomethane solution [2] for 10 min at room temperature. After evaporation the residue was additionally derivatized with 100 µL of acetic acid anhydride-pyridine (3:2, v:v) for 30 min at 60°C. After evaporation the extract was dissolved in 100 µL of methanol containing 0.5 mg/mL methaqualon as internal standard. 1 µL of this solution was injected into the GC-MS (conditions c.f. [4]).

The GC-MS peak areas were determined by autointegration of the peaks in the mass chromatograms of the following selected ions: m/z 235 for methaqualon, m/z 161 for methylated ibuprofen, m/z 232 for methylated phenobarbital, m/z 109 for acetylated paracetamol, m/z 343 for acetylated dihydrocodeine and m/z 339 for perazine. A methanolic solution containing 250,000 ng/mL of each drug was used as external standard. Dilutions of this methanolic standard were used to determine the limits of detection of the analytes.

3. Results and discussion

3.1 Usage stability

As shown in Fig. 1, a decrease of both enzyme activities could be observed down to 60-80% of the initial value determined as described above (c.f. 2.1). Nevertheless, the amount of free morphine yielded after 1h of incubation did not alter over the whole 70 hours. This demonstrated that the column had enough hydrolyzing capacity even if some enzyme was lost. The immobilizate columns could thus be reused for at least 70 incubations giving a reproducible hydrolysis yield of morphine conjugates of 97.1±5.1 % (n=30).

3.2 Determination of analyte carry over

Ibuprofen, paracetamol, dihydrocodeine, phenobarbital and perazine were chosen as they are widely used and applied in high doses. Furthermore, in our experience they all are known for analyte carry over. Simple elution with 10 mL of standard acetate buffer was not sufficient to completely remove these substances from the immobilizate column. As shown in Table 1, washing with 30 mL of 20 % methanol in standard acetate buffer was sufficient to completely remove the test substances from the column.

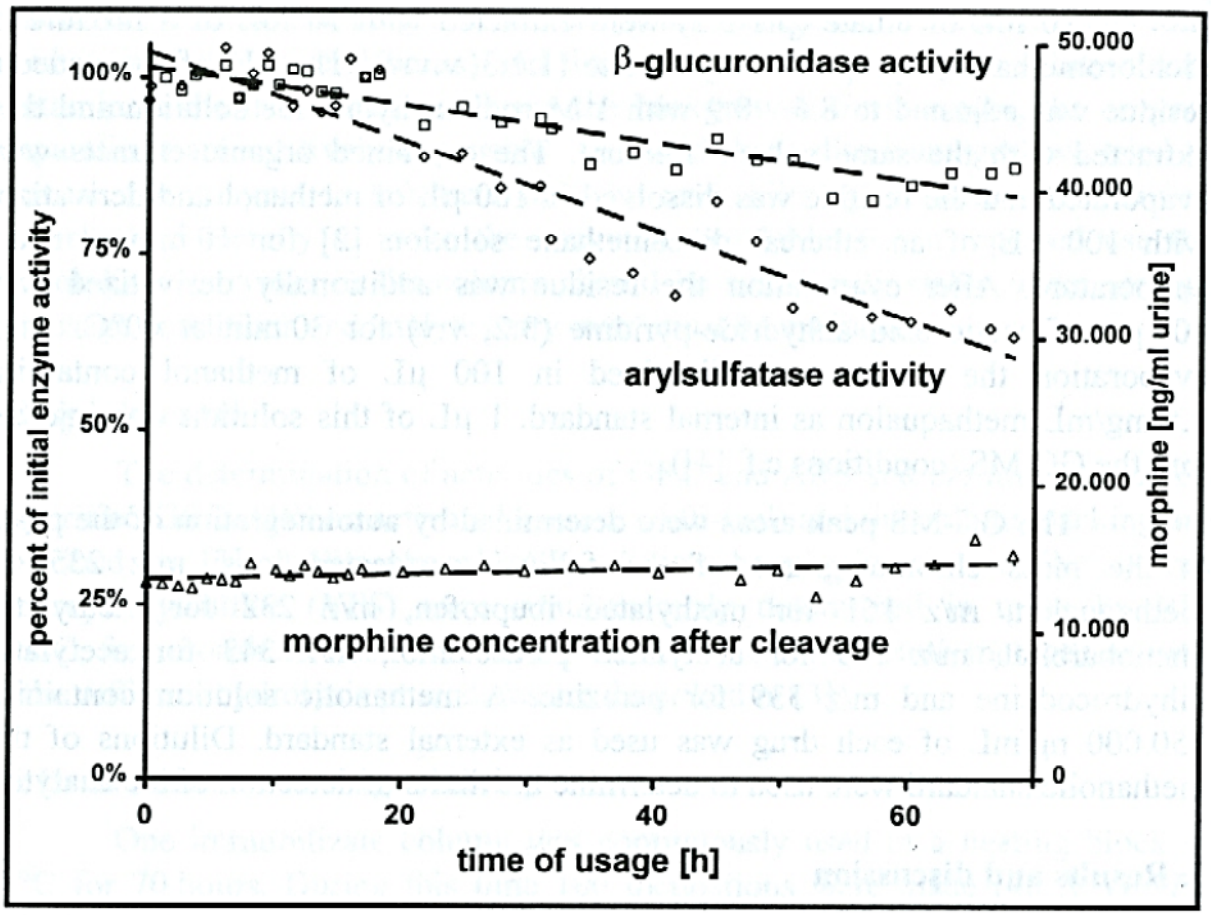

Fig. 1. Usage stability of the column packed immobilizate

Table 1. Analyte carry over after washing with different volumes of 20 % methanol in acetate buffer

Substance	urine conc.	LOD	analyte carry over [%] after washing with MeOH 20 % in acetate buffer		
	[ng/ml]	[ng/mL]	10 mL	20 mL	30 mL
Ibuprofen	25,000	< 10	nd	nd	nd
Paracetamol	250,000	< 1000	nd	nd	nd
Dihydrocodeine	250,000	< 500	nd	nd	nd
Phenobarbital	250,000	< 10	0.04 %	nd	nd
Perazine	125,000	< 250	1.3 %	0.08 %	nd

nd = not detected

4. Conclusions

Column packed immobilizates of purified GRD and ARS have proved to be nearly as fast as acid hydrolysis for the cleavage of 4-nitrophenol conjugates. Even the relatively stable conjugates of morphine could almost completely be cleaved within 1 h. The columns could be reused for at least 70 incubations without relevant loss of hydrolysis yields. Analyte carry over could be excluded with a washing step. Therefore, cleavage of conjugates by recyclable immobilized *GRD/ARS* may be an alternative for cleavage of conjugates and may be useful in automation of sample preparation.

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6. References

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