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USE OF A POST-COLUMN IMMOBILIZED β-GLUCURONIDASE ENZYME REACTOR FOR THE DETERMINATION OF DIASTEREOMERIC GLUCURONIDES OF FENOLDOPAM IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A post-column enzyme reactor, containing β-glucuronidase immobilized on controlled-pore glass beads, was developed for use in the high-performance liquid chromatographic (HPLC) analysis of glucuronide metabolites using electrochemical detection. The reactor performance was evaluated with glucuronide conjugates of the new antihypertensive agent, fenoldopam [6-chloro-2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-1H-3-benzazepine-7,8-diol]. These conjugates, which are electrochemically inactive at 0.6 V *vs.* Ag/AgCl, were separated by HPLC and passed directly into the post-column β-glucuronidase reactor, which converted the glucuronides to their electrochemically active aglycone, fenoldopam. The enzyme reactor converted > 80% of the entering glucuronide to fenoldopam and produced a linear response for fenoldopam glucuronide in the range 0.4–200 ng injected on-column. The reactor performance was optimal when the mobile phase (methanol–acetate buffer) contained 0–25% methanol, but the efficiency gradually declined thereafter until, at 50% methanol, the reactor was inactive. The working pH range for the mobile phase was 5.5–8.0, with a performance optimum at pH 6.0. The reactor displayed marked stability during usage (> 4 months) and during storage (> 6 months). The reactor did not hydrolyze the 8-O-sulfate conjugate of fenoldopam but did convert the 1(*R*) and 1(*S*) diastereomers of fenoldopam-7-O-β-glucuronide and 1(*S*)-fenoldopam-8-O-β-glucuronide to fenoldopam. An assay was developed for 1(*R*)-fenoldopam-7-O-β-glucuronide in plasma and urine by using the deschloro, des-4'-hydroxy analogue of fenoldopam glucuronide as the internal standard. The assay was linear in the range 4–1600 ng/ml. The within-day and between-day coefficients of variation for the method were less than 7% at three plasma fenoldopam glucuronide concentrations.

INTRODUCTION

Conjugation of drugs with D-glucuronic acid is an important metabolic mechanism in humans and is frequently the final step in many metabolic transformations.

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Biliary excretion of these glucuronide conjugates may occur, followed by subsequent hydrolysis via gut microflora and reabsorption into the portal and systemic circulation[1]. This enterohepatic recirculation has proven to be significant for many drugs. In order to have a better understanding of these biotransformation and excretory processes, sensitive analytical methods are required for measurement of these compounds and their glucuronide conjugates in biological systems. Glucuronide conjugates are usually analyzed in an indirect fashion by chemical or enzymatic hydrolysis, followed by subsequent quantitation of the aglycones. The hydrolysis techniques present several problems that must be considered before use, including: the introduction of impurities into samples for trace analysis, lengthy incubation times, undesirable chemical reactions, the occasional presence of enzyme inhibitors in biological samples, and variability in the extent of hydrolysis. In addition, hydrolysis techniques conducted prior to chromatography preclude the analysis of regio- and diastereomeric glucuronides. Therefore, the use of a post-column enzyme reactor, containing immobilized β-glucuronidase, for on-line hydrolysis of glucuronide conjugates in a high-performance liquid chromatographic (HPLC) system was explored.

Fenoldopam I (Fig. 1) [SK&F 82526, 6-chloro-2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-1H-benzazepine-7,8-diol] is a new antihypertensive agent, which is currently undergoing clinical trials[2–4]. Fenoldopam contains three potential sites for O-glucuronidation and also contains an asymmetric center at C-1, which gives rise to various diastereomeric glucuronide metabolites. These diastereomers were readily separated by reversed-phase HPLC; however, their detection was severely limited by the high potential equired for their oxidation compared to the parent aglycone. The existence of diastereomers and the necessity of a hydrolysis step to liberate the parent aglycone made these glucuronides ideal subjects for evaluation of the post-column β-glucuronidase reactor performance. In addition, fenoldopam is metabolized in part to O-sulfate conjugates, thereby providing another analyte for which the specificity of the enzyme reactor could be evaluated. This report describes the preparation and optimization of the immobilized β-glucuronidase enzyme reactor and its adaptation to a post-column HPLC system for the on-line determination of the diastereomeric glucuronide conjugates of fenoldopam in plasma and urine in conjugation with the electrochemical detector.

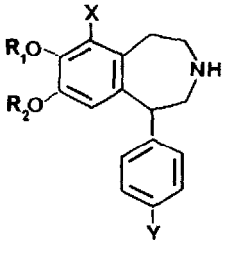


Fig. 1. Structures of fenoldopam, fenoldopam glucuronide and internal standard.

	R_1	R_2	X	Y
Fenoldopam	H	H	Cl	OH
1(*R*)- or 1(*S*)-fenoldopam-7-O-β-glucuronide	$C_6H_9O_6$	H	Cl	OH
1(*S*)-fenoldopam-8-O-β-glucuronide	H	$C_6H_9O_6$	Cl	OH
Internal standard (I.S.)	$C_6H_9O_6$	H	H	H

MATERIALS AND METHODS

Chemicals

1(*R*)-Fenoldopam-7-O-β-glucuronide (7-GLU), 1(*S*)-fenoldopam-7-O-β-glucuronide, 1(*S*)-fenoldopam-8-O-β-glucuronide and the internal standard (I.S.) were synthesized by incubation with immobilized rat-liver UDP-glucuronyl transferase according to methods to be published elsewhere[5]. HPLC-grade methanol was used (Fisher Scientific, Pittsburgh, PA, U.S.A.). All other chemicals were of analytical grade and were obtained from commercial suppliers. β-Glucuronidase (Type IX, from *Escherichia coli*) and glutaraldehyde were obtained from Sigma (St. Louis, MO, U.S.A.). Aminopropyl controlled-pore glass (500 Å pore diameter, 125–177 μm particle size) was obtained from Pierce (Rockford, IL, U.S.A.).

Mobile-phase buffers

Citrate–acetate buffer (pH 4.0). Sodium acetate trihydrate (11.0 g), citric acid monohydrate (10.5 g), sodium hydroxide (4.9 g), disodium EDTA (0.335 g) and acetic acid (37.5 ml) were dissolved in 1 l of deionized water.

0.2 M Acetate buffer (pH 6.0). After dissolving 54.4 g of sodium acetate trihydrate in 2 l of distilled water, the pH was adjusted to 6.0 with 0.2 *M* acetic acid.

Mobile phases

Fenoldopam assay. Methanol–citrate-acetate buffer (pH 4.0) (20:80, v/v).

7-GLU assay. Methanol–0.2 *M* acetate buffer (pH 6.0) (6:94, v/v).

Enzyme immobilization. Aminopropyl Controlled-Pore Glass (CPG) beads (3.0 g) were mixed under vacuum at 25°C for 1 h with 9 ml of a 2.5% (v/v) solution of glutaraldehyde in 0.05 *M* potassium phosphate (pH 7.5). The activated beads were washed (5 times) with cold phosphate buffer (0.05 *M*, pH 7.5) and mixed with 10 ml of a solution of β-glucuronidase (400 mg) in 0.05 *M* phosphate buffer (pH 7.5). The slurry was rotated under vacuum at 4°C for 16 h. The support was washed five times with 0.1 *M* Tris–HCl buffer (pH 7.5) and finally suspended in the same buffer. The amount of protein coupled to the support was determined by the difference in protein concentration of β-glucuronidase solution used before and after the immobilization process. The CPG-bound enzyme was slurry-packed by hand into a stainless-steel column (50 × 2.1 mm I.D.). The column reactor was placed in a continuous-flow HPLC system.

Standard solutions. Stock standard solutions (417 μg/ml for 7-GLU or 117 μg/ml for the I.S.) were prepared in methanol by adding a few drops of concentrated ammonia. Appropriate dilutions of the stock solutions were made with 0.2 *M* acetate buffer (pH 6.0). All stock and working standard solutions were stored at 4°C.

Collection of clinical samples. Seven healthy male volunteers received an oral dose of 100 mg (free base) of fenoldopam mesylate. Blood samples were collected in heparinized Vacutainers (Becton-Dickinson, Rutherford, NJ, U.S.A.) and centrifuged at 3000 *g*. Samples of plasma (4.75 ml) were transferred to polypropylene tubes, containing 0.25 ml of 10% ascorbic acid (prepared daily), mixed, and stored immediately at −20°C. The urine was collected in polypropylene containers, containing sufficient ascorbic acid to give an approximate final concentration of 0.5% (w/v). The urine volume was measured, and aliquots were stored at −20°C.

Measurement of enzyme activity. 1(*R*) Fenoldopam-7-O-β-glucuronide (7-GLU) was incubated with immobilized β-glucuronidase at room temperature under various conditions of pH and methanol content to determine the optimum conditions for glucuronide hydrolysis. CPG-bound β-glucuronidase (200 μl) was washed (at least 3 times) with 3 ml of distilled water and then once with the appropriate buffer prior to use. One ml of 7-GLU (417 ng/ml in the appropriate buffer) was then added to the immobilized enzyme, the mixture was swirled for 2 min, and 20 μl of the solution were injected into the fenoldopam HPLC system. To determine the trace sulfatase activity present in the immobilized β-glucuronidase mixture, 200 μl of CPG-bound enzyme were washed with water and then with acetate buffer (0.2 *M*, pH 6.0). One ml of fenoldopam-8-O-sulfate solution (480 ng/ml in 0.2 *M* acetate, pH 6.0) was added to the washed enzyme and swirled for 2 min. The supernatant (20 μl) was analyzed for fenoldopam.

Extraction of 7-GLU from plasma

C_{18} Sep-Pak cartridges (Waters Assoc., Milford, MA, U.S.A.) were prepared by successively passing 10 ml of methanol, 20 ml of water and 2 ml of phosphate buffer (1.0 *M*, pH 7.5) through them. A 0.5-ml aliquot of plasma, 50 μl of 0.05 *M* acetic acid (containing standards when preparing standard curve), 100 μl of I.S. (11.7 μg/ml) and 0.5 ml of phosphate buffer (1.0 *M*, pH 7.5) were mixed in a polypropylene tube (100 × 17 mm I.D.). The sample was then poured into a syringe barrel, attached to a previously conditioned Sep-Pak cartridge, and vacuum was applied. After passage of the sample through the cartridge, the packing was rinsed twice with 10-ml portions of water, and the cartridge was then centrifuged to remove residual traces of water. The cartridge was eluted with 2 ml of 0.3 *M* acetic acid in methanol, the methanol was evaporated to dryness under nitrogen at 40°C, and the residue was dissolved in 200 μl of 0.05 *M* acetate (pH 6.0), containing 6% methanol. The aqueous phase was then transfered to an Autosampler vial (Sun Brokers, Wilmington, NC, U.S.A.) and 20–50 μl-aliquots of the final extracts were injected onto the 7-GLU HPLC system.

For measurement of glucuronide conjugates of fenoldopam in urine, the urine was diluted 1:100 with distilled water, and a 50-μl aliquot of the diluted urine was injected directly into the HPLC system.

Chromatography

A Model 110A HPLC pump (Beckman, Palo Alto, CA, U.S.A.) and an autoinjector (WISP Model 710B, Waters Assoc.) were used. Separations were carried out on Ultrasphere octadecyl silica columns (Beckman): 75 × 4.6 mm I.D. (3 μm particle size) for 7-GLU, and 250 × 4.6 mm I.D. (5 μm particle size) for fenoldopam. The electrochemical detector consisted of a single glassy carbon electrode, an Ag/AgCl reference electrode and an LC-4A amperometric controller (Bioanalytical systems, West Lafayette, IN, U.S.A.). A potential of +0.6 V for 7-GLU or +0.65 V for fenoldopam was maintained relative to the reference electrode. The mobile phase for 7-GLU was methanol–acetate (0.2 *M*, pH 6.0; mixed at a ratio of 6:94, v/v) and for fenoldopam, methanol–citrate-acetate buffer (pH 4.0; mixed at a ratio of 20:80, v/v). The mobile phases were pumped at a flow-rate of 1 ml/min. Degassing was achieved by filtering the solution through a 0.45-μm membrane filter (Type HA, Millipore, Bedford, MA, U.S.A.).

Quantitation

An 8-point calibration curve (0, 8.3, 20.9, 41.7, 83.4, 208.5, 417 and 834 ng/ml) was prepared daily for each set of samples. Peak-height data were collected with a Computer-Automated Laboratory System (Computer Inquiry Systems, Waldwick, NJ, U.S.A.), and the peak-height ratios of 7-GLU *vs.* I.S. were calculated. The following equation was used to quantitate 7-GLU: $[7\text{-GLU}] = (c/d - a)/b$ where b = slope of the regression line, a = y-intercept of the regression line, c = peak height of 7-GLU, d = peak height of the I.S.

Validation procedures

Three pools (low, medium and high concentrations) of the fenoldopam glucuronide were prepared by dissolving weighed amounts in known volumes of drug-free plasma. Five replicate samples from each pool were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve, prepared on the day of analysis. Since the urine assay involves direct analysis without sample preparation, no formal validation was considered to be necessary.

RESULTS

This section was divided into two parts, the first part deals with the evaluation of the enzyme column and the second part deals with the evaluation of analytical methods for fenoldopam-7-O-β-glucuronide. Unless otherwise specified, the 1(*R*)diastereomer of fenoldopam-7-O-β-glucuronide (7-GLU) was employed as the substrate for immobilized β-glucuronidase.

Evaluation and optimization of the enzyme reactor

Effect of pH. The effect of pH on the activity of the immobilized enzyme was examined in the pH range 3–8 (Fig. 2). Direct incubation of CPG-bound β-glucuronidase with 7-GLU in buffers (0.2 *M*, no methanol, at 28°C) of varying pH values showed optimal activity at pH 5.0 and no significant activity at pH 3.0. The experiment was repeated at 2 and 24 h after the initial testing of enzyme activity by incubating the same immobilized enzyme (after buffer wash) with fresh substrate. Results from the latter incubations (2 and 24 h) exhibited a marked loss of enzyme activity at lower pH values and a slight increase in activity at higher pH values. A pH of 6.0 was chosen for the HPLC mobile-phase buffer.

Choice of buffers. In addition to acetate, two additional buffers, monochloroacetate and citrate–acetate, were studied. Monochloroacetate buffer is commonly used in HPLC–ED systems, while citrate–acetate has previously been found to be the buffer of choice for fenoldopam analysis. The experiments were conducted by incubating the β-glucuronidase and 7-GLU in either monochloroacetate or citrate–acetate buffers. The immobilized enzyme was irreversibly inactivated in the presence of both monochloroacetate (0.015 or 0.15 *M*, pH 3.0) and citrate–acetate (0.15 *M*, pH 4.0) buffers.

Effect of methanol. CPG-bound β-glucuronidase was incubated with 7-GLU in acetate buffer (0.2 *M*, pH 6.0, 28°C), containing various percentages (v/v) of methanol (Fig. 3). In the range 0–25% methanol, the immobilized enzyme retained 100%

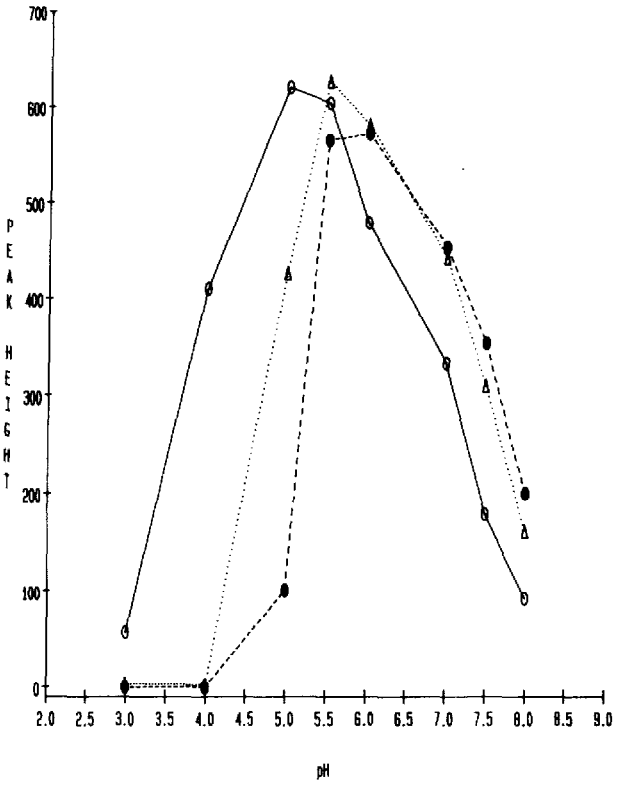


Fig. 2. Effect of pH on the stability of immobilized β-glucuronidase on CPG. 7-GLU was incubated for 2 min with CPG-bound β-glucuronidase after 0 (○), 2 (△) and 24 (●) h of exposure to buffer, the liberated fenoldopam being measured under the HPLC conditions described for fenoldopam assay.

activity. Above 25% methanol, the β-glucuronidase activity gradually declined until, at 50% methanol, no activity could be detected.

Effect of temperature. The analytical column, enzyme reactor and detector were placed in an incubator, and the temperature was varied between 23 and 37°C to determine the effect of temperature on β-glucuronidase activity. A standard solution of 7-GLU (8.34 ng) was injected in triplicate at each of three temperatures (23, 30 and 37°C). Since the temperature changes influenced the peak-height measurements by changing the retention times, peak areas were used to correlate enzyme activity with temperature. The enzyme reactor displayed an increase in β-glucuronidase activity as the temperature was increased (Fig. 4). Although the enzyme reactor exhibited the highest activity at 37°C, this temperature also resulted in a shortening of column life. For routine analyses, the enzyme reactor was operated at 28°C. Operation of the system at this temperature did not significantly change the enzymatic activity, even after four months of use.

Efficiency of the enzyme reactor. The efficiency of the enzyme reactor was determined in a two-step process. 7-GLU (125 ng) was injected into an HPLC system equipped with the enzyme reactor. The standard mobile phase (0.2 *M* acetate, pH 6.0, 6% methanol, 28°C) was used. The fraction corresponding to the glucuronide

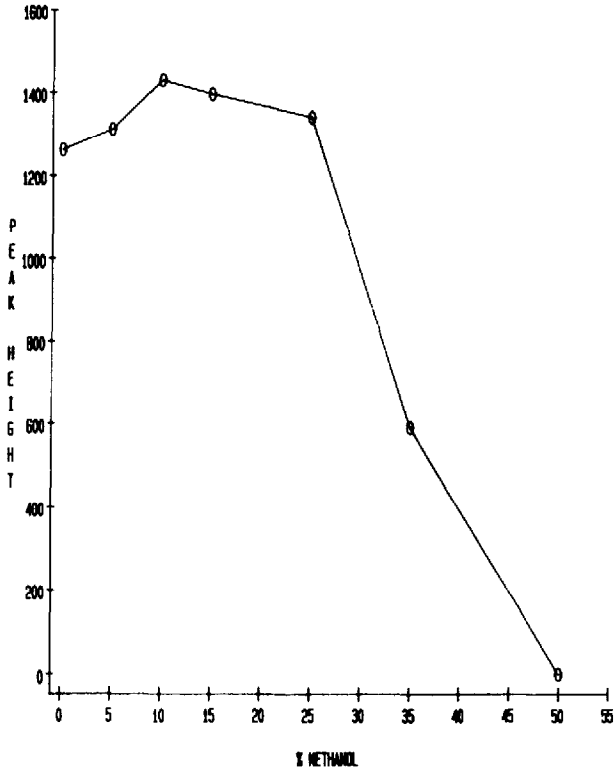


Fig. 3. Effect of methanol on enzyme activity of immobilized β-glucuronidase. 7-GLU was incubated for 2 min with CPG-bound enzyme in acetate buffer (0.2 *M*, pH 6.0), containing various amounts of methanol. The liberated fenoldopam was measured under the HPLC conditions described for fenoldopam assay.

peak was collected in a 5-ml volumetric flask. After adjusting the volume to 5 ml, an aliquot (50 μl) was analyzed for the parent aglycone, fenoldopam, with the fenoldopam HPLC–ED assay. The molar ratio of aglycone recovered to the conjugate injected was used to determine reactor efficiency. The post-column reactor converted 83% of the fenoldopam glucuronide to the aglycone. Ideally, reactor efficiency could have been determined by injecting fenoldopam and 7-GLU into the same HPLC system. However, the prolonged retention time of fenoldopam under the chromatographic conditions for glucuronide precluded the direct approach.

Linearity of reactor response. The linearity of the post-column β-glucuronidase reactor was evaluated by injecting increasing quantities of 7-GLU into the HPLC–reactor–ED system. The enzyme reactor displayed remarkable linearity in hydrolyzing fenoldopam glucuronide conjugates in the range 0.4–200 ng on-column (Fig. 5). Injection of 7-GLU in amounts greater than 200 ng produced a non-linear response, due to the saturation of detector electronics.

Lower limit of detection. The lower limit of detection for 7-GLU in HPLC with the enzyme reactor and electrochemical detector was 100 pg. This quantity produced a signal-to-noise ratio of 5:1.

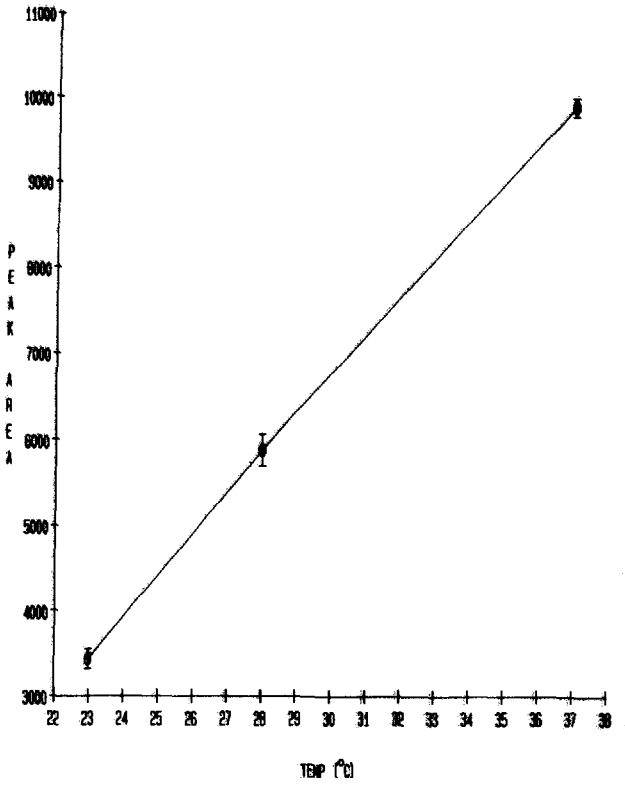


Fig. 4. Effect of temperature on the β-glucuronidase activity of the post-column reactor. 7-GLU (8.34 ng) was injected into the HPLC–reactor–ED system using the standard mobile-phase conditions described under Methods. Peak areas were determined as the temperature of the HPLC column and reactor were increased from 23 to 37°C.

Reproducibility (precision). The precision of the enzyme reactor was determined by repeated injection of a standard solution of 7-GLU (8.34 ng) into the column and measuring the peak heights of the liberated aglycone. The enzyme reactor displayed excellent reproducibility, yielding a coefficient of variation (C.V.) of 1.32% (Table I).

Specificity of immobilized β-glucuronidase. Since many drugs that are metabolized to glucuronides are also subject to conjugation with sulfate, the specificity of the enzyme reactor for glucuronides was examined. Both 7-GLU and fenoldopam-8-sulfate were incubated with immobilized β-glucuronidase in acetate buffer (0.2 *M*, pH 6.0). Under these conditions, fenoldopam was liberated in quantitative yields from the glucuronide conjugate, but no fenoldopam was released from its sulfate conjugate under identical incubation conditions (Fig. 6). The enzyme reactor displayed no specificity for the regio- and diastereomeric fenoldopam-O-glucuronides. The 1(*R*)- and 1(*S*)-fenoldopam-7-O-β-glucuronides as well as the 1(*S*)-8-O-β-glucuronide of fenoldopam were extensively hydrolyzed by the immobilized enzyme.

Particle size. It was anticipated that the use of smaller particles of CPG with a narrow size distribution would result in increased resolution. Immobilization of

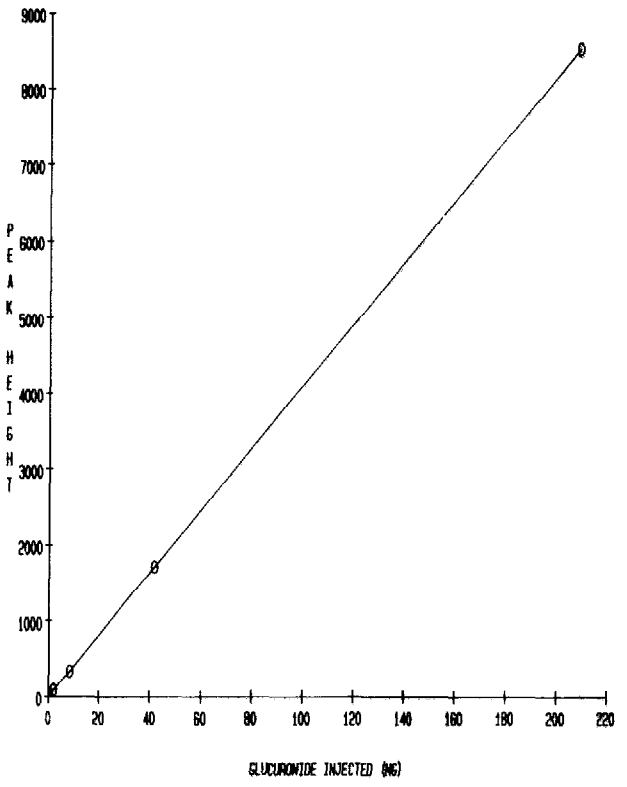


Fig. 5. Linearity of the β-glucuronidase activity of the post-column reactor. 7-GLU was injected in increasing quantities into the HPLC–reactor–ED system under the standard conditions described under Methods.

β-glucuronidase on CPG with smaller particle size (37–74 μm instead of 125–177 μm) yielded a support with the same enzyme activity, based on *in vitro* incubations. However, packing this support into a 50 × 2.1 mm I.D. stainless-steel column and placing it in line with the analytical column resulted in broader peaks. This result may be attributed to the difficulty experienced in packing particles of smaller diameter into the column. Future studies with smaller particles would require packing the column with a slurry-packing machine.

Operational and storage stability. the pH of the mobile phase, and the temperature of the enzyme reactor were optimized for enzyme activity, as well as for operational stability, as described above. The routine working conditions were as follows: mobile phase, 6% methanol in acetate buffer (0.2 *M*, pH 6.0); 28°C for column and enzyme reactor. During analysis of non-biological standard solutions, the enzyme reactor displayed no loss in enzyme activity, even after four months of continuous operation. During analysis of plasma extracts containing fenoldopam glucuronides, peak broadening was observed after 400–500 samples had been injected. The peak shape returned to normal when the enzyme reactor was replaced. CPG-bound β-glucuronidase was prepared in batches and stored at 4°C until needed. After

TABLE I

REPRODUCIBILITY OF ENZYME REACTOR

*No. of injection**	*Peak height*
1	201.3
2	204.2
3	202.9
4	199.9
5	198.5
6	199.1
7	200.9
8	198.8
9	198.4
10	198.9
11	197.3
12	195.9
13	194.8
14	196.3
15	198.9
	Mean = 199.1
	S.D. = ±2.63
	C.V. = 1.32%

* An 8.34-ng aliquot of 7-GLU was injected into the HPLC–reactor–ED system.

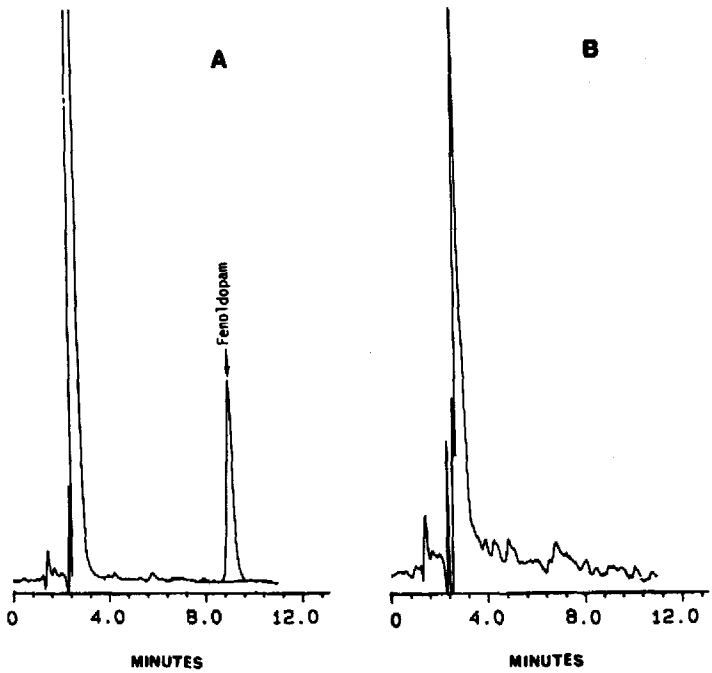


Fig. 6. Specificity of immobilized β-glucuronidase. Chromatograms resulting from the injection of fenoldopam standard solution (A) and fenoldopam-8-O-sulfate standard solution (B), after incubation with β-glucuronidase immobilized on CPG, to show the specificity of the enzyme reactor.

six months of storage at 4°C, the immobilized β-glucuronidase retained >90% of its original activity.

Evaluation of 7-GLU assay

A chromatogram, illustrating the separation of 1(*R*)- and 1(*S*)-fenoldopam-7-O-β-glucuronides and 1(*S*)-fenoldopam-8-O-β-glucuronide, is shown in Fig. 7. The diastereomeric 7-glucuronides are incompletely separated under these conditions, whereas the regioisomers are distinctly separated. Neither of the fenoldopam-O-β-glucuronides are electrochemically active at 0.6 V. However, the aglycone, fenoldopam, produced by hydrolysis in the enzyme column, undergoes oxidation to an orthoquinone at this potential. Based on these findings, an assay was developed for 1(*R*)-fenoldopam-7-O-β-glucuronide (7-GLU) in plasma. 7-GLU and I.S. (deschloro, des-4′-hydroxy-fenoldopam-7-O-β-glucuronide) were extracted from plasma on C_{18} Sep-Pak cartridges, eluted with the mobile phase, and analyzed with the HPLC–reactor–ED system.

Typical chromatograms for extracts of human plasma before and after oral administration of fenoldopam mesylate are shown in Fig. 8. The retention times for 1(*R*)-fenoldopam-7-O-β-glucuronide (7-GLU), 1(*S*)-fenoldopam-7-O-β-glucuronide, and I.S. were 6.7, 7.5 and 19.4 min, respectively. The retention time for 1(*S*)-fenoldopam-8-O-β-glucuronide was 8 min. The peak at 20.2 min is due to the diastereomer

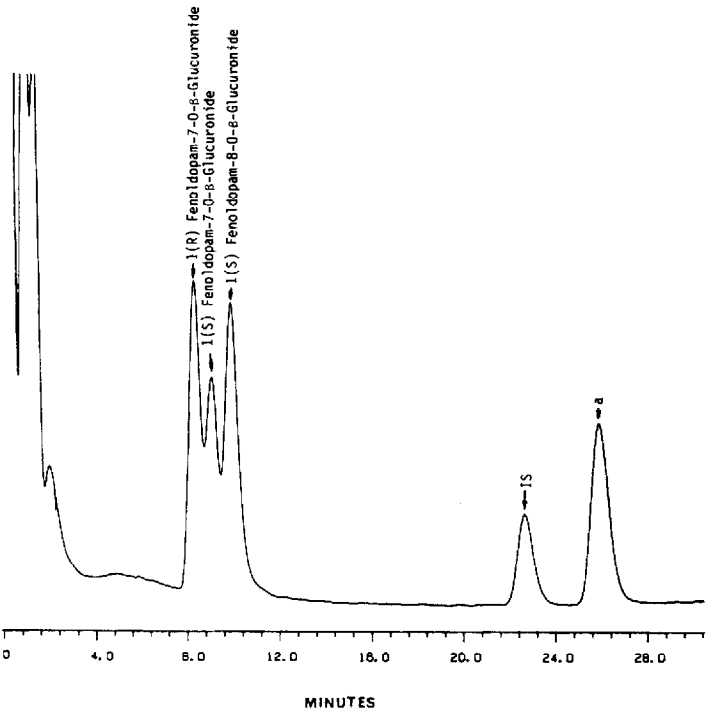


Fig. 7. Chromatogram of an aqueous standard solution of 1(*R*) and 1(*S*) diastereomers of fenoldopam-7-O-β-glucuronide, 1(*S*)-fenoldopam-8-O-β-glucuronide and I.S.; (a) is the diastereomer glucuronide conjugate of I.S. present in the solution.

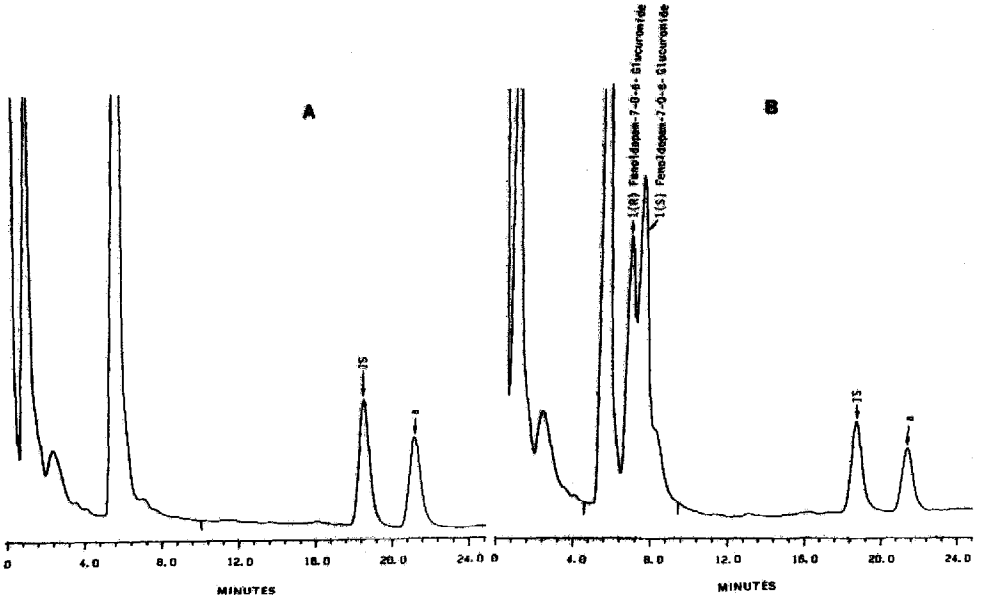


Fig. 8. Chromatograms of plasma extracts from a human subject before (A) and 0.5 h after (B) oral administration of fenoldopam. The concentrations of 1(*R*)- and 1(*S*)-fenoldopam-7-O-β-glucuronide are 156 and 160 ng/nl, respectively.

of glucuronide I.S., which was present in the I.S. solution. The glucuronides of fenoldopam and I.S. were adequately separated from one another, and the sulfate and O-methyl metabolites of fenoldopam did not interfere with glucuronide determination. The lower limit of detection and quantitation for fenoldopam glucuronides in human plasma were 1 and 2.5 ng/ml, respectively. Extraction recovery was determined by comparing the detector response to standards injected directly onto the column to standards extracted from the plasma. The mean recovery for 7-GLU and I.S. were 57 and 67%, respectively. Linear responses in 7-GLU *vs.* I.S. peak-height ratios were observed over the concentration range 4–1600 ng/ml plasma. Correlation coefficients were typically 0.99 for all plasma standard curves. The within-day and

TABLE II

ACCURACY AND PRECISION DATA FOR 7-GLU IN PLASMA

Concentration (ng/ml)	*Assay conc.* Mean ± S.D.*	*Within-day C.V.***	*Between-day C.V.****
16.68	18.04 ± 1.58	6.69	1.40
166.80	172.80 ± 8.42	6.07	4.88
834.00	839.00 ± 77.98	5.30	3.83

* Mean value on a single day.

** (Day 1 C.V. + Day 2 C.V. + Day 3 C.V.)/3.

*** $\frac{\text{Standard deviation of daily mean concn.}}{\text{Mean of individual daily mean concn.}} \times 100.$

TABLE III

SUMMARY OF CALIBRATION DATA FOR 7-GLU OBTAINED DURING THE ROUTINE ANALYSIS OF CLINICAL SAMPLE OVER FOUR DAYS

	Actual concentration (ng/ml)						
	8.3	*20.9*	*41.7*	*83.4*	*208.5*	*417*	*834*
n	4	4	5	4	4	4	4
Mean concn. found (ng/ml)	7.7	19.3	40.4	85.5	212.9	414.9	822.8
S.D.	0.32	1.09	1.73	3.89	12.03	5.43	23.2
C.V.(%)	4.1	5.6	4.3	4.5	5.6	1.3	2.8

between-day precision of the plasma methods are indicated by the C.V. shown in Table II.

Table III summarizes the mean, S.D. and C.V. data for 7-GLU calibration standards run during the routine analysis of clinical samples. The mean plasma concentrations of 1(*R*)-fenoldopam-7-O-β-glucuronide (7-GLU) and 1(*S*)-fenoldopam-7-O-β-glucuronide obtained after administration of fenoldopam mesylate (100 mg as base) to seven male volunteers are shown in Fig. 9.

In urine, 7-GLU was measured directly by injecting the diluted urine into the

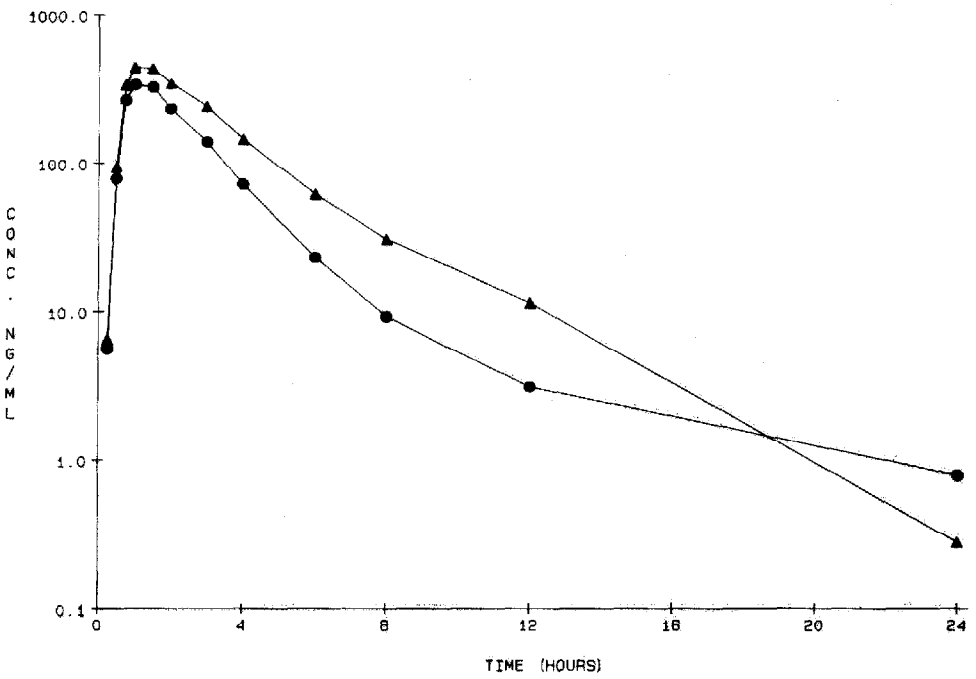


Fig. 9. Mean plasma concentrations of 1(*R*)-fenoldopam-7-O-β-glucuronide (●) and 1(*S*)-fenoldopam-7-O-β-glucuronide (▲) obtained after oral administration of 100 mg (as base) of fenoldopam mesylate to seven human male volunteers.

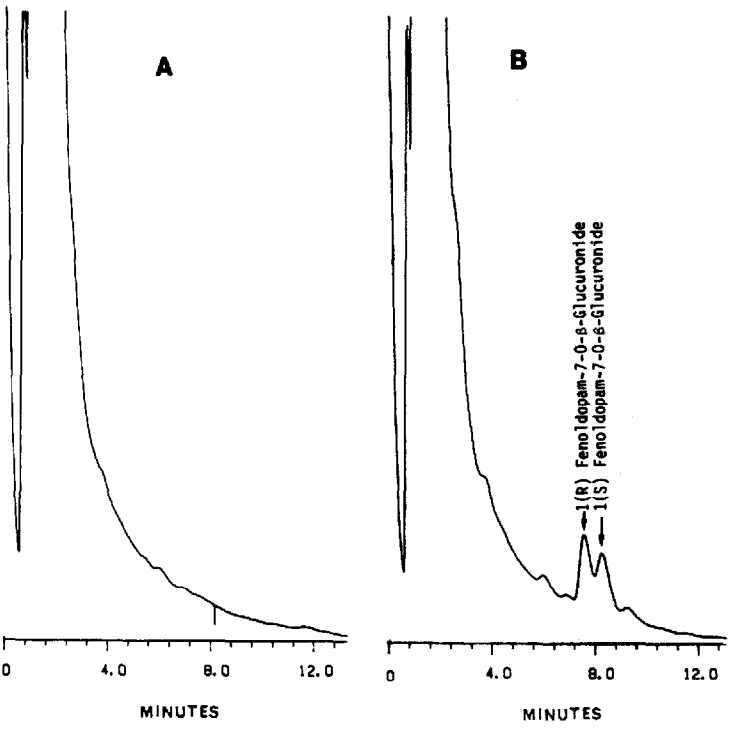


Fig. 10. Chromatograms of urine from a human subject before (A) and 0–24 h after (B) ingestion of fenoldopam. The concentrations of 1(*R*)- and 1(*S*)-fenoldopam-7-O-β-glucuronides are 3.51 and 2.14 μg/ml, respectively.

column and comparing the response with a standard solution of 7-GLU. Chromatograms obtained by injection of diluted urines before and after administration of fenoldopam mesylate to a human subject are shown in Fig. 10. Urinary concentrations of 7-GLU ranged from 3 to 34 μg/ml in this subject.

DISCUSSION

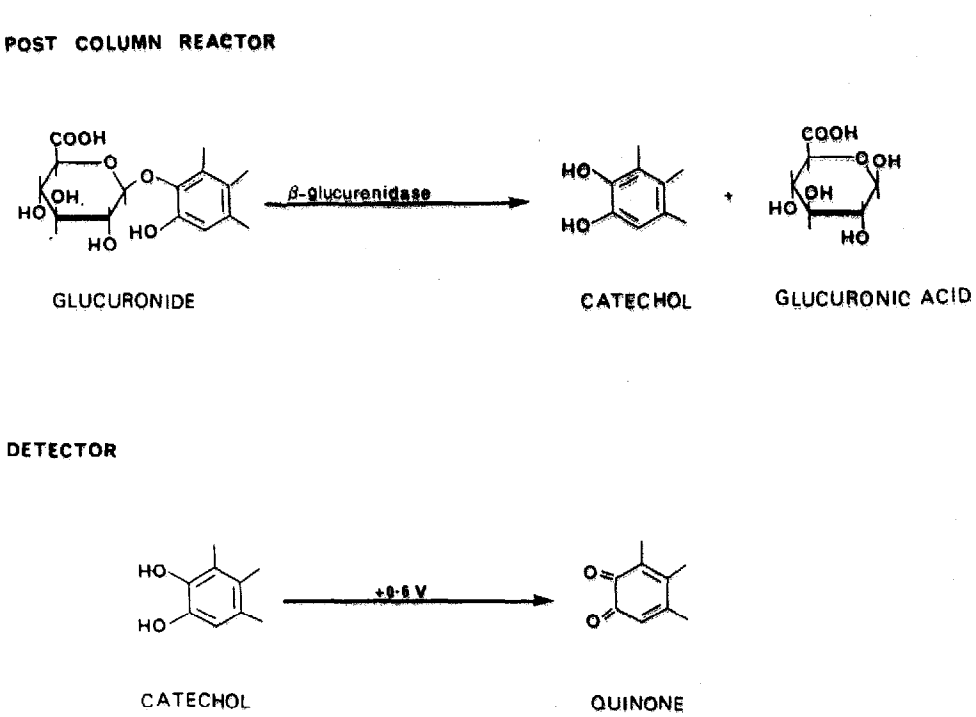
Although immobilized enzymes have become increasingly popular as analytical tools[6], the use of immobilized β-glucuronidase as an on-line reactor in conjunction with reversed-phase HPLC has not been extensively investigated as a routine analytical tool. β-Glucuronidase has been immobilized on the inner surface of Tygon tubing[7] and also on CPG[8]. Bowers and Johnson employed immobilized β-glucuronidase as an on-line pre-column reactor for the qualitative determination of steroid glucuronide conjugates[8]. Since the pre-column use of β-glucuronidase precludes the study of regio- and diastereomeric glucuronides, we employed this enzyme reactor in the post-column position to allow the study of diastereomeric fenoldopam glucuronides.

Application of immobilized enzyme technology to HPLC presents several advantages and also unique problems. The stability of the enzyme reactor in the mixed non-aqueous–aqueous mobile phases, pH of buffer, the type and ionic strength of

buffers, the temperature and the extra-column effects on chromatographic resolution are some of the primary issues that one must address before applying this technique to routine analysis of glucuronide conjugates[9]. In the present report, we have described the preparation of an immobilized β-glucuronidase enzyme reactor, its optimization and adaptation to the quantitative HPLC–ED analysis of fenoldopam glucuronide conjugates. β-Glucuronidase was attached by covalent linkage to the glutaraldehyde-derivatized aminopropyl CPG. The mechanical and chemical stability of CPG in packed-bed reactors is well suited to its use in HPLC. The duration of activation of aminopropyl CPG with glutaraldehyde is a critical step in the immobilization procedure. Under the experimental conditions employed, 47 mg of protein were immobilized per gram of support, yielding a coupling efficiency of *ca.* 70%. Activation of the support with glutaraldehyde for more than 60 min may result in excessive cross-linkage of amino groups of CPG, thus yielding a support with low protein binding and low enzyme activity.

The performance of the β-glucuronidase post-column reactor was evaluated by using the fenoldopam glucuronides as substrates for the enzyme. These glucuronides are difficult to analyze in low concentrations by HPLC–ED due to the high oxidation potential (> 1.0 V) required to oxidize them to the orthoquinone form. Operation of the electrochemical detector at 1.0 V and above has often resulted in rapid passivation of the electrode surface due to impurities present in the mobile phase, in complex chromatograms due to oxidation of endogenous components present in the plasma extracts, and in excessive baseline noise. However, fenoldopam, which contains a catechol moiety, is readily oxidized ay +0.6 V; a potential where oxidation of many extractable plasma components is minimized. In an effort to make use of these differences in oxidation potentials between fenoldopam and its glucuronide conjugate, the practicality of the on-line post-column β-glucuronidase reactor was explored. The immobilized β-glucuronidase reactor was positioned between the analytical column and an electrochemical detector (+0.6 V *vs.* Ag/AgCl) so that electrochemically inactive glucuronides eluted from the analytical column would be converted to the electrochemically active aglycone upon passing through the enzyme reactor and be oxidized to the orthoquinone form at the thin glassy carbon electrode, operating at a potential of +0.6 V *vs.* Ag/AgCl (Scheme 1). This system proved to be a practical, reliable and, in many ways, ideal approach to the analysis of fenoldopam glucuronide conjugates. Thousands of clinical samples have been successfully analyzed by this methodology. In contrast to the more traditional techniques, involving β-glucuronidase incubations or chemical hydrolysis, the enzyme reactor minimized sample manipulation, avoided introduction of impurities into the sample, eliminated chemical degradation, minimized variability and permitted the study of regio- and diastereomeric glucuronide conjugates.

A recent report from this laboratory has demonstrated the utility of an electrochemical post-column reactor (dual-electrode electrochemical detector) for the determination of drug conjugates[10]. In this approach, fenoldopam-8-O-sulfate and O-methyl fenoldopam conjugates were analyzed by HPLC in combination with a dual-electrode electrochemical detector. These conjugates were oxidized to the orthoquinone at the first electrode and quantitated at the second electrode upon reduction back to the catechol. Fenoldopam-7-O-β-glucuronide was isolated by the fenoldopam-8-O-sulfate extraction procedure, but the dual-electrode electrochemical



Scheme 1. Schematic representation of reactions occurring in the post-column β-glucuronidase reactor and at the thin-layer glassy carbon electrode of the electrochemical detector in the HPLC–reactor–ED system.

detector lacked the sensitivity necessary for measuring plasma levels of the glucuronide in clinical samples. The sensitivity problem was solved by hydrolyzing the glucuronide conjugates with soluble β-glucuronidase and subsequent quantitation of the aglycone. However, the HPLC–reactor–ED system has proven superior to the latter approach and is the preferred method for glucuronide analysis. In fact, the sulfate and glucuronide conjugates of fenoldopam are extracted simultaneously from plasma and the extract is analyzed twice: once for sulfate conjugates by the HPLC–dual-electrode ED system and then for glucuronide conjugates with the HPLC–reactor–ED system. In conclusion, we have successfully immobilized β-glucuronidase on CPG, optimized its operational stability, demonstrated its use as a post-column reactor for the hydrolysis of fenoldopam glucuronide conjugates, and utilized this novel approach for the quantitation of the 1(*R*)-diastereomer of fenoldopam-7-O-β-glucuronide in plasma and urine by HPLC–ED.

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