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SIMPLIFIED PROCEDURES FOR THE DETERMINATION OF FENOLDOPAM AND ITS METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION: COMPARISON OF MANUAL AND ROBOTIC SAMPLE PREPARATION METHODS

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SUMMARY

Quantitative analytical methods, based on high-performance liquid chromatography with electrochemical detection, were developed for fenoldopam and its metabolites in human plasma. Two extraction methods, a liquid–liquid extraction method for fenoldopam and its methoxy metabolites and a liquid–solid extraction procedure for the sulfate and glucuronide conjugates of fenoldopam were developed. The extractions can either be performed manually or by robot. The limit of detection for fenoldopam, its sulfate and methoxy metabolites was 0.025, 2 and 0.5 ng/ml, respectively, at a signal to noise ratio of 4. The intra-assay and inter-assay coefficients of variation for both manual and robotic extraction procedures were comparable. These methods were suitably selective and sensitive for pharmacokinetic and metabolic studies of fenoldopam

INTRODUCTION

Fenoldopam (SK&F 82526; 6-chloro-2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-1H-3-benzazepine-7,8-diol; Fig. 1), a potent agonist at peripheral dopamine-1 (DA-1) receptors, has been shown to produce beneficial effects including an improvement in renal blood flow and reduction in blood pressure following intravenous and oral administration [1,2]. Pharmacokinetic studies in healthy subjects have shown that following oral administration, fenoldopam undergoes extensive first-pass, as well as systemic metabolism, to a variety of sulfate, glucuronide and methoxy metabolites [3,4].

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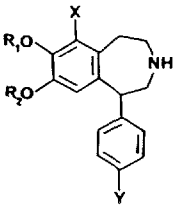

Compound	X	Y	R_1	R_2
Fenoldopam	Cl	OH	H	H
7-SO_4	Cl	OH	SO_3^-	H
8-SO_4	Cl	OH	H	SO_3^-
7-MEO	Cl	OH	CH_3	H
8-MEO	Cl	OH	H	CH_3
7-GLU	Cl	OH	$C_6H_9O_6$	H
IS-A*	H	H	H	H
IS-B*	H	H	SO_3^-	H
IS-C*	H	H	H	CH_3

* Internal standards

Fig. 1. Structures of fenoldopam, its metabolites and the internal standards.

Analytical methods previously developed [5] for the quantitation of fenoldopam and its metabolites in biological fluids were not fully validated for fenoldopam-7-sulfate (7-SO_4), 7-methoxyfenoldopam (7-MEO) and 8-methoxy-fenoldopam (8-MEO) in plasma due to a lack of availability of authentic standards. In addition, the plasma fenoldopam assay suffered from variations in total recoveries of compounds due to changes in extraction pH, as a consequence of variability in the addition of small volumes (26 μl) of sodium hydroxide. Moreover, the individual extraction procedures themselves were laborious and required large amounts of plasma to measure fenoldopam and its metabolites. In order to eliminate these problems, we modified the extraction methods. This report describes two extraction procedures that were developed for fenoldopam and its metabolites from plasma: a liquid–liquid extraction method for the extraction of fenoldopam and its methoxy metabolites and a liquid–solid extraction method for the extraction of sulfate and glucuronide conjugates of fenoldopam. These modified extraction procedures were also automated using a laboratory-based robot in an effort to increase sample throughput, precision and overall productivity.

EXPERIMENTAL

Chemicals

Fenoldopam, its metabolites and internal standards were obtained from Drug Substances and Products, SK&F Laboratories (Swedeland, PA, U.S.A.). HPLC-grade methanol, acetonitrile, ethyl acetate and Prep-Sep C_{18} columns for the robotic method were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other chemicals were of analytical grade and were obtained from commercial suppliers.

Internal standards

Three internal standards, IS-A, IS-B and IS-C, were employed for the quantitation of fenoldopam and its metabolites (Fig. 1). In the fenoldopam/methoxy metabolites assay, the internal standard solution contains both IS-A and IS-C at a concentration of 0.2 and 2.06 μg/ml, respectively. IS-B was employed in the sulfate metabolite assay. The concentration of IS-B was 5.85 μg/ml.

Reagents and standard solutions

Citrate–acetate buffer, pH 4.0, monochloroacetate buffer, pH 3.2, and stock standard solutions were prepared according to the methods described previously [5].

Mobile phases

Mobile phase A was methanol–citrate–acetate buffer, pH 4.0 (18:82, v/v), mobile phase B methanol–monochloroacetate buffer, pH 3.2 (18:82, v/v), and mobile phase C acetonitrile–methanol–monochloroacetate buffer, pH 3.2 (18:4:78, v/v).

Collection of clinical samples

Seven normal male volunteers (mean age 25 years, range 22–32 years) participated in the study. The subjects were in good health as determined by physical examination and standard laboratory tests and none had received any medication in the two weeks before the study. Written consent was obtained from each subject before participation in the study. After an overnight fast each subject received a single 100-mg (free base) tablet of fenoldopam mesylate. Blood samples were collected in heparinized Vacutainers (Becton-Dickinson, Rutherford, NJ, U.S.A.) and centrifuged at 3000 *g* for 10 min at 4°C. Samples of plasma (4.75 ml) were transferred to polypropylene tubes, containing 0.25 ml of 10% (w/v) ascorbic acid solution (prepared daily), mixed and stored immediately at −20°C.

Robotic instrumentation

The robotic apparatus used was the Zymate II laboratory automation system (Zymark, Hopkinton, MA, U.S.A.). The components include: microprocessor–controller with Easy-lab software, laboratory robot, general purpose (GP), syringe (SYR), blank (BLK) and dual function (DF) hands. The DF hand was capable of performing both pipetting and holding tasks. The BLK hand was used for liquid transfers. The master laboratory station (for liquid dispensing), capping station, centrifuge, linear shaker, evaporator, vortex mixers, solid-phase extraction station and other miscellaneous accessories (Fig. 2) were all obtained from Zymark. The system was configured in order to allow for both liquid–liquid and liquid–solid extractions.

Fenoldopam/methoxy metabolites (liquid–liquid) extraction by robot

Plasma (1 ml) and 100 μl of 0.05 *M* acetic acid (containing analytical standards when preparing standard curves) were manually transferred to a 116 mm × 16 mm conical polypropylene tube. After capping, the tube was placed in

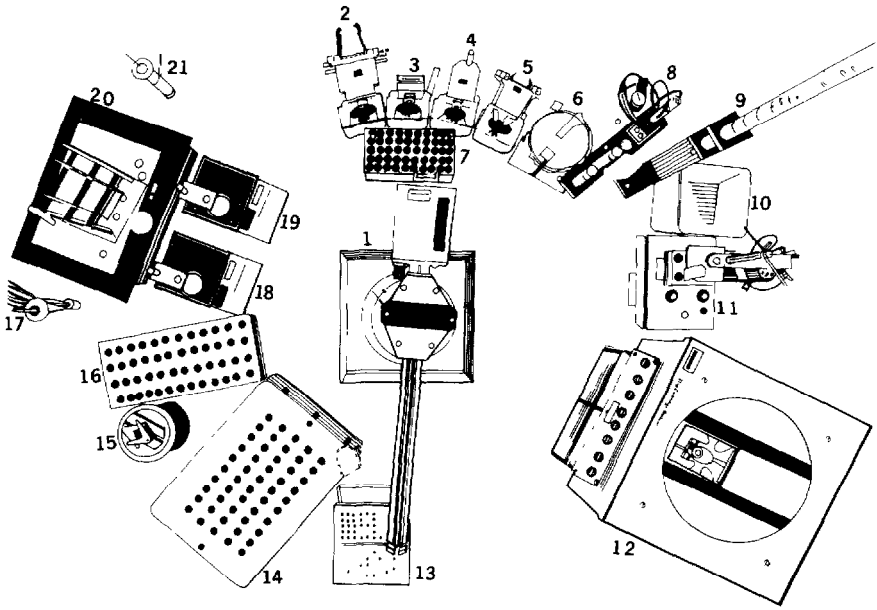

Fig. 2. Schematic diagram of the robotic layout for liquid–liquid and liquid–solid extraction methods. 1 = Robot arm; 2 = DF hand; 3 = BLK hand; 4 = SYR hand; 5 = GP hand; 6 = washing station; 7 = WISP vial rack; 8 = solid-phase extraction station; 9 = column dispenser; 10 = waste container; 11 = evaporator; 12 = centrifuge; 13 = pipette tip rack; 14 = sample rack A; 15 = capping station; 16 = sample rack B; 17 = solvent dispensing station; 18 and 19 = vortex mixers; 20 = linear shaker; 21 = mobile phase dispensing station.

sample rack A immersed in a cooling bath maintained at −4 °C. Since the robot programming allows for serial processing of the samples, the extraction process was carried out by the robot in four separate steps.

In the first step, the robot attached the DF hand, removed the sample tube from sample rack A and transferred it to the capping station. After uncapping the tube, 50 μl of internal standard solution (containing IS-A and IS-C) were added and the tube was transferred to a vortex mixer. After mixing for 10 s the tube was moved to a dispensing station, where 5 ml of ethyl acetate and 0.5 ml of dibasic sodium phosphate solution (0.5 *M*) were added. After mixing for 10 s, the tube was returned to the capping station, capped and placed in a linear shaker for 10 min.

In the second step, the robot removed the tube from the shaker and placed it in the centrifuge tube rack. After removing the previously processed tube from the centrifuge and placing it in the centrifuge tube rack, the incoming tube was placed in the centrifuge and spun at 1500 *g* for 5 min at room temperature.

In the third step, the robot took the previously centrifuged sample tube from the centrifuge rack, uncapped the tube and placed the tube in a temporary holding station. The robot removed an empty tube from sample rack B, uncapped it and left the tube in the capping station. It then attached the BLK hand and transferred 4.5 ml of ethyl acetate from the sample tube to the blank tube in the capper.

The robot washed the cannula with methanol (10 ml) and parked the hand. It attached the DF hand, took the tube which now contained the ethyl acetate phase from the capper to a dispensing station where 0.5 ml of citrate–acetate buffer was added. The tube was capped and vortex-mixed for 2 min. The tube was returned to the capping station and uncapped. The robot then attached the BLK hand, aspirated the top organic layer to waste and once again rinsed the cannula with methanol. It attached the DF hand and placed the tube in the nitrogen evaporator maintained at 40 °C.

In the fourth step, after evaporation of trace amounts of ethyl acetate (approximately 20 min), the robot removed the tube from the evaporator and placed it in the vial holding station. The SYR hand was attached and 200 μl of the residual extract were transferred to an autosampler vial. After rinsing the syring with methanol, the SYR hand was returned to the parking station. The DF hand was reattached and the empty tube returned to the sample rack B. The sample was then ready for injection into the appropriate high-performance liquid chromatographic (HPLC) system for analysis of fenoldopam or its methoxy metabolites.

Fenoldopam sulfate/glucuronide metabolite extraction (liquid–solid) by robot

The station required by the robot to perform solid-phase extractions consisted of a column holder, pneumatically controlled polypropylene lid (containing five tubes for dispensing liquids and to introduce air through the column) and a shuttle-like apparatus located directly under the extraction station. The shuttle apparatus moved in and out and consisted of a tube holder and a waste receptacle (Fig. 3). Plasma (0.5 ml) and 50 μl of 0.05 *M* acetic acid (containing standards when preparing standard curves) were manually transferred to a 125 mm × 16

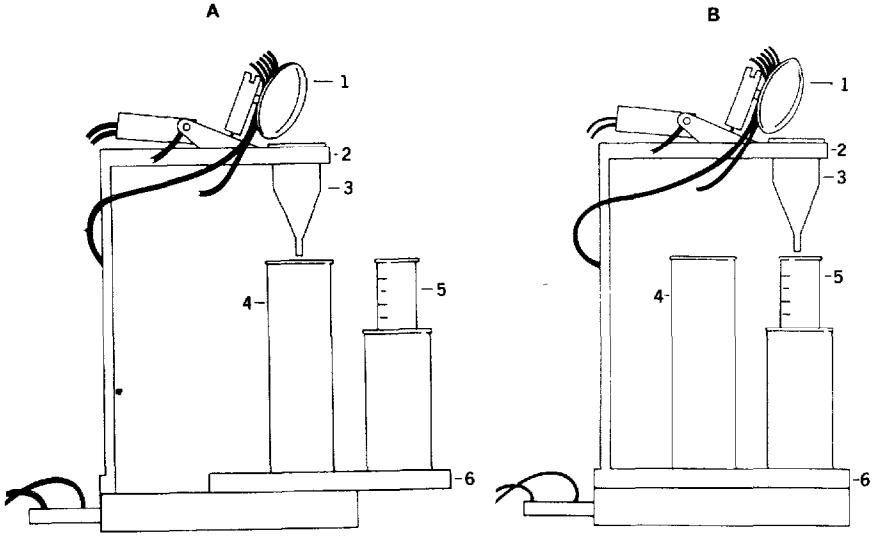

Fig. 3. Schematics of solid-phase extraction station. (A) Position during conditioning and sample application phase. (B) Position during elution phase. 1 = Polypropylene lid; 2 = column holder; 3 = column; 4 = waste-receptacle; 5 = collection tube; 6 = shuttle apparatus.

mm polypropylene tube and placed in the sample rack A. The sample preparation was then carried out by robot in two steps.

In the first step, the robot attached the GP hand, removed a C_{18} column from a dispenser and placed it in the extraction station. The robot then attached the DF hand, removed a blank tube from sample rack B and placed it in the shuttle tube holder of the extraction station. It removed the sample tube from sample rack A and placed it in the capping station. IS-B solution (50 μl) was added and the tube moved to a solvent dispensing station, where 0.5 ml of potassium phosphate solution (0.5 *M*, pH 7.5) was dispensed. The tube was then vortex-mixed for 10 s. After conditioning the C_{18} column by successively passing 3 ml of methanol, 3 ml of water (twice) and 2 ml of phosphate buffer (pH 7.5) through the column, the robot transferred the plasma sample to the C_{18} column and forced the sample through it by applying positive pressure by introducing air onto top of the column. The tube was rinsed twice with 3 ml of water and the washings were also passed through the column and allowed to go to waste. After washing the column with an additional 5 ml of water, 2 ml of methanolic acetic acid (0.3 *M*) were added to the column and the effluents were collected in the tube placed underneath it. The tube was then placed in a nitrogen evaporator maintained at 55°C (for 60 min).

In the second step, the robot removed the tube from the evaporator, took it to the solvent dispenser and dispensed 200 μl of mobile phase B. After mixing, the robot transferred 200 μl of the extract to an autosampler vial where it was ready for injection into the appropriate HPLC system for analysis of either fenoldopam sulfate or glucuronide metabolites.

Manual extraction procedures

The extraction procedures used for the manual method were similar to those described under robotic methods, except for the following: 1.0 *M* dibasic sodium phosphate (liquid–liquid extraction) was substituted for 0.5 *M* solution and Sep-Pak C_{18} columns (Waters Assoc., liquid–solid extraction) were used in place of Prep-Sep columns. These changes were made during the automation of manual sample preparation methods to suit the robotic application. The sodium phosphate solution (1.0 *M*) used in the liquid–liquid extraction method caused salt crystallization at the end of dispensing tube in the robotic method and substitution of dilute solution (0.5 *M*) corrected the problem without altering the recoveries of compounds. The Sep-Pak columns were unsuitable for the robotic method and were replaced with the Prep-Sep columns specifically designed for the robot.

Chromatography

All three chromatographic systems employed a Model 110A HPLC pump (Beckman, Palo Alto, CA, U.S.A.), an autoinjector (WISP Model 710B, Waters Assoc., Milford, MA, U.S.A.) and a coulometric electrochemical detector (ESA, Bedford, MA, U.S.A.). All separations were carried out on an Ultrasphere 5-μm octadecyl silica column (25 cm×4.6 mm I.D., Beckman) maintained at 30°C using a column heater (Rainin Instruments, Woburn, MA, U.S.A.). The potentials used for the fenoldopam asay were W1 = +0.15V and W2 = −0.15 V; for

methoxy and sulfate metabolites the potentials were W1 = +0.65 V and W2 = −0.15 V. All mobile phases, A for fenoldopam, B for sulfates and C for methoxy metabolites, were used at a flow-rate of 1 ml/min. Prior to use, the mobile phases were degassed by filtering through a 0.45-μm membrane filter (Type HA, Millipore, Bedford, MA, U.S.A.).

Quantitation

An appropriate standard curve was prepared for each set of samples. Peak-height data were collected with a computer-automated laboratory system (CIS-Beckman, Waldwick, NJ, U.S.A.) and peak-height ratios, analyte to internal standard, were calculated. The equation for the linear regression line ($y = a + bx$, fitted by least squares) of concentration versus peak-height ratio was obtained for the standard curve. Analyte concentrations for samples were calculated from the following equation:

$$\text{concentration of analyte } (x) = (y - a)/b$$

where b = slope of the regression line, a = y-intercept of regression line and y = peak-height ratio of analyte to internal standard.

Validation procedures

Three pools of plasma containing low, medium and high concentrations of analyte were prepared by dissolving weighed amounts in known volumes of drug-free plasma and stored at −20°C. On three separate days, at least five replicate samples from each pool were extracted and analyzed. Concentrations were determined by comparison with a standard curve, prepared freshly on the day of analysis. Validation of assays using clinical samples could not be carried out due to difficulties in obtaining enough pool of plasma from subjects dosed with fenoldopam.

RESULTS

Fenoldopam assay

An aliquot of the final plasma extracts obtained after the robotic or manual methods was first injected into the fenoldopam HPLC system. Typical chromatograms of plasma extracts obtained before and after oral administration of fenoldopam to a healthy volunteer are shown in Fig. 4. The retention times for fenoldopam and internal standard (IS-A) were 8.7 and 16.6 min, respectively. None of the known metabolites of fenoldopam or the endogenous substances interfered either with fenoldopam or IS-A. Using 1 ml of plasma, the limit of detection for fenoldopam was 25 pg/ml at a signal-to-noise ratio of 4. Extraction recovery was determined by comparing detector response to standards injected directly on the column with the response to standards extracted from plasma. The mean recovery of fenoldopam from plasma by the present method was 81%. Linear responses in fenoldopam-to-internal standard peak-height ratios were noted for plasma concentrations from 0.025 to 100 ng/ml. Excellent correlation coefficients

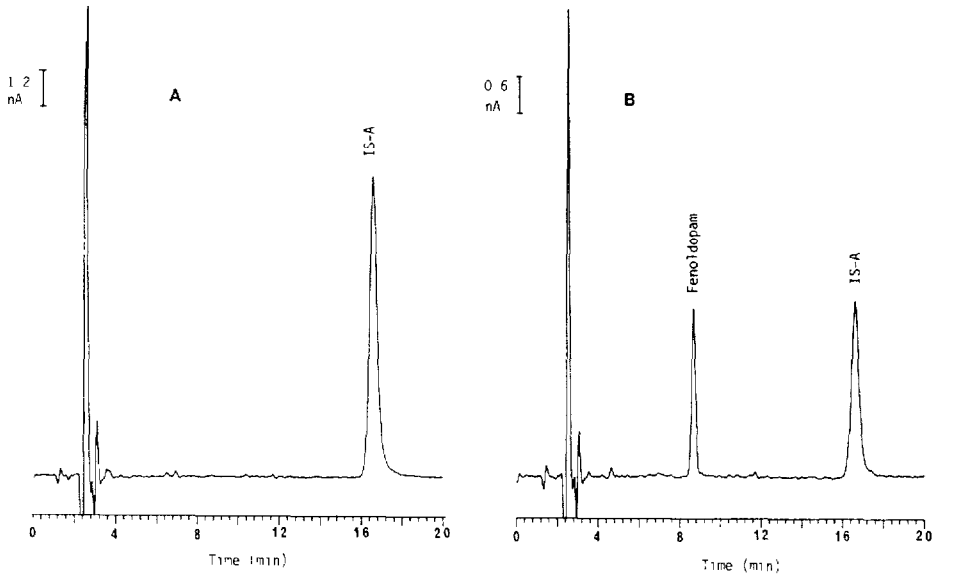

Fig. 4. Specific analysis of fenoldopam. Chromatograms of plasma extracts (extracted by robot) from a human subject before (A) and 1 h after (B) oral administration of fenoldopam. The concentration of fenoldopam was 4.3 ng/ml.

TABLE I

PRECISION DATA FOR FENOLDOPAM AND ITS METHOXY METABOLITES IN PLASMA

Analyte	Theoretical concentration (ng/ml)	Assay concentration[a] (mean ± S.D.) (ng/ml)		Coefficient of variation (%)			
				Intra-assay[b]		Inter-asssay[c]	
		Robot ($n=6$)	Manual ($n=6$)	Robot ($n=6$)	Manual ($n=6$)	Robot ($n=18$)	Manual ($n=18$)
Fenoldopam	0.25	0.25 ± 0.01	0.22 ± 0.03	4.0	13.6	8.4	11.3
	2.5	2.58 ± 0.12	2.40 ± 0.06	4.6	5.4	3.2	4.4
	50.0	51.43 ± 1.35	53.08 ± 2.22	2.6	2.9	2.6	4.9
7-MEO	2.3	2.46 ± 0.10	2.77 ± 0.61	4.1	22.0	9.7	21.1
	11.5	11.81 ± 0.32	11.04 ± 0.45	2.7	4.1	6.6	3.2
	57.5	59.04 ± 1.34	56.25 ± 2.65	2.3	4.7	6.5	4.3
8-MEO	2.3	2.38 ± 0.08	2.77 ± 0.42	3.4	15.2	6.7	21.5
	11.5	11.79 ± 0.20	11.16 ± 0.32	1.7	2.9	4.4	4.5
	57.5	59.06 ± 1.25	55 41 ± 2.88	2.1	5 2	5.5	3.5

[a] Mean value on a single day.
[b] (S.D./mean) × 100 in same assay.
[c] (S.D./mean) × 100 in three different assays.

(> 0.999) were obtained for all plasma standard curves extracted by both robotic and manual methods. The intra-assay and inter-assay precision data obtained by manual and robotic methods were in close agreement, however, the automated method was found to improve the precision at the lower concentration range (Table I).

Methoxyfenoldopam assay

After assaying the plasma extracts for their fenoldopam content, an aliquot of the sample was injected into the methoxyfenoldopam HPLC system within 48 h (stored at 4 °C) after their initial extraction. Typical chromatograms of plasma extracts obtained before and after oral administration of fenoldopam to a healthy volunteer are shown in Fig. 5. The retention times for IS-A, 7-MEO, 8-MEO and IS-C were 7.3, 9.1, 12.4 and 17.9 min, respectively. The chromatograms displayed no interfering peaks related to endogenous substances or to the fenoldopam and its sulfate and glucuronide conjugates close to the compounds of interest. The limite of detection for these metabolites was 0.5 ng/ml at a signal-to-noise ratio of 4. Mean recoveries for 7-MEO and 8-MEO were 36.2 ± 3.1 and 46.5 ± 2.8%, respectively. Linear responses in 7-MEO- and 8-MEO-to-IS-A peak-height ratios with plasma concentrations from 1 to 58 ng/ml were observed. Correlation coefficients exceeded 0.99 for all plasma standard curves. The intra-assay and inter-assay precision for robotic and manual methods, indicated by the coefficients of variation, were in close agreement except in the lower concentration range, where, again, the robotic method yielded better precision (Table I).

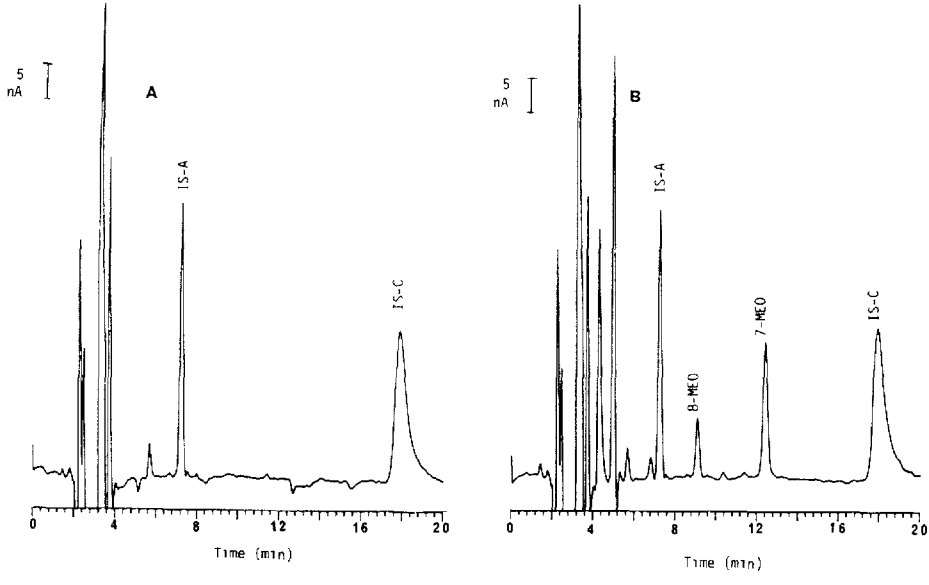

Fig 5. Specific analysis of methoxy metabolites of fenoldopam. Chromatograms of plasma extracts (extracted manually) from a human subject before (A) and 1.25 h after (B) oral administration of fenoldopam. The concentrations of 7-MEO and 8-MEO were 14.2 and 3.4 ng/ml, respectively.

Fenoldopam sulfate assay

Plasma extracts obtained after the liquid–solid extraction method contained both the sulfate and glucuronide metabolites of fenoldopam. Formal validation for the glucuronide metabolites of fenoldopam was not conducted because of insufficient quantities of pure glucuronide metabolite standard. If the need arises

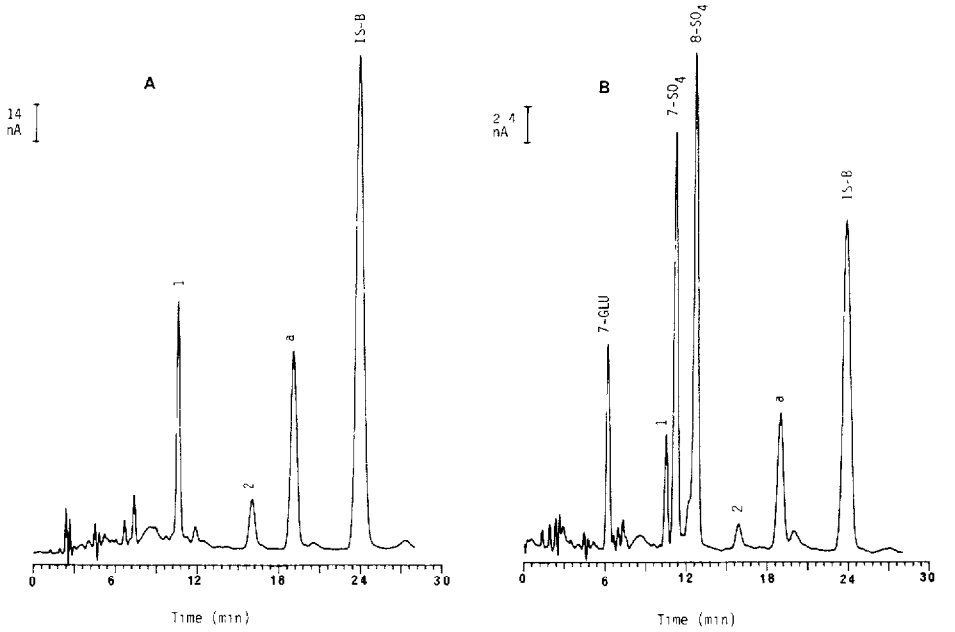

Fig. 6. Specific analysis of sulfate metabolites of fenoldopam. Chromatograms of plasma (extracted by robot) from a human subject before (A) and 1.5 h after (B) oral administration of fenoldopam. The concentrations of 7-SO_4 and 8-SO_4 were 454.6 and 278.4 ng/ml, respectively. Peaks 1 and 2 were of endogenous origin, peak a was the 8-sulfate isomer of IS-B, which was present as an impurity. The peak at 6.0 min was due to the 7-glucuronide metabolite of fenoldopam (7-GLU).

TABLE II

PRECISION DATA FOR 7-SO_4 and 8-SO_4 METABOLITES OF FENOLDOPAM IN PLASMA

Analyte	Theoretical concentration (ng/ml)	Assay concentration (mean ± S.D.) (ng/ml)		Coefficient of variation (%)			
				Intra-assay		Inter-asssay	
		Robot ($n=5$)	Manual ($n=5$)	Robot ($n=5$)	Manual ($n=5$)	Robot ($n=15$)	Manual ($n=15$)
7-SO_4	11.5	10.6 ± 0.4	11.1 ± 0.2	3.8	1.4	9.9	9.3
	230.0	214.1 ± 1.4	231.3 ± 3.4	0.7	1.5	5.9	4 1
	1380.0	1285.4 ± 37.0	1441.1 ± 37.3	2.9	2.6	4.9	3.8
8-SO_4	11.5	11.0 ± 0.9	11.0 ± 0.5	8.2	4.2	5.5	3.9
	230.0	222.5 ± 2.2	215.8 ± 3.4	1.0	1.6	7.2	2.8
	1380.0	1365.6 ± 41.3	1295.3 ± 23.8	3.0	1.8	3.2	2.4

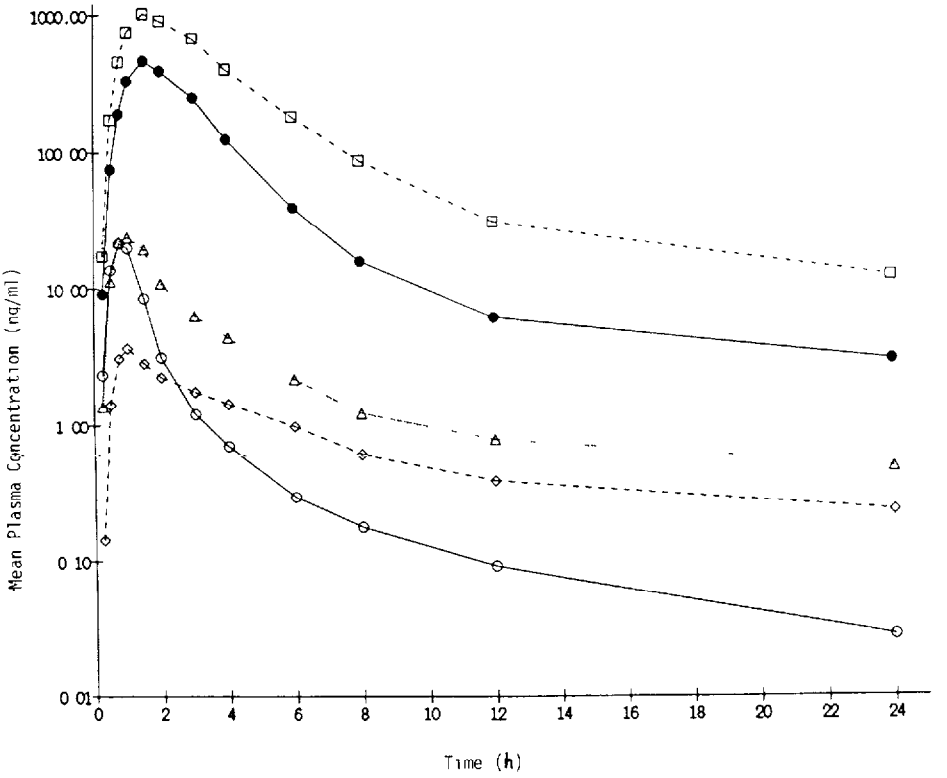

Fig. 7. Mean plasma concentrations of fenoldopam (○), 7-MEO (△), 8-MEO (◇), 7-SO_4 (□) and 8-SO_4 (●) following oral administration of 100 mg of fenoldopam to seven healthy male human volunteers aged 22–32 years

and standard compound is available, quantification of these conjugates can be achieved by incorporating the glucuronide internal standard in the extraction procedure and injecting the samples on to the fenoldopam glucuronide HPLC system described previously [6]. Typical chromatograms of plasma extracts obtained before and after oral administration of fenoldopam to a healthy volunteer are shown in Fig. 6. The retention times for 7-SO_4, 8-SO_4 and IS-B were 11.4, 12.8 and 24.0 min, respectively. No endogenous or drug-related peaks interfered with the 8-SO_4 peak. A small endogenous peak eluted very close to the 7-SO_4 peak. This peak was resolved from the 7-SO_4 peak by slight manipulation of the methanol content of mobile phase. Using 0.5 ml of plasma, the limit of detection for these metabolites was 2 ng/ml at a signal-to-noise ratio of 4. Linear responses in 7-SO_4- and 8-SO_4-to-IS-B peak-height ratios with plasma concentrations from 2 to 1380 ng/ml were observed. Correlation coefficients exceeded 0.99 for all plasma standard curves. Mean recoveries for 7-SO_4 and 8-SO_4 were 66.8 ± 7.2 and $78.0 \pm 11.0\%$, respectively. The intra-assay and inter-assay precision for the robotic and manual methods for 7-SO_4 and 8-SO_4 are shown in Table II. The precision obtained by both the robotic and manual method were in close agreement.

Analysis of clinical samples

Plasma samples collected from human subjects after administration of fenoldopam mesylate (100 mg as base) were analyzed for fenoldopam and its metabolites. The mean plasma concentrations of fenoldopam and its sulfate and methoxy metabolites from 0 to 24 h following fenoldopam administration are shown in Fig. 7. These analytical methods displayed sufficient sensitivity and selectivity to measure the parent drug and metabolite levels even 24 h following the dose.

DISCUSSION

Fenoldopam and its metabolites differ greatly in their chemical stability and polarity, thus necessitating the development of different extraction methodology specific for each class of compounds. Detection of fenoldopam and its metabolites at the picogram levels, observed following therapeutic dosing, required the sensitivity provided by electrochemical detection. The use of such a detector at the highest sensitivity setting, however, in gradient HPLC is a very difficult task due to the inherent problem of shifting baseline and noise during gradient analysis. In view of these problems, we developed two independent extraction methods and three isocratic chromatographic systems in order to quantitate fenoldopam and its metabolites accurately and precisely in human plasma samples. The present sample preparation method requires only 1.5 ml of plasma to measure fenoldopam and six of its known metabolites. The methods can be performed manually or by robot.

Fenoldopam/methoxy metabolites assay

The plasma fenoldopam assay reported previously [5] was used in our laboratory for the past two years, however, difficulties were experienced in other laboratories trying to reproduce the assay. These failures were mainly attributed to the addition of small volumes (26 μl of 1.0 *M* solution) of sodium hydroxide and to the presence of impurities in the diethyl ether employed in the extraction procedures. These small variations in the addition of sodium hydroxide influenced the recovery of the compounds, thereby limiting their detection at low plasma concentrations. The impurities present in some commercial sources of diethyl ether gave rise to chromatographic interferences and also affected the stability of fenoldopam. Substitution of dibasic sodium phosphate solution (pH 9.3) in place of sodium hydroxide adequately controlled the plasma pH to 7.5. The reason for choosing the dibasic sodium phosphate solution (0.5 *M*) in place of a buffer solution was mainly the ease of solution preparation. Elimination of the diethyl ether wash step by back-extracting the compounds into citrate–acetate buffer assured the stability of fenoldopam and the internal standard in the final extract and provided a much cleaner extract chromatographically. Chromatographic analysis of hundreds of drug-free plasma samples from various phases (I to III) of clinical studies of fenoldopam showed that the present extraction method was free from any interferences related to substances of either endogenous or environmental origin. The modified extraction procedures described here for fenol-

dopam were also found to be suitable for the simultaneous extraction of the methoxy metabolites of fenoldopam.

The extraction procedure described in this report for fenoldopam was evaluated against the previous method [5] by simultaneously analyzing two sets of plasma samples spiked with fenoldopam (50 ng/ml). The present method exhibited less intra-assay variation compared to the previous method, as indicated by their coefficients of variation (2.4 versus 8.1%). The extraction recoveries were also improved in the present method for fenoldopam (81 versus 69%) and IS-A (93 versus 58%), thereby enhancing the sensitivity of the assay.

The hydrolysis of sulfate conjugates of fenoldopam during the extraction procedure was also examined by extracting the plasma samples spiked with 50 μg/ml of either 7-SO_4 or 8-SO_4 and analyzing for their fenoldopam content. Results from this study indicated that the percentage of these conjugates hydrolyzed during the extraction procedure was ca. 0.007 for 7-SO_4 and 0.003 for 8-SO_4 and this conversion was not significant for the clinical samples. Repeated thawing and freezing of authentic samples may result in slightly higher values for fenoldopam. For this reason clinical samples should be analyzed first for their fenoldopam content.

The other modifiations that were made in the method were the replacement of the amperometric electrochemical detector with a coulometric detector (with two electrodes in series) and the inclusion of a second internal standard. By monitoring the reduction reaction at the second electrode following the oxidation of fenoldopam at the first electrode, the coulometric detector increased selectivity and enhanced the overall sensitivity of the method. A second internal standard was included in the extraction procedure mainly due to long-term instability of IS-A. If the samples were not injected within 48 h after extraction (stored at 4°C), the peak height of IS-A was found to be reduced. Although the use of IS-A was suitable for the majority of work, the second internal standard (IS-C) was used for quantitation when the samples were injected more than 48 h following the extraction.

Fenoldopam sulfate assay

Analytical methods for fenoldopam sulfate conjugates did not differ from the previous methods except in the use of a coulometric detector in place of an amperometric detector. The amperometric detector used in the previous method for the detection of sulfate metabolites rapidly lost sensitivity for 7-SO_4 while retaining its sensitivity for 8-SO_4. Polishing the thin-layer cell did not restore the sensitivity for the 7-SO_4. The reason for this phenomena is unknown, however, this differential loss in sensitivity made it difficult to routinely measure both of these metabolites over prolonged periods. Replacing this detector with the coulometric detector gave equal sensitivity for both 7-SO_4 and 8-SO_4 for at least 600 injections of plasma extracts. With time this detector also lost sensitivity for 7-SO_4, but it was easily regained by cleaning the cell with nitric acid.

Robotic sample preparation

Transfer of a manual extraction procedure to a robot necessitates some changes in order to suit the robot's specific needs. Since the robot processes samples in a

serial manner, unlike the batch mode during manual operation, the plasma samples (48 samples) had to remain at room temperature for 16–20 h before they were processed. This caused a significant stability problem during the analysis of plasma samples for their fenoldopam content. Due to this long waiting period, authentic plasma samples containing high fenoldopam sulfate levels (> 2000 ng/ml) when extracted at the end of a robotic run yielded higher fenoldopam levels (ca. 13%), due to breakdown of the sulfate metabolites as compared to the samples processed in the beginning. Since the combined concentration of sulfate metabolites (7-SO_4 and 8-SO_4) was approximately 1000-fold higher than fenoldopam levels, even conversion of less than 1% of these metabolites to fenoldopam can result in misleading clinical data. Keeping the samples at $-4\,^{\circ}C$ by circulating a coolant around the sample rack and extracting clinical samples both at the beginning and at the end of robotic operation resulted in only a 6.5% increase in fenoldopam levels. This increase in fenoldopam levels may be due to the intra-assay variation (coefficient of variation) associated with the fenoldopam method (Table I). Extraction of plasma samples even after 48 h storage at $-4\,^{\circ}C$ did not result in a further increase in the fenoldopam levels suggesting that samples were actually stable at this temperature.

Extraction of drug-free plasma samples following the plasma samples containing high concentrations of fenoldopam (100 ng/ml) or sulfate metabolites (2 μg/ml) resulted in positive values for fenoldopam or sulfates indicating the carry-over of drug from the previously processed sample. This carry-over corresponds to about 0.5%. The major contributors to this problem were identified as the cannula of the BLK hand, which was used for the transfer of organic extracts and aspirations, and the SYR hand, which was used for the transfer of final extracts into the autosampler vials. Washing the lumen of the cannula by dispensing 10 ml of methanol and rinsing the syringe four times with methanol after each operation minimized the carry-over to less than 0.05%.

Compared to manual methods, the robotic method can be reproduced with relative ease. Since the entire method is stored on a floppy disk it eliminates the variations routinely faced in the implementation of manual methods. Interchangeability between the liquid–liquid and the solid-phase extraction procedures is instantaneous through the use of appropriate software changes. Chemical incompatability between solvents and possible chromatographic interferences are minimal through the use of a purging program using the appropriate solvents prior to switching the assays. The sample turn around time and the precision of the robotic method was very similar to the one obtained by the manual method. In conclusion we have developed simplified manual and automated extraction procedures, that enable the measurement of fenoldopam and its metabolites in plasma with just two separation methods and require less than 2 ml of plasma.

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