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BIMODAL COLUMN SWITCHING LIQUID CHROMATOGRAPHIC ASSAY OF SIX METABOLITES OF [^{14}C] FELODIPINE IN RAT URINE*

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SUMMARY

A liquid chromatographic method using bimodal column switching is presented which allows for the separation of six urinary metabolites of [^{14}C] felodipine and their quantification using an on-line radioactivity detector. Evaluation of the chromatographic conditions was performed with non-labelled reference compounds and UV detection. Pre-separation of the metabolites into two groups, one consisting of carboxylic acid metabolites and the other of the hydroxylated analogues, was performed on underivatized silica. The mobile phase used was optimized with respect to pH and the character of quaternary ammonium ion, and was 0.01 *M* tetrapropylammonium in 5% (v/v) methanol in 0.05 *M* phosphate buffer (pH 5.0). Each group was introduced and separated, after band compression, by a gradient of increasing methanol concentration on an octyl-bonded column. The analysis time was 70 min. The method was applied to urine collected from rats (n = 4, 0—24 h) after oral dosing of [^{14}C] felodipine (5 μmol/kg). The urine was analysed with no pre-treatment other than slight dilution. The six metabolites accounted for 58% of the excreted amount (13% of the dose).

INTRODUCTION

Felodipine (3,5-pyridinedicarboxylic acid, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl, 5-ethyl 3-methyl ester, Fig. 1, I) is a calcium antagonist that lowers blood pressure by reducing peripheral resistance by means of a direct, selective action on smooth muscle in arterial resistance vessels [1]. The drug is metabolized extensively and the urinary metabolic pattern has been

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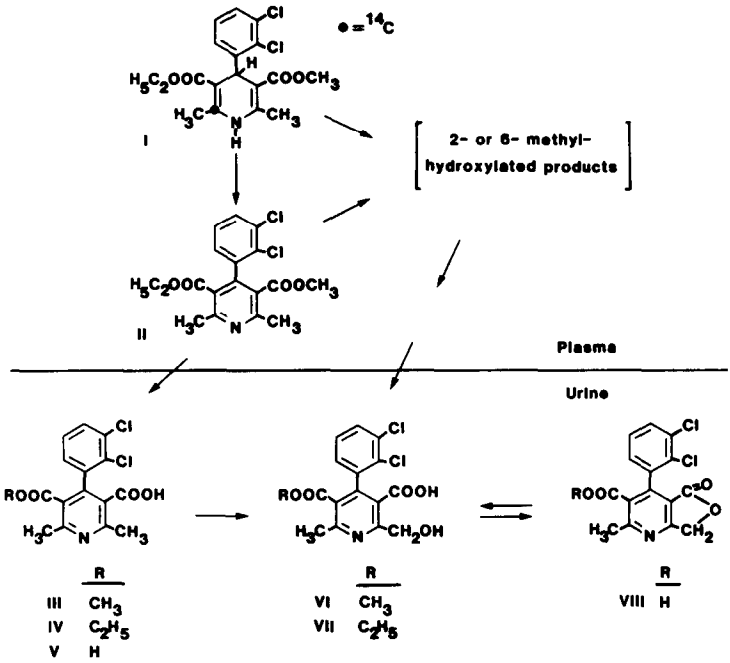

Fig. 1. Molecular structures of felodipine (I), oxidized felodipine (II) and urinary metabolites (III—VIII). (Short names: III = methyl monoacid; IV = ethyl monoacid; V = diacid; VI = methyl hydroxy acid; VII = ethyl hydroxy acid; VIII = acid lactone).

studied in humans, dogs, rats and mice [2], and eight urinary metabolites excreted by the rat have been identified [3].

Quantitative investigations of urinary metabolites formed from drugs of the 1,4-dihydropyridine class have been reported for SK&F 24260 by thin-layer chromatography (TLC) [4] and gas chromatography (GC) [5], for nifedipine by TLC [6], GC [7] and high-performance liquid chromatography (HPLC) [8] and for nicardipine by GC and HPLC [9]. These methods all involve solvent extraction of the metabolites giving non-quantitative yields, as determined with material obtained after dosing with radioactively labelled compounds. Furthermore, the GC methods involve derivatization of the formed acids with diazomethane, which decreases the selectivity of the method as different metabolites can give identical derivatives when methylated (cf. Fig. 1 and ref. 3).

The chromatographic system used in ref. 2 failed to resolve two carboxylic acid metabolites from their methylhydroxylated analogues for proper quantification. Attempts to improve this reversed-phase single-column separation by modification of the mobile phase or by the use of other alkyl-bonded adsorbents were not successful. The approach for the development of the bimodal separation method reported here was based on the physicochemical properties of the metabolites reported in refs. 10 and 11, and the resolution could be increased to facilitate quantification by an on-line radioactivity detector. Slightly diluted urine was injected directly into the system without pre-treatment or derivatization, allowing for the majority of the excreted metabolites to be separated and quantified.

EXPERIMENTAL

Chemicals

Felodipine, [6-^{14}C]felodipine (sp. radioactivity 811 MBq/mmol (57.1 μCi/mg) and radiochemical purity 96.0% by HPLC), reference compounds of metabolites II–VIII (Fig. 1) and quaternary ammonium hydrogen sulphates or bromides were synthesized at the Department of Organic Chemistry at Hässle. Methanol (p.a., E. Merck, Darmstadt, F.R.G.) was used without further purification. Deionized water was obtained from a Milli-Q^{TM} water purification system (Millipore, Milford, MA, U.S.A.). Sodium phosphate salts (E. Merck) were of analytical grade.

Animal experiment design

Four male Sprague-Dawley rats (270–330 g) (Möllegaard, Denmark) were fasted overnight and dosed orally by gavage with a solution of [^{14}C]felodipine (5 μmol/kg) in absolute ethanol–poly(ethylene glycol) 400–water (1:1:2, v/v). The radioactive dose was 1.2–1.4 MBq per rat. Urine and faeces were collected separately at 24-h intervals for 72 h, and the samples were stored at −18°C until analysis of the ^{14}C content as reported previously [2].

Apparatus

The chromatographic system (Fig. 2) consisted of two Altex 110 A pumps and a Model 421 system controller (Altex, Berkeley, CA, U.S.A.). Samples were introduced with a Rheodyne Model 7125 valve (Cotati, CA, U.S.A.) (valve S) equipped with a 162-μl sample loop. Pneumatically operated Rheodyne Model 7030 valves (valves V1 and V2) were used for column switching. The valve configurations permitted valves V1 and/or V2 to be switched in line or both V1 and V2 to be switched off line for fast flushing of

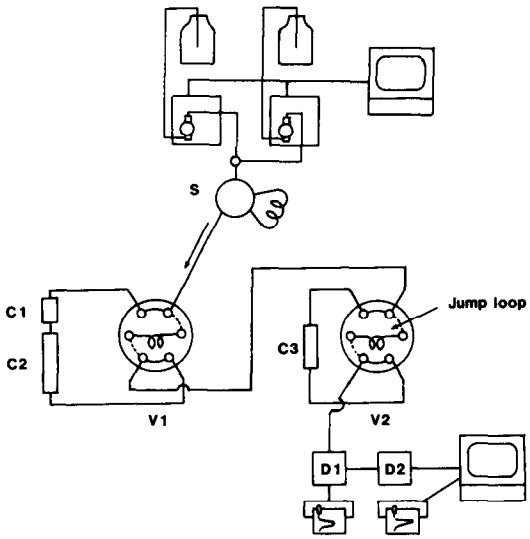

Fig. 2. Schematic representation of the chromatographic system and the switching valve configurations. See text for further explanation.

the capillaries, connections and detectors. The valves were activated or deactivated by the system controller, which had built-in external flags for the control of external devices. The eluent was monitored with a Lambda-Max Model 480 UV detector (Waters) set at 280 nm (D1), and an HPLC radioactivity monitor LB 503 (Berthold, Wildbad, F.R.G.) equipped with a G 200 solid scintillator cell (D2). Data were collected and analysed with an LB 500 M radiochromatography evaluation system (Berthold).

Chromatographic system

Analytical columns (stainless steel, 150 mm × 4.5 mm I.D. or 300 mm × 4.5 mm I.D.) were packed with LiChrosorb Si 60, 5 μm (E. Merck) or Nucleosil 50-7, 7 μm (Macherey-Nagel, Düren, F.R.G.) in the ascending mode with methanol as slurry medium and mobile phase at a pressure drop of 400 bar. Bonded-phase columns (LiChrosorb RP-8, 7 μm, E. Merck) were packed in the descending mode with 2-propanol—methylene chloride (1:2, v/v) as slurry medium and methanol—water (6:4) as the mobile phase at 400 bar.

Guard columns (50 mm × 3 mm I.D.) were slurry-packed with LiChrospher Si 4000, 10 μm (E. Merck).

The complete microprocessor-regulated program for the bimodal separation method is given in Table I. The mobile phases were 0.01 *M* tetrapropylammonium hydrogen sulphate (TPrA) in mixtures of 0.05 *M* phosphate buffers (pH 5.0)—methanol (phase A 95:5, phase B 1:2, v/v).

TABLE I

MICROPROCESSOR-REGULATED EVENTS (CF. FIGS. 2 AND 6)

0% B = 0.01 *M* TPrA in 0.05 *M* phosphate buffer (pH 5.0)—methanol (95:5); 100% B = 0.01 *M* TPrA in 0.05 *M* phosphate buffer (pH 5.0)—methanol (1:2). The activation of a valve switches the column in-line.

Time (min)	Function	Value	Duration (min)
0.0	Flow (ml/min)	0.8	
0.0	Valve V1	Active	
0.0	B (%)	0	
7.3	V2	Active	
9.0	V1	Inactive	
16.0	B (%)	50	
16.1	B (%)	80	8.9
29.5	B (%)	0	1
34.5	Flow	1.5	
37.0	Flow	0.8	
37.2	V1	Active	
42.0	V1	Inactive	
42.1	B (%)	85	0.9
53.0	B (%)	100	
56.0	B (%)	0	1
60.0	Flow	1.5	
64.0	Flow	0.8	
64.1	V1	Active	
64.2	V2	Inactive	

Sample pre-treatment

Urine collected during the 0—24 h interval was thawed and shaken thoroughly. An aliquot was transferred to a centrifuge tube and spun at 1000 *g* for 10 min. Typically 1.6 ml of clear urine was mixed with 0.3 ml of 0.05 *M* TPrA in 1 *M* phosphate buffer (pH 3.2, giving pH 5—5.5 after mixing) and 0.1 ml of methanol to match the sample composition with mobile phase A before analysis.

RESULTS AND DISCUSSION

Single-column chromatography

The separation and quantification of urinary felodipine metabolites [2] was achieved with a chromatographic system similar to the one used for the separation shown in Fig. 3. Chromatography of synthetic reference compounds revealed that the hydroxy acids VI and VII eluted very close to their non-hydroxylated analogues III and IV (UV trace). The separation was not improved by pH changes (range 3.5—7) of the mobile phase, omission of the counter-ion, changes in gradient profile or use of other adsorbents (e.g. LiChrosorb RP-18 or RP-2).

The reference mixture separated in Fig. 3 contained the acidic compounds III—VIII, but also three neutral potential metabolites, i.e. II and the lactones of VI and VII. However, compound II has previously been reported [2] not to

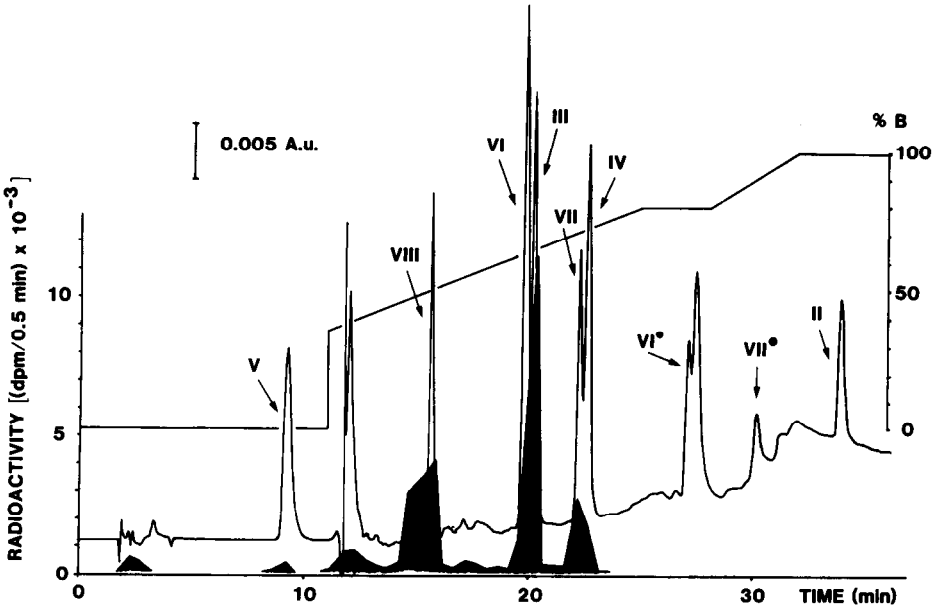

Fig. 3. Single-column gradient-elution separation. Column, LiChrosorb RP-8, 7 μm (150 mm × 4.5 mm I.D.); mobile phases, 0.01 *M* TPrA in 0.05 *M* phosphate buffer (pH 5.0)—methanol, (A) 95:5 (v/v) and (B) 1:2 (v/v); flow-rate, 1.2 ml/min. The UV trace is for reference compounds of metabolites II—VIII (VI* and VII* are the lactone forms of VI and VII, respectively); injected amounts, 0.4—0.9 μg of each. The lower filled trace shows the ^{14}C content of the 0.5-min eluate fractions after injection of 100 μl of rat urine in mobile phase A (1:2 v/v) (36 000 dpm). Gradient profile is given as %B.

be a urinary metabolite in the rat, and the equilibrium of the ester lactones (VI' and VII') at urinary pH strongly favours the open form [3, 10]. This is indicated in Fig. 3 by the lack of radioactivity in the eluate at the retention times corresponding to these reference compounds.

The radioactivity trace in Fig. 3 resulted from manual collection of 0.5-min eluate fractions after injection of a urinary sample from a [^{14}C] felodipine-dosed rat. As indicated in the figure, this method for quantification of the metabolites does not resolve compounds VI and III or compounds VII and IV.

One of the aims of this study was to quantify the eluting radioactivity with an on-line detector. The cell volume in on-line radioactivity detectors is a compromise between sensitivity, efficiency of radioactivity counting and resolution [12]. The cell volume recommended for our instrument, for the injected amount of ^{14}C, was 200 μl, which impaired the resolution between the methyl analogue pair (VI, III) and the ethyl analogue pair (VII, IV) compared with UV detection. Therefore a new approach was chosen to find a selective method for the chromatographic separation of the compounds.

Bimodal column-switching separation

Pre-separation on bare silica. The aim of this part of the study was to find a chromatographic system giving higher resolution, especially between the methyl analogues III and VI and between the ethyl analogues IV and VII. If this could not be achieved in a single-column system, the aim was to develop a separation method giving a different separation pattern, and combining it with the bonded-phase system used so far. A prerequisite for this was to use a low methanol content in the mobile phase in the first separation system to obtain on-column concentration in the second column.

It has been shown that the majority of urinary felodipine metabolites have acidic properties, owing to the hydrolysis of one or both ester functions [2]. Studies on the physico-chemical properties of compounds III—VII have demonstrated that the pK_a values of the hydroxy acids are ca. 0.5 log units lower than those of the monoacids [10, 11]. To exploit this difference, a number of silica-based anion-exchange packing materials were investigated (LiChrosorb-NH_2, LiChrosorb AN and Nucleosil $N(CH_3)_2$). Different elution orders were obtained, e.g. the diacid (V) was the most retained compound, but the resolution was not improved.

The use of ion-pair adsorption chromatography with aqueous mobile phases on bare silica has been described in several reports [e.g. refs. 13–16], and an analogous phase system was developed for chromatography of the compounds III—VIII. An aqueous mobile phase containing an ion-pairing quaternary ammonium compound and a low content of methanol provided a different retention order compared with the bonded-phase system. In the latter, resolution seemed to be based primarily on hydrophobic interactions between the adsorbent and the solutes, i.e. the methyl analogues III and VI eluted together and were well separated from the ethyl analogues IV and VII. However, in the bare silica system, elution seemed to be more dependent on the pK_a values, and the least acidic compounds, III and IV (p$K_a \approx 3.6$), were retained most strongly and were well separated from the more acidic compounds V—VIII (p$K_a \leq 3.1$).

The influence of the size and character of the counter-ion was evaluated with LiChrosorb Si 60, 5 μm, at pH 4.5 with 5% methanol and 0.01 *M* counter-ion as the mobile phase. The capacity factor (k') and the resolution between the methyl monoacid and the ethyl hydroxy acid ($R_{s(III/VII)}$), i.e. those compounds determining the "space" between the two groups, were calculated with standard methods [e.g. ref. 17], and are given in Fig. 4. (In this part of the study, the acid lactone (VIII) was not available as a reference compound, but was later found to elute in the first group.) The symmetrical TPrA and TBA gave the widest "space" between the two groups, and TPrA was arbitrarily chosen for further studies of this phase system.

To increase further the resolution between the two groups, the final evaluations were performed with a 300 mm × 4.5 mm I.D. column and Nucleosil 50-7 as the adsorbent. The back-pressure was somewhat lower with this packing material, and the peak symmetry improved as compared with LiChrosorb Si 60. The optimum pH of the mobile phase was evaluated with 0.05 *M* sodium hydrogen phosphate after adjustment of pH with sodium hydroxide or phosphoric acid in the pH range 3.5—6 and was found to be ca. 5.0. Above pH 5 the capacity factors and resolution decreased and below pH 4 the elution order between III and VII was even reversed. This phase system was sensitive to changes in mobile phase composition, and the column was equilibrated overnight, after change of counter-ion or mobile phase pH, to obtain constant chromatographic parameters [cf. refs. 15 and 16]. After some 20—30 injections on

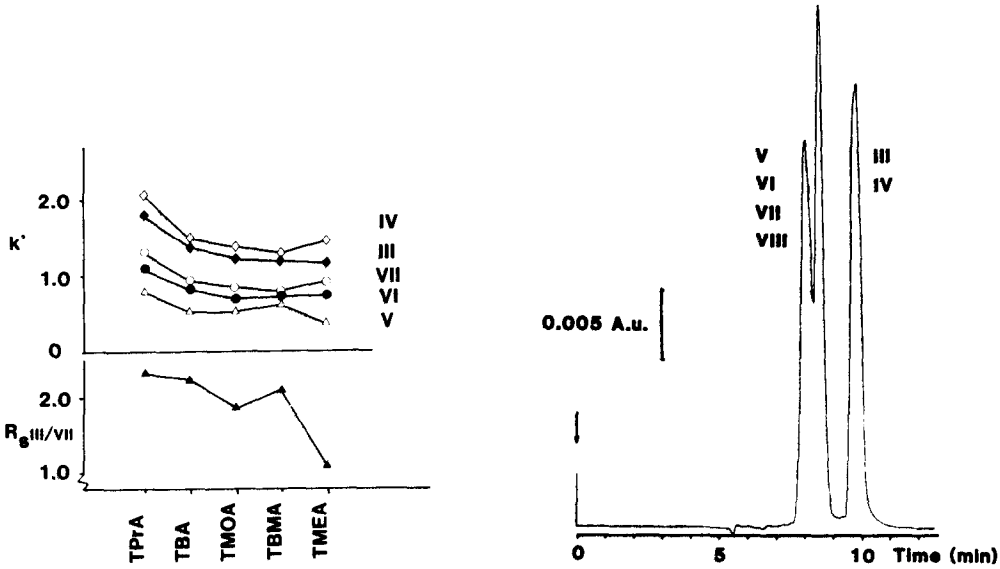

Fig. 4. Retention (k') of metabolites III—VII and resolution (R_s) between metabolites III and VII with 0.01 *M* counter-ion in 5% v/v methanol in 0.05 *M* phosphate buffer (pH 4.5) on LiChrosorb Si 60, 5 μm (150 mm × 4.5 mm I.D.). TPrA = tetrapropylammonium, TBA = tetrabutylammonium, TMOA = trimethyloctylammonium, TBMA = tributylmethylammonium, TMEA = trimethylethylammonium, all as salts with HSO_4^- except for $TMOA^+Br^-$.

Fig. 5. Group separation of reference compounds III—VIII on columns C1 and C2 with mobile phase A.

this column the resolution within the two groups decreased slightly, but the resolution between the groups was not affected. A chromatogram showing the group separation with optimum pH and counter-ion (mobile phase A) is given in Fig. 5.

A guard column packed with LiChrospher Si 4000 was used to protect the analytical silica column. The commercially available Brownlee Spheri-5 Si or dry-packed Kieselgel 63—200 μm guard columns impaired the resolution on the Nucleosil column significantly. However, the LiChrospher Si 4000 guard column acted as a mere filter for microparticulates, highly lipophilic compounds and urinary macromolecules, and did not contribute to the band broadening to a measurable extent.

Column switching separation of reference compounds. The separation method with the bare silica and the bonded-phase system was based on a "multi-cut" principle. The two groups of metabolites (V—VIII and III—IV) were to be introduced sequentially and separated by gradient programmes on the bonded-phase column.

The microprocessor-controlled events regulating the separation method are given in Fig. 6. Before injection, columns C1 and C2 were in line and equilibrated with mobile phase A at 0.8 ml/min. At time zero the injection was performed and the group separation achieved on C2. Then column C3 was switched in line to receive the first group of metabolites (V—VIII), following which C1 and C2 were switched off line, and a gradient programme was performed on C3. The gradient was reversed and re-equilibration with mobile phase A was brought about by increasing the flow-rate from 0.8 to 1.5 ml/min. Columns C1 and C2 were switched back in line and the remaining fraction of metabolites (III—IV) was introduced into C3. Columns C1 and C2 were switched off line again, and the two compounds were separated on C3 by a second gradient programme. The gradient was reversed by using the higher flow-rate as before, and the columns were switched back to the starting positions, i.e. C1 and C2 in line and C3 off line. This configuration was maintained for 10—20 min to wash columns C1 and C2 before injection of the next sample. The total analysis time was ca. 70 min. The actual gradient profile

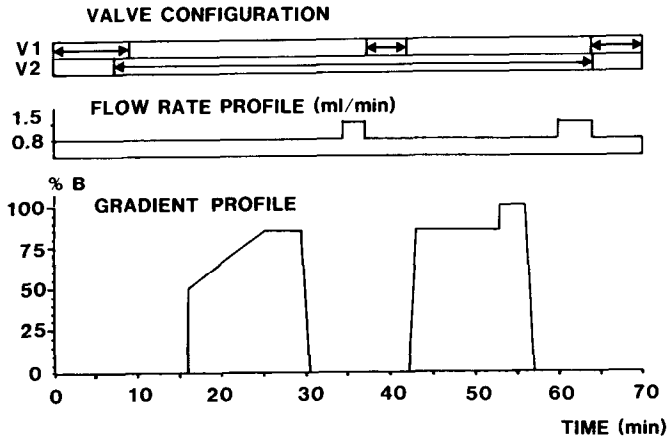

Fig. 6. Microprocessor-controlled events for the bimodal separation method.

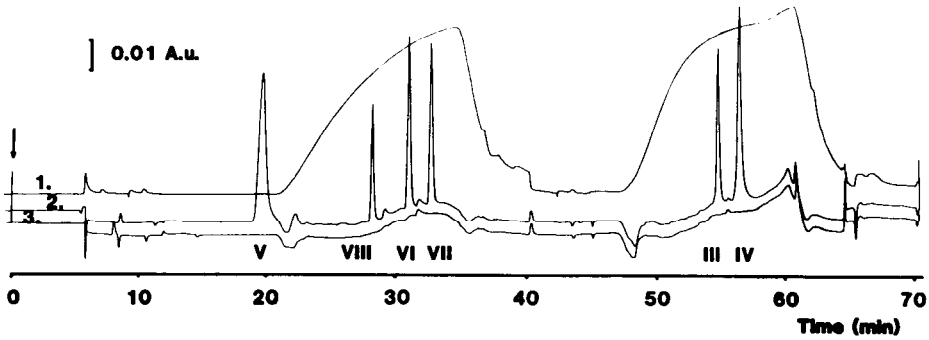

Fig. 7. Column switching separation of reference compounds III—VIII. Trace 1 = actual gradient profile (0.5% acetone in mobile phase B, 312 nm); trace 2 = reference compounds III—VIII (0.8—1.1 μg of each); trace 3 = blank gradient run.

TABLE II

TOTAL EXCRETION OF ^{14}C AFTER ORAL ADMINISTRATION OF [^{14}C]FELODIPINE TO THE RAT*

Collection interval (h)	Urine (% dose ± S.E.M.)	Faeces
0—24	22.18 ± 1.12	38.89 ± 3.16
24—48	2.99 ± 0.10	13.70 ± 3.08
48—72	0.97 ± 0.05	2.71 ± 0.92
0—72	26.14 ± 1.16	55.30 ± 4.53

*Dose, 5 μmol/kg; n = 4.

registered by the UV detector as visualized by acetone in phase B, a separation of the six reference compounds III—VIII and the full programme run without injection of sample, are shown in Fig. 7 (traces 1, 2 and 3, respectively).

Quantification of the metabolites in rat urine

Details of the excretion of total radioactivity after oral [^{14}C]felodipine administration to the rat are given in Table II. The urinary fraction collected during the first 24 h post dose was used for quantification of the six metabolites.

A radio-chromatogram of a sample injected on columns C1 and C2 is shown in Fig. 8. The capacity factor of the least retained reference compound (V, cf. Fig. 5) was 0.4 (2 min from the front of the chromatogram), but in the actual sample, radioactivity eluted from the front (5.5 min) and on. This was not due to column overloading, since a sample diluted five-fold with the mobile phase gave the same pattern. Consequently, the radioactive fraction eluting between 5.5 and 7.5 min contained metabolites other than III—VIII. This unknown fraction was vented directly to the radioactivity detector for quantification, so as to avoid interference with the separation of the known metabolites V—VIII on C3.

The separation of all six metabolites is shown in Fig. 9. The metabolites

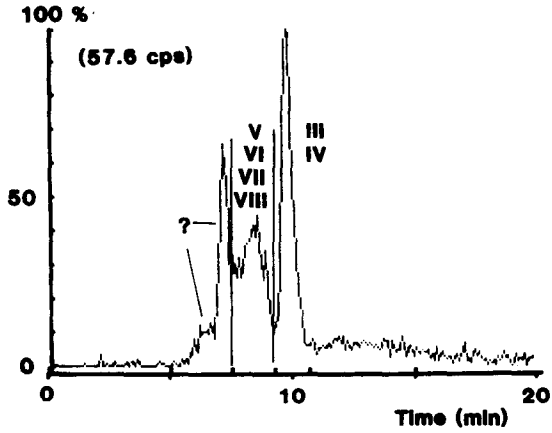

Fig. 8. Group separation of ^{14}C metabolites in rat urine. Mobile phase A and columns C1 and C2; sample volume, 100 μl (104 000 dpm).

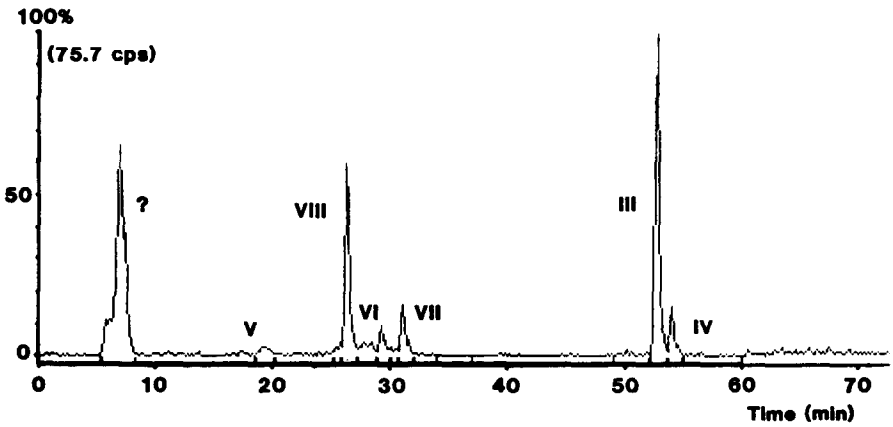

Fig. 9. Column switching separation of ^{14}C metabolites in rat urine. Sample volume, 162 μl (169 000 dpm).

were well separated and quantification was performed with the microprocessor-based evaluation system. The radioactivity level between the two gradients (run time 35—50 min) was used as the background level and subtracted from the amount of radioactivity integrated in each peak.

It was not possible to determine the accuracy or the precision of the method, since ^{14}C-labelled reference compounds of the metabolites were not available. Neither was it meaningful to estimate these "parameters" with non-labelled compounds and UV detection, since the endogenous background made quantification of reference compounds added to blank urine impossible.

The relative standard deviation (R.S.D.) for quantification of ^{14}C with the on-line detector was estimated by analysing the urine from the individual rats in triplicate or quadruplicate. For the least abundant metabolite (V), the R.S.D. ranged from 2.9 to 34%, and for the most abundant (III), from 2.5 to 8.9%. The rather large variation is most likely due to difficulties in

TABLE III

QUANTITATIVE PATTERN OF [^{14}C] FELODIPINE METABOLITES EXCRETED IN 24-h RAT URINE

Roman numerals refer to metabolites in Fig. 1.

	Front fraction*	V ± S.D.	VIII ± S.D.	VI ± S.D.	VII ± S.D.	III ± S.D.	IV ± S.D.
$Mean_{tot}$**	29.8 ± 2.3	1.3 ± 0.4	13.7 ± 1.4	3.7 ± 0.1	5.2 ± 0.8	28.8 ± 3.8	5.0 ± 0.7
Percentage of dose	6.6	0.3	3.0	0.8	1.2	6.4	1.1

*Metabolites of unknown identity.

**$Mean_{tot}$ = mean value of the means from three or four quantifications of each individual rat (n = 4).

the manual selection of the limits for integration of the radioactivity content in the peaks.

The amount of radioactivity corresponding to the diacid (ca. 1% of the injected amount) was considered as the minimum determinable concentration with this assay and corresponded to (22—45) · 10^{-6} M in the original urine (27—58 pmol per injection), depending on the concentration of ^{14}C in each urinary sample.

The yield of ^{14}C from the columns was 100 ± 2% (n = 6), as determined by the ratio of the known injected amount in a sample to the amount in the eluate collected after radioactivity detection for 70 min post injection.

The amounts of the metabolites excreted by the rat are given in Table III. The values given are the mean and standard deviation of the means of three or four determinations for each rat. The diacid (V) was found to be a minor metabolite, corresponding to 1.3% of the excreted amount. This finding is contradictory to our earlier report, in which the diacid was stated to be one of the main metabolites [2]. Possible explanations might be that the single-column method used did not provide resolution between the diacid and other metabolites, most probably belonging to the unidentified front fraction in the method reported here. Also, the mobile phases used in the earlier work were of a different composition (pH 7 vs. 5 and 10% vs. 5% methanol in phase A), which might have caused co-elution of compounds that were better resolved by the present method.

The methyl monoacid (III) was found to be the main metabolite excreted in urine by the rat (28.5%). This is in agreement with the earlier findings, in which the fraction containing both III and VI was found to be the major one [2]. The metabolites separated and quantified in this study constituted 57.7% of the excreted amount in urine during the first 24 h post dose. This corresponded to 12.8% of the dose.

CONCLUSION

Earlier reports on liquid chromatographic methods for metabolites of 1,4-dihydropyridine analogues have not commented on the problem of separating the hydroxy acid metabolites from the corresponding monoacids. In two methods for the quantification of unchanged drug and plasma metabolites of nifedipine [18] and of nitrendipine [19], separation was achieved on alkyl-bonded silica, using isocratic elution with more than 50% organic modifier

in the mobile phase. As regards the carboxylic acid metabolites, these could not be quantified since they were only slightly retained and eluted close to the chromatographic front.

Recently, an ion-pair liquid chromatographic method was published for quantification of the monoacid metabolite of nifedipine in human urine by isocratic elution on bonded silica [8]. Acetonitrile was used as organic modifier, and the method was stated to be selective without interference from other metabolites. However, chromatographic data supporting these conclusions were missing and it is not unlikely that the excreted amount of monoacid metabolite reported (60% dose) is actually the sum of the monoacid and the corresponding hydroxy acid metabolite. In the study reported here, it was found that mobile phases containing acetonitrile instead of methanol as the organic modifier, failed to resolve the hydroxy acids from the monoacids on bonded silica.

A different approach for the separation would be to convert the hydroxy acid metabolites into lactones by acidification and heat treatment [10]. This method was applied to a rat urine sample, transforming the ethyl and methyl hydroxy acids quantitatively into the corresponding lactones, which were well resolved from the monoacids in a single-column system (cf. Fig. 3). Compound VIII, which is already a lactone at physiological pH [3], was not affected by the acidic heat treatment. However, some unidentified metabolites were transformed and interfered with the quantification of the monoacids, thereby limiting the utility of this method.

Since single-column systems did not provide sufficient resolution between the compounds, a combination of packing materials with different selectivity was used in a column switching method, giving the necessary resolution for on-line quantification of radioactivity.

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