Journal of Chromatography, 414 (1987) 109–120
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3430

LIQUID CHROMATOGRAPHIC DETERMINATION OF DILTIAZEM AND ITS METABOLITES USING *trans* ISOMERS AS INTERNAL STANDARDS, WITH DYNAMIC MODIFICATION OF THE SOLID PHASE BY ADDITION OF AN AMINE TO THE MOBILE PHASE

PETER HÖGLUND* and LARS-GÖRAN NILSSON

Department of Clinical Pharmacology, University Hospital of Lund, S-221 85 Lund (Sweden)

(First received June 20th, 1986; revised manuscript received September 23rd, 1986)

SUMMARY

A selective and sensitive reversed-phase high-performance liquid chromatographic method for simultaneous analysis of diltiazem and its five metabolites known to occur in man is presented. Different C_{18} columns are compared. The influences of organic co-solvent, pH, ionic strength and the addition of various amines are studied. The use of *trans*-diltiazem as internal standard is advocated. UV detection at 237 nm is used for plasma analyses. The possibilities of electrochemical detection are also discussed. Before solvent extraction, plasma samples are saturated with sodium chloride and made alkaline. Triethylamine is also added. The use of the method in pharmacokinetic applications is exemplified.

INTRODUCTION

Diltiazem, *cis*-(+)-3-acetyloxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-one hydrochloride (Fig. 1), is a calcium entry blocker which is currently used in the treatment of angina pectoris, hypertension and supraventricular arrhythmias [1]. The bioavailability of an oral solution of diltiazem or a loosely filled capsule is only about 40% [1], mostly due to presystemic metabolism. The systemic clearance is predominantly due to hepatic metabolism [2]. At present five metabolites have been identified in humans [3]. The pharmacokinetics of these metabolites in humans is, however, poorly understood.

Earlier methods of analysis for diltiazem have been based on thin-layer chromatography (TLC) [4], gas chromatography (GC) using a nitrogen-sensitive [5] or an electron-capture detector [2,6], in some methods after derivatisation [5,6], or high-performance liquid chromatography (HPLC) [7–10]. The only

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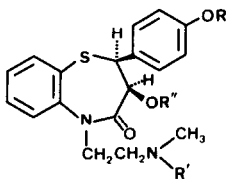

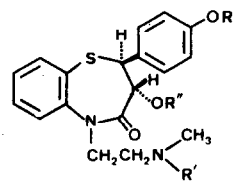

	R	R′	R″		R	R′	R″
Dtz	CH_3	CH_3	$COCH_3$	trans-Dtz	CH_3	CH_3	$COCH_3$
M_A	CH_3	H	$COCH_3$	trans-M_A	CH_3	H	$COCH_3$
M_1	CH_3	CH_3	H				
M_2	CH_3	H	H				
M_4	H	CH_3	H				
M_6	H	H	H				
PD	CH_3	CH_3	$COCH_2CH_3$				

Fig. 1. Molecular structures of diltiazem (Dtz), five of its metabolites and three potential internal standards.

metabolite measured in the GC assays is the deacetylated form of diltiazem. The HPLC methods generally involve a simpler pre-chromatographic step, give better possibilities to detect and quantify more metabolites but are, in most cases, slightly less sensitive than GC. At present no HPLC method has been described which permits the simultaneous determination of all the five identified metabolites in human plasma. The objective of this study was to develop such a method.

EXPERIMENTAL

Materials

Acetonitrile (Rathburn Chemicals, Walkerburn, U.K.) was of HPLC grade. 1-Dimethylaminododecane (DMDA), practical grade, was obtained from Fluka (Buchs, Switzerland) and triethylamine (TEA), synthetic grade, was purchased from Merck (Darmstadt, F.R.G.). All other solvents and chemicals were of analytical, reagent or synthetic grade (Merck, Fluka or Fisons, Loughborough, U.K.). For aqueous solutions, water was made organic free by purification in a Milli-Q/Organex-Q system (Millipore, Neu Isenburg, F.R.G.). Diltiazem·HCl, *cis*-(+)-deacetyldiltiazem·HCl (M_1), *cis*-(+)-N-demethyldiltiazem fumarate (M_A), *cis*-(+)-N-demethyldeacetyldiltiazem (M_2), *cis*-(+)-O-demethyldeacetyldiltiazem·HCl (M_4), *cis*-(+)-N,O-didemethyldeacetyldiltiazem·HCl (M_6), *trans*-(+)-diltiazem·HCl (*trans*-diltiazem) and *trans*-(±)-N-demethyldiltiazem·HCl (*trans*-M_A) were generously donated by Tanabe Seiyaku (Japan). The structures of these compounds are given in Fig. 1. Further, *cis*-(+)-3-propionyloxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-one oxalate (PD, Fig. 1) was synthesized in our labo-

ratory [11], with diltiazem as starting material. A mass spectrum of the product showed a molecular ion at 428 *m/z* and the prominent ions at *m/z* 206, 178, 150, 136, 121, 71 and 58.

Apparatus

Two different liquid chromatographic (LC) systems for solvent delivery and injection were used: a Waters M-45 pump with a programmable automatic injector (WISP 710 B, Waters Assoc., Milford, MA, U.S.A.) or an SP 8700 XR pump with an SP 8780 XR autosampler (Spectra-Physics, Darmstadt-Kranichstein, F.R.G.). Both systems were used together with a Spectroflow 783 UV detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) set at 237 nm. When studying electrochemical detection, a Waters M450 variable-wavelength UV detector was used in series with an LC-4 amperometric detector with a TL-5 glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.). For evaluation of retention times and peak areas or heights an SP 4290 integrator (Spectra Physics) was used.

Columns

Three different reversed-phase LC columns were used. Nucleosil C_{18}, 5 μm, was packed in a stainless-steel column 200×4.6 mm I.D., by a slurry-technique. Its performance was tested by injection of a standard test mixture. Spherisorb ODS-2, 3 μm, 150×4.6 mm I.D. was obtained from HPLC Technology (Macclesfield, U.K.) and Novapak C_{18}, 150×3.9 mm I.D. from Waters.

Chromatography

Acetic acid and phosphoric acid were adjusted to the desired pH with 5 mol/l sodium hydroxide or with isomolar solutions of the appropriate acids or bases. The ionic strength was varied by altering the molar concentration of buffer salts or by adding sodium sulphate. All buffer solvents were filtered prior to use and vacuum filtration or helium bubbling was used for degassing. The composition of the mobile phases is expressed in v/v. Since some of the mobile phases used required long equilibration times, the solvent was often recycled. All separations were performed at ambient temperature, at a flow-rate of 1.0 ml/min. Column pressure depended on the type of column used, but normally ranged between 100 and 200 bar.

Standard solutions

Stock solutions of diltiazem or metabolites were prepared in water at concentrations of 10^{-2} mol/l or 10^{-3} mol/l (M_A) and frozen at −20 °C. Prior to use they were diluted with water.

Extraction methods

Plasma (normally 1 ml) was extracted with 5 ml extraction solvent — diethyl ether, diisopropyl ether, *tert.*-butyl methyl ether or hexane–2-propanol (95:5) — after addition of 100 μl carbonate buffer (1.0 mol/l, pH 9) and finally 0.5 g sodium chloride and/or 5 μl TEA. After gentle rocking for 10–45 min, the organic phase

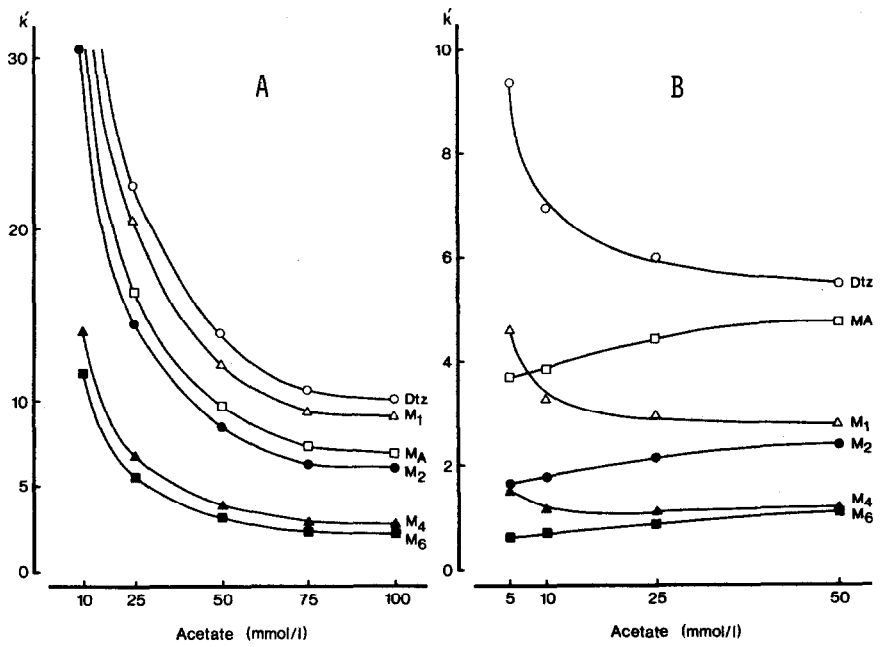

Fig. 2. Dependence of the retention of diltiazem (Dtz) and its metabolites on ionic strength without and with amine additive. Column: Spherisorb ODS-2, 3 μm, 150×4.6 mm I.D.. Mobile phase: acetate buffer pH 4.5–acetonitrile (45:55); (A) without amine, (B) with 1.0 mmol/l DMDA.

was transferred to a new test tube and evaporated to dryness under nitrogen or back-extracted into 0.01 mol/l hydrochloric acid. The solution was then made alkaline and extracted with a new 5-ml portion of extraction solvent, which then was evaporated to dryness. The residue was redissolved in mobile phase (normally 150–250 μl) of which 25–100 μl was injected into the HPLC system.

RESULTS AND DISCUSSION

HPLC separation

Diltiazem was easily separated from its metabolites on all columns tested. Likewise, the metabolites were generally well separated due to the differences in their hydrophobicity. As could be expected for weak amines, the retention was strongly pH-dependent in the pH region 3.5–7.5, i.e. increasing the pH of the mobile phase resulted in longer retention times. Like most other reversed-phase separations, the retention was influenced by ionic strength (Fig. 2A) and the concentration of organic co-solvent, e.g. acetonitrile, in the eluent, i.e. higher acetonitrile contents resulted in shorter retention times.

The separation was, however, little affected by these parameters and the resolution was not always satisfactory because of broad and asymmetrical peaks. Both the Spherisorb and the Novapak columns gave poor peak shapes regardless of the above-mentioned changes in the composition of the mobile phase (Fig. 3A). Addition of an amine (DMDA or TEA) brought about a large improvement

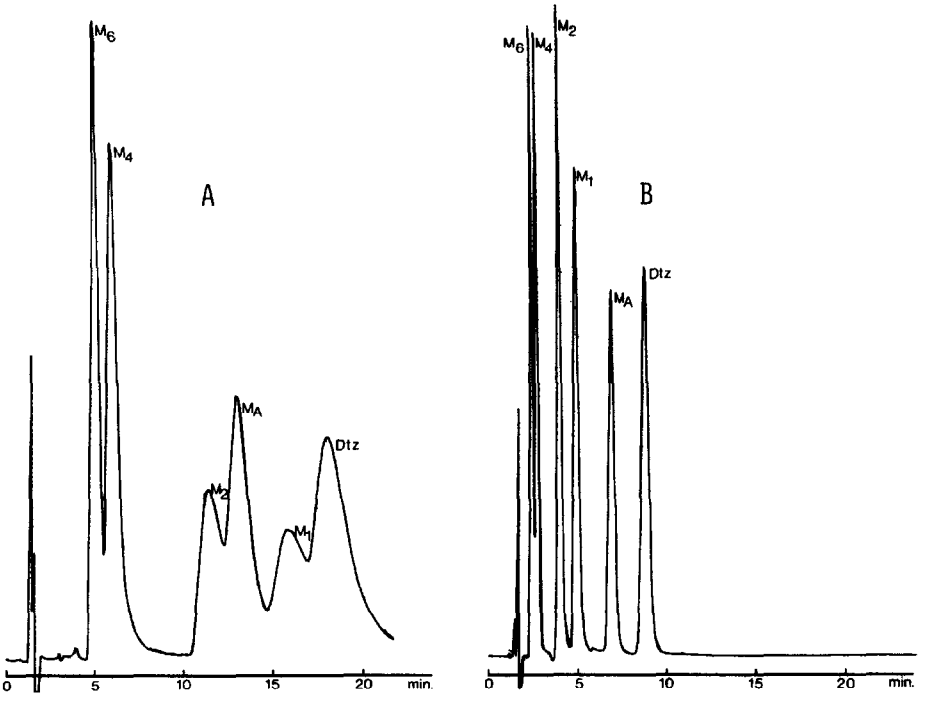

Fig. 3. Separation of diltiazem (Dtz) and its metabolites on a Spherisorb ODS-2, 3 μm, 150×4.6 mm I.D. column. Mobile phase: (A) 50 mmol/l acetate buffer, pH 4.5-acetonitrile 45:55; (B) 25 mmol/l acetate buffer, pH 4.5-acetonitrile (90:10) containing 1.0 mmol/l DMDA.

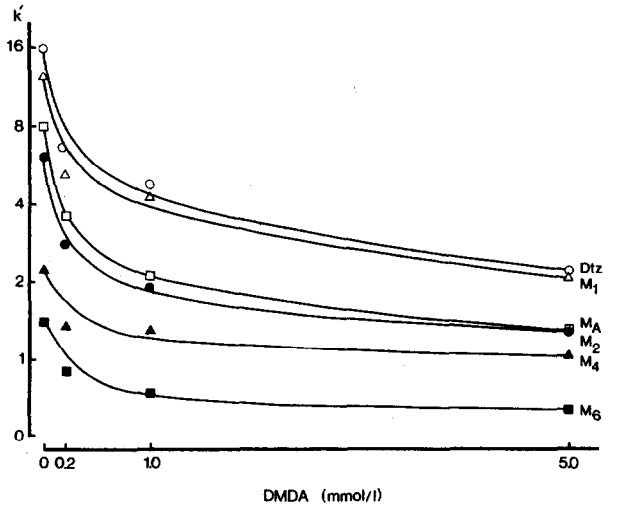

Fig. 4. Effect of DMDA addition on the retention of diltiazem (Dtz) and its metabolites. Column: Spherisorb ODS-2, 3 μm, 150×4.6 mm I.D. Mobile phase: 10 mmol/l phosphate buffer containing 20 mmol/l sodium sulphate, pH 4.5-acetonitrile (45:55) containing varying amounts of DMDA.

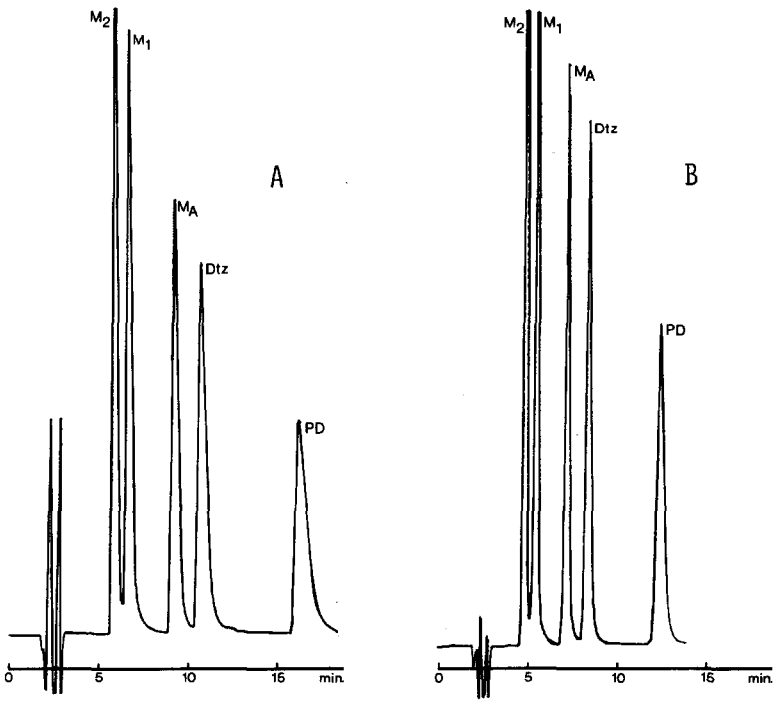

Fig. 5. Separation of diltiazem (Dtz) and its metabolites on a Nucleosil C_{18}, 5 μm, 200×4.6 mm I.D. column. Mobile phase: 50 mmol/l acetate buffer, pH 4.0–acetonitrile (60:40); (A) without amine, (B) with 5.0 mmol/l TEA.

in chromatographic performance. The former, in a concentration of 1.0 mmol/l mobile phase, increased the apparent number of theoretical plates of the Spherisorb column from 2000 to 30 000 plates per meter and decreased the asymmetry factor from 2.5 to 1.2 (Fig. 3B). DMDA also had a drastic effect on the retention, necessitating a decrease in acetonitrile content of the mobile phase in order to get acceptable retention times (Fig. 4). Similar effects, although less drastic, were obtained for the Nucleosil column by the addition of TEA, 5 mmol/l mobile phase (Fig. 5). The same effects of amine additives have been described, for example, for tricyclic antidepressant drugs [12]. As without the addition of an amine, the retention could be controlled by varying the pH, the ionic strength or the concentration of acetonitrile in the mobile phase. Changes in ionic strength of the DMDA-containing eluents resulted in large changes in the selectivity. The retention of diltiazem and its non-N-demethylated metabolites decreased upon increasing the ionic strength, whereas the opposite took place for the N-demethylated metabolites (Fig. 2B). Since the use of long-chain dimethylalkylamines needs relatively long equilibration times, several hundred milliliters of DMDA-containing eluent had to be pumped through the column before a stable distribution between stationary and mobile phase was achieved. Increasing the acetonitrile content of the mobile phase therefore resulted in two events: first, a rapid decrease in retention (i.e. the expected effect of increasing the amount of organic

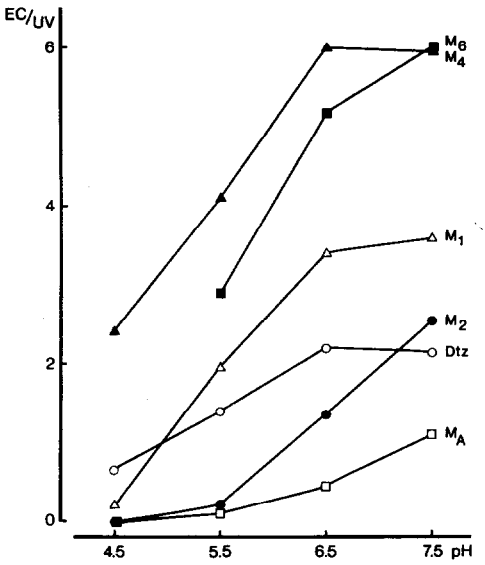

Fig. 6. Electrochemical response versus UV absorbance for diltiazem (Dtz) and its metabolites as a function of pH. EC/UV = ratio of the peak height of the electrochemical signal (potential, +1.10 V vs. Ag/AgCl; 50 nA f.s.) and that of the UV signal (237 nm; 0.04 a.u.f.s.). Column: Nucleosil C_{18}, 5 μm, 200×4.6 mm I.D.. Mobile phase: 10 mmol/l phosphate buffer containing 20 mmol/l sodium sulphate at various pH–acetonitrile (55:45).

co-solvent) and then a slow increase in retention due to a decreased adsorption of the amine on the stationary phase. The net effect of an increase in the acetonitrile content was a small decrease in retention.

Addition of heptanesulphonate (5 mmol/l) to the mobile phase at pH 4.5 also decreased retention, but had a less pronounced effect on peak symmetry than the amines.

To sum up, columns like Spherisorb ODS-2, 3 μm, and Novapak C_{18} are not suitable for sensitive determinations of diltiazem and its metabolites unless the mobile phase is modified, for example by addition of a long-chain dimethylalkylamine such as DMDA. A suitable concentration of amine would be 1.0 mmol/l in acetonitrile–25 mmol/l phosphate buffer, pH 4.5 (15:85). Ionic strength and acetonitrile content could then be varied according to the above procedure in order to obtain an optimal separation. The phosphate buffer could be replaced by an acetate buffer, but then the most polar metabolites M_6 and M_4 are more difficult to separate from the chromatographic front.

HPLC detection

Diltiazem is generally monitored by UV detection around its absorption maximum at 237 nm. However, diltiazem and its metabolites could also be detected electrochemically. The hydrodynamic voltammograms differed between substances and were also dependent on the pH of the mobile phase. However, an oxidative potential of around 1 V had to be applied in all cases. The O-demethyl-

ated metabolites were most easily oxidized and also produced the highest signals (Fig. 6). At pH 7–8, electrochemical detection could prove valuable as a complement to UV detection. However, the required high working potential led to a high background current and a decreased selectivity. The addition of DMDA to the mobile phase increased the electrochemical background to such an extent that its effect on the signal of the analytes could not be studied.

Extraction

Extraction of diltiazem and its metabolites from plasma at alkaline pH was tested using different extraction solvents. The recovery was affected not only by the nature of the solvent but also by the pH, the time for extraction and the addition of salt and amines. Saturating the plasma with sodium chloride increased the hexane–2-propanol (95:5) extraction recovery from 26% to 62%, from 55% to 99%, from 8% to 57% and from less than 5% to 33% for M_A, M_1, M_2 and M_4, respectively ($n=3$). A subsequent addition of TEA, tended to increase the recovery further for these metabolites whereas the addition of TEA without salt had little or no effect. Diltiazem was almost completely extracted (94%) even without salt addition, while M_6, the most polar metabolite studied, still could not be extracted with hexane–2-propanol. Instead, the ethers gave acceptable extractions for all metabolites after salt addition, recoveries ranging from 65% for M_A to 80–97% for the other metabolites and 98% for diltiazem. For diethyl ether an extraction time of 30 min seemed necessary, while 15 min was found to give complete extraction for the other solvents. A carbonate buffer of pH 9 was chosen to alkalinize the plasma samples since a lower pH was found to give lower recoveries and a higher pH may catalyze deacetylation of diltiazem and M_A.

At some occasions during the method development the N-demethylated metabolites, M_A, M_2 and M_6, gave spuriously low extraction recoveries in spite of the salt addition, whereas it was found to be stable (coefficient of variation $<10\%$) at other times. The reason for this is unclear but adsorption may play a role since hydrophobic secondary amines are known to be specially prone to this.

The different types of extraction solvents also affected the blank chromatogram obtained from the extraction of a drug-free plasma sample. The ethers especially gave extra peaks in the chromatogram. Some of these peaks could be eliminated by performing a backextraction into a weak acid, whereas other peaks seemed to originate from the ether, their appearance and size depending on the quality and age of the solvent.

One problem that must be taken into consideration is the risk for deacetylation of diltiazem and M_A as well as of *trans*-diltiazem, *trans*-M_A and PD. pH values above 9 and below 3 are known to catalyze this deacetylation. For diltiazem, 1 mmol/l in 0.1 or 0.9 mol/l hydrochloric acid, a hydrolysis to M_1 of about 4% and 25%, respectively, was found after 2 h at room temperature. In 0.01 mol/l hydrochloric acid negligible amounts of M_1 (less than 0.5%) were formed during the same period. The use of strong acid for back-extraction should therefore be avoided. Further, we found a deacetylation to M_1 in methanol–water (50:50) solutions (4% in 24 h at ambient room temperature) while water solutions were found stable at least for 24 h. This indicates that methanol solutions should be avoided.

Choice of internal standard

The compound chosen as internal standard should have physicochemical properties as close to those of the analyte as possible. We therefore examined the stereoisomer *trans*-diltiazem for use as internal standard. *trans*-Diltiazem was less retained than diltiazem on all LC systems tested, its retention relative to diltiazem being approximately 0.9 and 0.6 without and with the addition of amine, respectively. *trans*-Diltiazem was mostly well separated from the metabolites. Extraction recoveries from plasma showed very good agreement with diltiazem, M_1 and M_4 but often deviated from the recoveries of M_6, M_2 and M_A. Thus, we examined *trans*-M_A for chromatographic and extraction performance. *trans*-M_A was less retained than *trans*-diltiazem, its relative retention times being 0.6 and 0.3–0.55 without and with amine, respectively. For extraction from plasma with hexane–2-propanol (95:5), *trans*-M_A behaved more similar to M_A and M_2 than *trans*-diltiazem, but further studies have to be performed to establish the suitability of *trans*-M_A as an internal standard. The different behaviour of diltiazem and its tertiary amine metabolites on the one hand and the secondary amine metabolites on the other hand creates a need for more than one internal standard. A similar case has been described for the analysis of zimelidine and its N-demethylated metabolite norzimelidine [13].

The great number of peaks may be a problem. Diltiazem, its five metabolites and two internal standards would have to be resolved in the chromatogram. Further, one must be assured of a sufficient separation from endogenous compounds as well as from the deacetylated products from *trans*-diltiazem and *trans*-M_A, which may be formed before or during sample work-up. PD, which was used as internal standard in one recently published method [10], is an alternative to *trans*-diltiazem but at least on the Spherisorb column after DMDA addition its retention time was unsatisfactorily long (2.0 relative to diltiazem). Further, one must be aware of the risk for deacetylation of PD to M_1, which may result in a falsely high concentration of this metabolite.

Application

The method has been used for evaluation of the pharmacokinetics of diltiazem and its metabolites in humans [14]. Fig. 7 shows chromatograms after a diethyl ether extraction from 1 ml plasma made slightly alkaline and saturated with sodium chloride, backextraction into weak acid followed by a further ether extraction and evaporation of the organic solvent. The samples in Fig. 7c and d were taken from a subject 4 and 4.5 h after administration of 90 mg diltiazem·HCl (Cardizem®) as a single-dose and after repeated administration (90 mg three times a day for 7 days), respectively. In this case M_4 was not resolved from an interfering peak, and could not be determined. The measured concentrations for the other metabolites at steady-state conditions were higher than hitherto believed. The further investigation of this is the scope of ongoing studies.

CONCLUSIONS

Diltiazem is extensively metabolised, and the pharmacokinetics of the metabolites and their contribution to the clinical effects are not known. An analytical

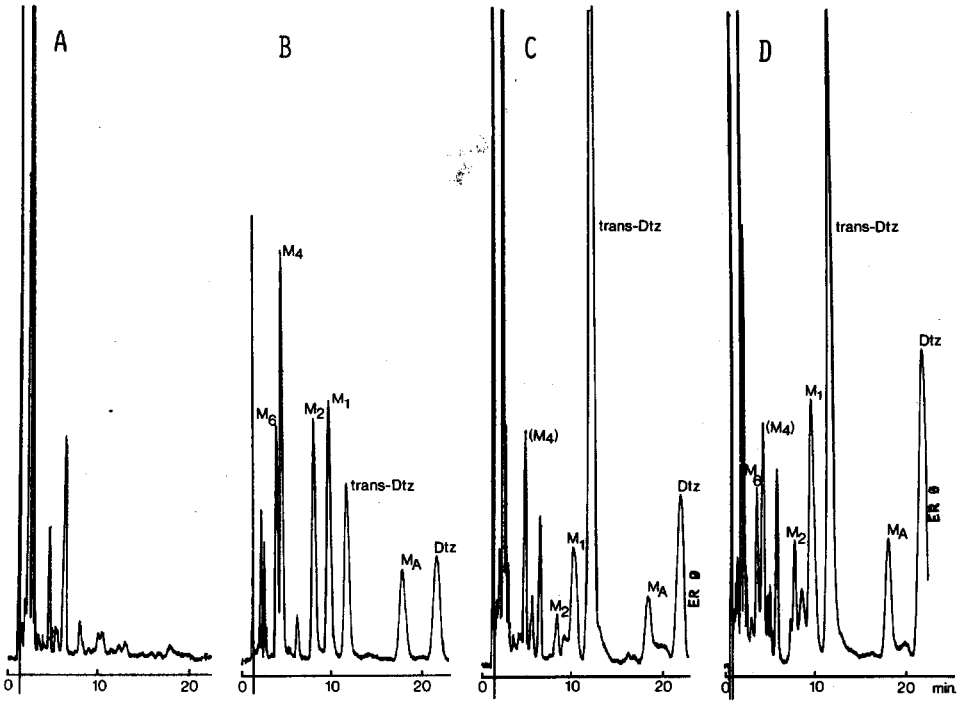

Fig. 7. Example of chromatograms obtained after diethyl ether extraction from human plasma. (A) Blank plasma; 100 µl injected. (B) Blank plasma with diltiazem and metabolites added, 500 nmol/l of each, together with *trans*-diltiazem; 25 µl injected. (C) Plasma from a subject 4 h after a single dose of 90 mg diltiazem·HCl (Cardizem®); 100 µl injected. Concentrations measured: diltiazem, 150 nmol/l; M_1, 39 nmol/l; M_A, 58 nmol/l; M_2, < 25 nmol/l; M_4 not determinable because of an interfering peak; M_6, < 25 nmol/l. (D) Plasma from the same subject 4.5 h after dose during steady-state conditions: 90 mg diltiazem·HCl (Cardizem®) three times a day; 100 µl injected. Concentrations measured: diltiazem 364 nmol/l; M_1, 122 nmol/l; M_A, 156 nmol/l; M_2, 62 nmol/l; M_4, not determinable because of an interfering peak; M_6, 83 nmol/l. Column: Novapak C_{18}. Mobile phase: 25 mmol/l phosphate buffer, pH 4.0–acetonitrile (90:10) containing 0.6 mmol/l DMDA. Detection: UV at 237 nm.

method for determination of the parent compound and its metabolites is a prerequisite for kinetic and concentration–response studies.

Reversed-phase liquid chromatography is the technique most often used, but some problems are associated with such analyses. The drug and at least some of its metabolites may differ considerably in polarity, which makes an isocratic separation difficult. Gradient elution, although theoretically a solution to this problem, demands instrumentation that is not generally available, and the results are often difficult to reproduce routinely. On the other hand, there might be metabolites that differ very little in polarity and therefore are difficult to separate. The extraction of the parent drug and the metabolites from plasma does not always yield the same recoveries, which makes it necessary to use more than one internal standard. A highly efficient LC separation system may be needed to resolve all peaks in the chromatogram. A stationary phase with small and narrowly distributed particles has to be used but even then the peak shape may be poor, as was

shown for diltiazem and its metabolites in this work. Amines are often difficult to handle because of strong interactions with the LC support, a phenomenon that could be eliminated with amine additives to the mobile phase. DMDA proved to be excellent but other long-chain dimethylalkylamines probably work equally well. With the DMDA-containing eluent we have shown that by a simple variation of the ionic strength one can control the selectivity in a way that is not easily achieved otherwise.

The pre-chromatographic sample work-up should ideally give full recoveries of the analytes and the exclusion of compounds that would otherwise interfere in the chromatography. This is hard to accomplish when substances of different polarity — like a drug and its metabolites — are to be analysed. We have found that extraction with an ether gives good recoveries of diltiazem and all its metabolites but unfortunately clean blank chromatograms were difficult to obtain. Back-extraction into 0.01 mol/l hydrochloric acid was used to reduce this problem.

Addition of an internal standard before extraction is often essential for obtaining a high precision in the analysis. The compound used should resemble the analyte, so that it can compensate for variations in extraction recoveries. *trans*-Diltiazem was found to be the ideal for diltiazem and M_1, while *trans*-M_A should be more accurate for the N-demethylated metabolites.

The sensitivity of an LC method depends on factors like the amount of sample and the analytical recovery, column performance, detection possibilities and also on the possibility to obtain clean blank chromatograms. With the conditions mentioned in this work, we found a practical limit of quantitation of about 25 nmol/l for all analytes with a 1-ml plasma sample. This concentration results in peak heights of 10 times the detector noise or more.

ACKNOWLEDGEMENTS

We are grateful to Tanabe Seiyaku, Japan, for generous supply of compounds, and to Ms. Gertrud Lundqvist and Ms. Gertrud Persson for skillful laboratory assistance.

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