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AUTOMATED PRE-COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE INVESTIGATION OF ADIBENDAN METABOLISM

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SUMMARY

An automated pre-column high-performance liquid chromatographic method has been developed for the isolation of adibendan and metabolites from biological fluids and for their simultaneous quantitative assay. High sensitivities were obtained by the use of a multiple-injection device allowing solid-phase extraction from several successive sample injections with enrichment of metabolite traces on the pre-column. Two metabolites in dog urine were identified as N-oxypyridine (M1) and 2-hydroxypyridine (M2) derivatives of adibendan, while the structure of M3 is still unknown. M1 and M2 are also metabolites in rats, rabbits and humans, and contribute to cardiovascular efficacy. The metabolic profiles were determined in plasma, urine and bile, as a function of dose, route of administration and sex, using radioactivity and ultraviolet detection of the eluates.

INTRODUCTION

Adibendan, 7,7-dimethyl-2-(4-pyridyl)-6,7-dihydro-3*H*,5*H*-pyrolo[2,3-*f*]-benzimidazol-6-one, has been shown to be a new potent agent of the class of tricyclic heterocycles with non-catecholamine and non-glycoside cardiotonic activity. Besides positive inotrope activity, it exhibits vasodilating and calcium-sensitizing efficacy at low doses in animals and in humans [1,2]. For pharmacokinetic studies a recently published [3] high-performance liquid chromatographic (HPLC) method allows the sensitive fluorometric determination of adibendan in plasma and urine.

Many authors have reported fully automated chromatographic procedures for drug analysis [4,5]. Sample preparation is often the most tedious and la-

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bour-intensive part of the whole analysis if carried out by manual means. Liquid extractions have been replaced by solid-phase extractions in pre-columns and cartridges [4,6,7]. These devices have been interfaced directly with the analytical column for subsequent analysis. The necessary loading, washing, eluting and regenerating are carried out by the use of microprocessor-controlled column-switching valves, allowing automatic and on-line operation of sample clean-up and analysis.

This paper describes the use of a multiple-injection device. The capacity is enhanced by repetitive injection of the sample followed by purging of the concentration column. Higher sensitivity is obtained for metabolite isolations and for their quantitative assay in metabolic profiling studies. This device is part of a modular HPLC system that has been established for studies on adibendan metabolism.

EXPERIMENTAL

Chemicals

Adibendan (BM 14.478), its ^{13}C- and ^{14}C-labelled derivatives and the potential metabolites BM 14.0506 (N-oxide, M1), BM 14.0518 (2-hydroxy derivative, M2), BM 14.0607 (3-hydroxypyridyl derivative) and BM 50.0308 (desmethyl compound) were synthesized in the Chemical Research Department of Boehringer Mannheim [8]. Chemical and/or radiochemical purity was greater than 98% for all synthesized compounds. The specific activity of [^{14}C]adibendan was 3.1 MBq/mg (23.3 mCi/mmol). Acetonitrile, methanol and ammonium acetate of AR quality (Baker Chemicals, Gross Gerau, F.R.G.) were used for the mobile phase.

Apparatus

The modular HPLC system consisted of two HPLC pumps (Model 64.00), two six-port switching valves (Rheodyne RH 7010) and a gradient programmer 50 B, all from Knauer (Berlin, F.R.G.). The system was completed by flow detectors for continuous monitoring of the UV absorption (spectrophotometer Model 87.00, Knauer) and ^{14}C radioactivity in the eluates (Model LB 504, Berthold, Wildbad, F.R.G.) with solid scintillator cells of different volumes, a fraction collector (SuperRac 2211, Pharmacia/LKB, Freiburg, F.R.G.), a recorder (Kompensograph, Siemens, Karlsruhe, F.R.G.) and an autoinjector (Perfusor, Braun, Melsungen, F.R.G.), comprising a dosing unit driven by an electric motor and a one-way plastic syringe as sample reservoir. A liquid scintillation spectrometer (Packard Instruments, Frankfurt, F.R.G.) was used for radioactivity measurements by liquid scintillation counting (LSC). HP 1000 and HP 3000 computers (Hewlett-Packard, Waldbronn, F.R.G.) were used for peak integration and raw data evaluation.

Animal studies

[^{14}C]Adibendan was administered to dogs (1 mg/kg orally, 0.5 mg/kg intravenously to three male and three female beagles), rats (100 mg/kg orally, 5.0 mg/kg into the duodenum of eight male and eight female Sprague–Dawley rats) and rabbits (50 mg/kg orally to six female Himalayans). Radioactivity was measured in plasma, urine, faeces and bile (rats) by LSC, and the pharmacokinetic data were evaluated up to seven days after dosage. The amounts of excreted radioactivity were calculated as a percentage of the dose for urine, faeces and bile. The composition of the radioactivity was determined by HPLC.

High-performance liquid chromatography

An automated pre-column HPLC method with on-line solid-phase extraction was used to isolate the parent drug and its metabolites and for their quantitative assay. The pre-column (17–40 mm×4.6 mm I.D.) was packed with

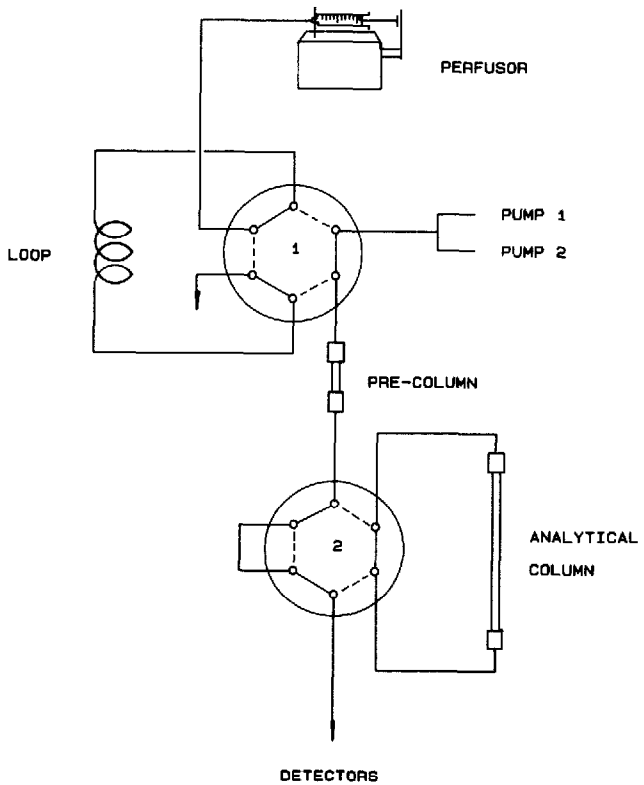

Fig. 1. Automated pre-column HPLC system with multiple-sample injection and column-switching devices. (1) Injection valve: (——) sample load; (---) sample injection onto the pre-column and pre-column purge. (2) Switching valve: (——) position load, purge phase is bypassing the analytical column; (---) position inject mobile phase, switching to the analytical column.

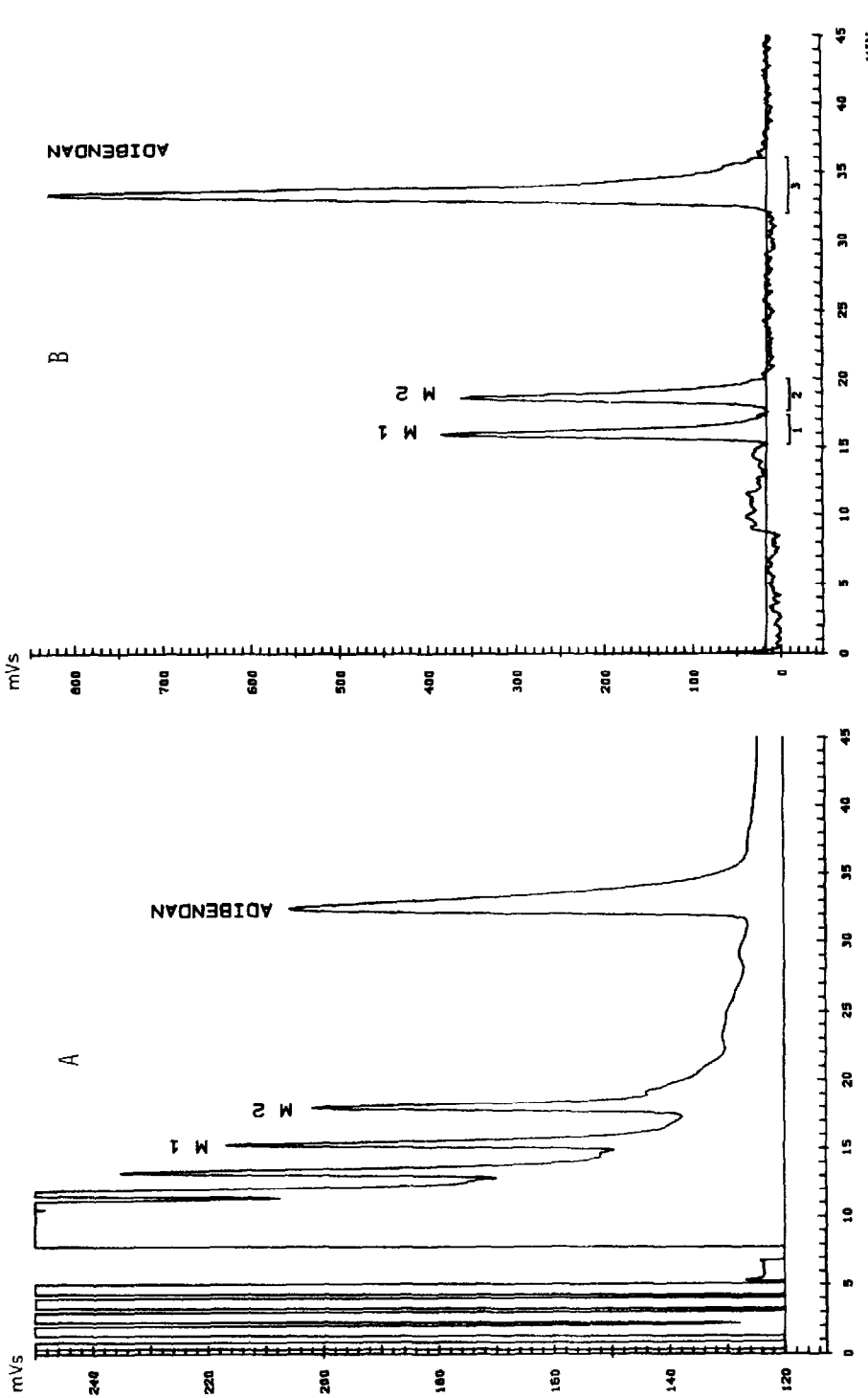

Fig. 2. Metabolic profiling using pre-column HPLC. Analysis of 0–48 h dog urine after oral administration of 1 mg/kg [^{14}C]adibendan. (A) On-line sample clean-up from five consecutive pre-column injections of 0.1 ml. Detection by UV at 230 nm. (B) On-line detection of radioactivity from the same HPLC run.

LiChroprep RP-18 (Merck, Darmstadt, F.R.G.), particle size 25–40 μm. The analytical column (125 mm×4.6 mm I.D.) was packed with Nucleosil $5C_{18}$ (Machery & Nagel, Düren, F.R.G.). The mobile phase was 0.05 *M* ammonium acetate buffer (pH 3.5)–methanol (60:40, v/v), at a flow-rate of 1 ml/min. The samples were injected onto the precolumn with the aid of an autoinjector and a sample loop (Fig. 1).

Isolation of metabolites. Multiple (up to ten) pre-column injections of up to 0.5 ml (urine, bile, plasma) per HPLC run were carried out using 40-mm pre-columns. Each injection was followed by a pre-column purge (water, delivered at 1 ml/min by pump 1). The mobile phase (delivered by pump 2) initiated the straight-flushing of the enriched parent drug and its metabolites from the pre-column to the analytical column for subsequent separation. The UV absorption was measured continuously at 230 nm in the eluates of the pre-column and the analytical column, immediately showing any interference in metabolite separation from run to run. The eluates of several HPLC runs were collected at 0.5-min intervals for 40 min, and aliquots were monitored off-line for radioactivity by LSC. The same tubes were used for each run. Appropriate fractions were pooled and lyophilized.

For determination of purity and identity, the isolated compounds were controlled by thin-layer chromatography (TLC), using TLC plates pre-coated with silica gel 60 F 254 (Merck). They were co-chromatographed with an authentic sample of unlabelled material with three different developing systems. A TLC-scanner (IM 3000, Isomess, Straubenhardt, F.R.G.) was used for radiodetection.

Quantitative analysis (metabolic profiling)

Depending on the actual counts per minute (cpm) of the sample, one to five pre-column injections of 0.1 ml were carried out per HPLC run, using analytical-size pre-columns (17 mm length). Radioactivity was monitored on-line in the eluates (RAM, Fig. 2B) or off-line in collected fractions by LSC (Fig. 3). The peak areas of the parent drug and its metabolites were determined by peak integration, and calculated as percentages of the total area for all radioactive compounds eluted. The injected radioactivity should be quantitatively recovered in the eluates.

RESULTS

An automated pre-column HPLC method has been established for the investigation of adibendan metabolism. The conditions are similar for metabolite isolations from biological samples and for their quantitative assay. They can easily be modified according to the desired application and sensitivity.

The mode of operation of the modular HPLC system is schematically illustrated in Fig. 1. Pre-columns packed with reversed-phase material are used for solid-phase extraction of the metabolites from the sample. Their capacity lim-

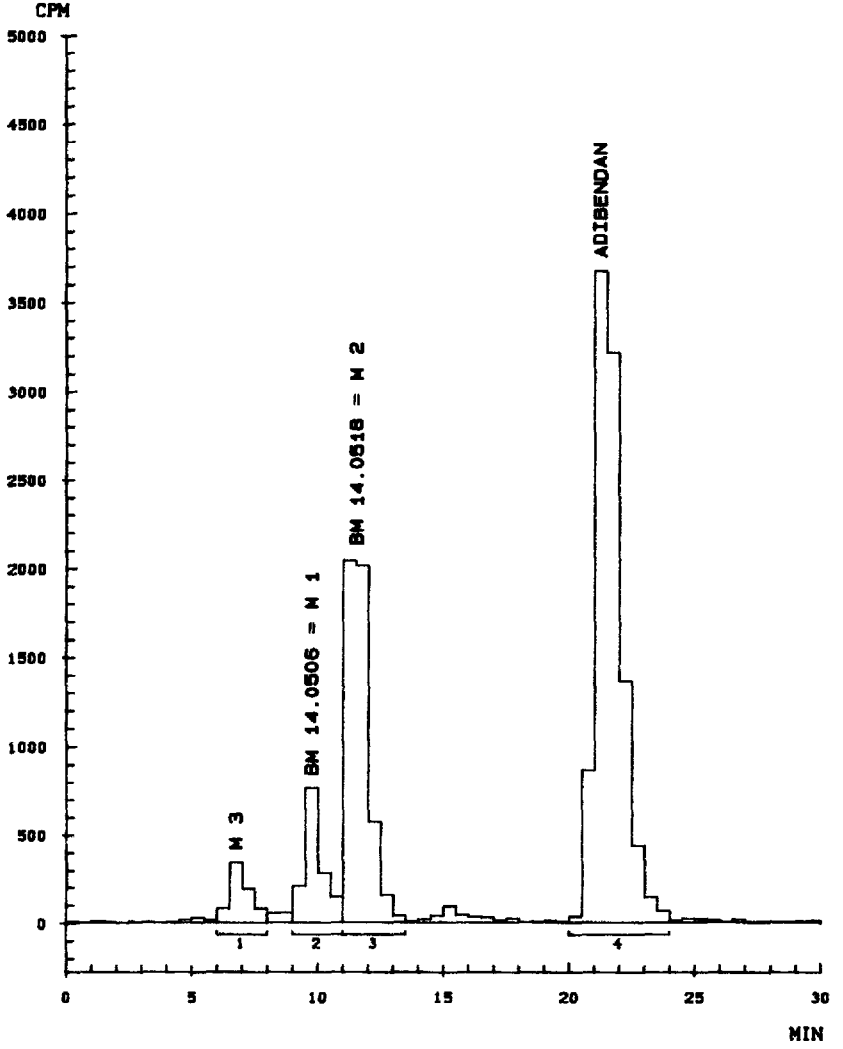

Fig. 3. Metabolic profile in rat bile, collected for 0.5–3 h after administration of 5 mg/kg [^{14}C]adibendan into the duodenum. Off-line detection of radioactivity by LSC.

its the sample volume and the number of injections onto the pre-column per HPLC run. Short (17 mm) columns are sufficient for analytical separation, and 40-mm columns are used for isolation.

Multiple (one to ten) pre-column injections of 0.1–0.5 ml are carried out. A multiple-injection device allows solid-phase extraction from several consecutive injections with a pre-column purge between the individual injection steps. Thus the degree of substance enrichment is increased compared with single

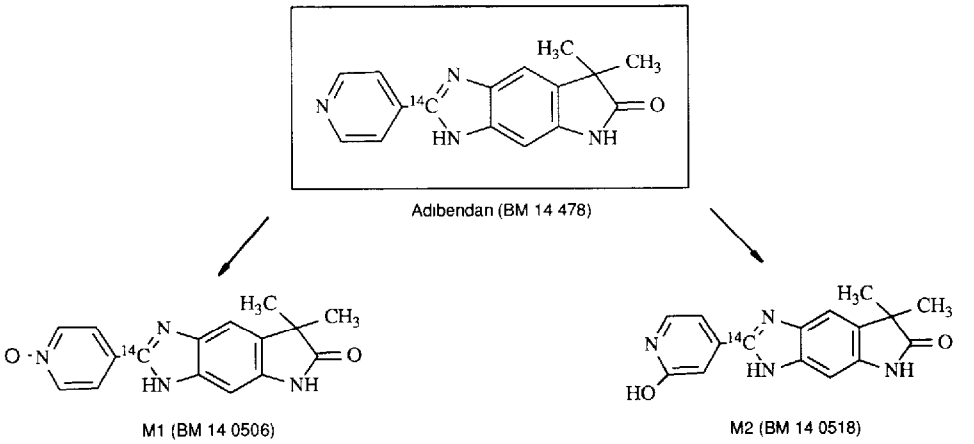

Fig. 4. Metabolic pathways of adibendan in rat, dog, rabbit and human.

injections. Higher yields are obtained for isolations, and the sensitivity is improved also for quantitative assays.

The pre-column is interfaced directly to the analytical column for subsequent separation. Injection, loading, washing and eluting of the pre-column and the analytical column are carried out by use of column-switching valves. The programmer is responsible for the sequence and timing according to the actual HPLC conditions, allowing on-line operation of the sample analysis.

Radioactivity and UV absorption are measured in the eluates of the pre-column and the analytical column in parallel. On-line radioactivity monitoring (RAM, Fig. 2B) and off-line detection in the tubes of a fraction collector by LSC (Fig. 3) can be used as alternatives.

The sensitivity of radiodetection by LSC is twenty times higher than by RAM. In our experiments at least 3000 cpm should be injected for the detection of a single peak by RAM, whereas 150 cpm are sufficient for LSC.

A comparison of UV absorption and RAM detection (Fig. 2) shows that the radioactivity from five consecutive pre-column sample injections is quantitatively retained on the pre-column, but most of the UV-absorbing material from the urine matrix is not. The total recovery of radioactivity in the eluates is near 100%.

The reproducibility of metabolite quantitation by pre-column HPLC was determined by repetitive analysis of a 0–24 h dog urine. The percentages of adibendan and of metabolites M1, M2 and M3 were calculated for six runs as the percentage of total radioactivity injected. The mean run-to-run coefficient of variation was ca. 5%.

In addition to unchanged adibendan, three metabolites (M1, M2 and M3) were isolated from dog urine. The chemical structures of M1 and M2 (Fig. 4) agree with those of the authentic synthesized compounds by mass spectro-

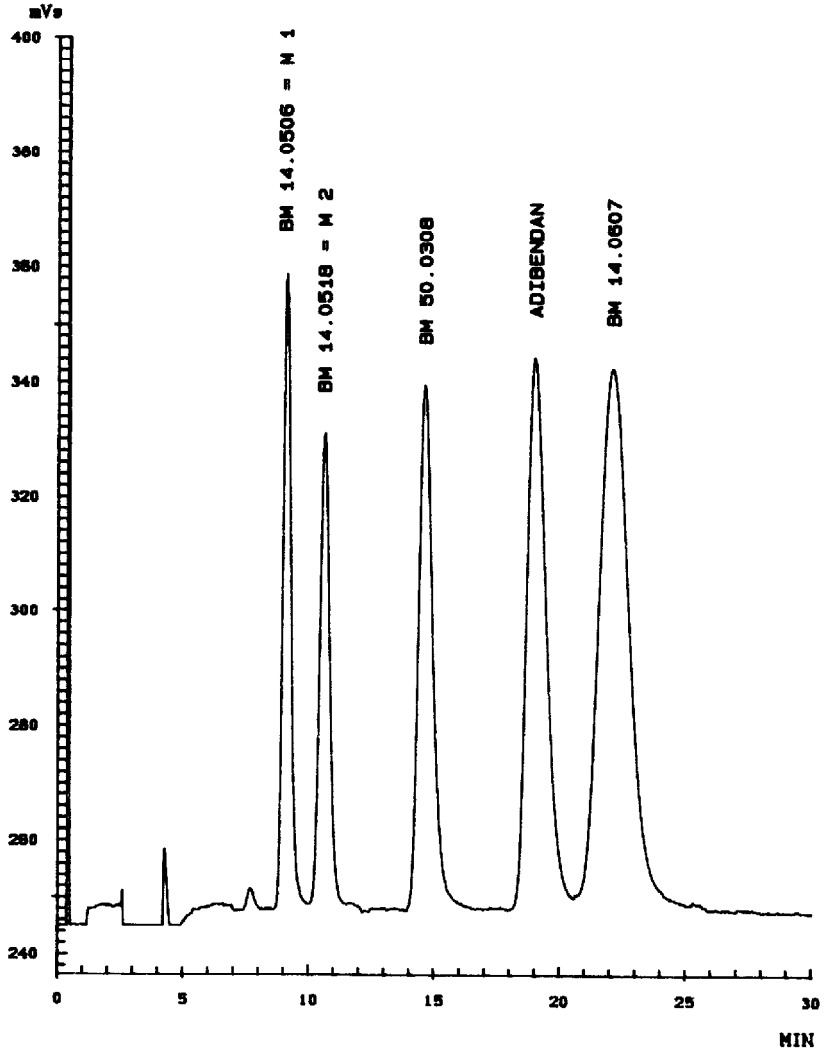

Fig. 5. Separation of adibendan metabolites and potential metabolites by pre-column HPLC with UV detection at 230 nm. Substance concentration: 2 μg per 0.1 ml and per run.

scopic, nuclear magnetic resonance and chromatographic (TLC and HPLC) data. The characterization of the metabolites by HPLC is shown in Fig. 5. BM 14.0607 and BM 50.0308 are not found as metabolites of adibendan, and the structure of M3 has not yet been clarified.

Metabolic profiling studies in dog, rat and rabbit showed that the parent drug is the major compound in all animal species. In dogs, the 0–24 h urine was analysed after oral and intravenous administration of [^{14}C]adibendan, separately for the males and females. Of the drug fraction excreted, 35–41% was

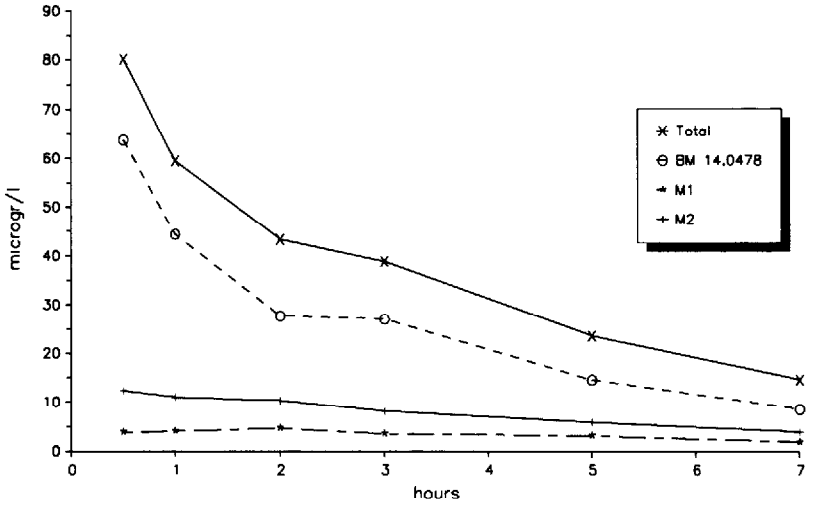

Fig. 6. Pharmacokinetics of (○) adibendan, (⋆) metabolite M1, (+) metabolite M2 and (×) total radioactivity in dog plasma after oral administration of 1 mg/kg [^{14}C]adibendan.

unchanged adibendan, 26–31% was M1, 23–27% was M2 and 5% was M3. These data were independent of the route of administration and of sex.

With the exception of M3, the chemical structures of almost 100% of the excreted radioactive drug fraction have been elucidated. Renal excretion amounts to 20% of the oral dose in dogs and 40% of the intravenous dose in dogs, with 63% and 54%, respectively, excreted via faeces.

The highest percentage of adibendan is found in the 0–7 h pool plasma of dogs (72%) and rats (92%) and in the 0–24 h urine of rats and rabbits (83%). The metabolic profile of adibendan in rat bile is shown in Fig. 3.

In a pharmacokinetic study with dogs the plasma concentrations of adibendan and its metabolites were determined (Fig. 6). The sum of M1, M2 and parent drug concentrations approaches the concentration of total drug fraction, measured as radioactivity by LSC for each point of the concentration–time curves, thus confirming the high identification rate also for plasma.

Metabolites M1 and M2 have also been identified as metabolites in humans. They were isolated from 0–6 h human urine of a volunteer who had received an oral dose of 5 mg of unlabelled drug. Metabolic profiling studies showed higher relative amounts of M1 (42%) in relation to adibendan (36%) compared with animals. The more extensive rate of metabolism may be explained by dose dependence. The effect of increasing percentages of adibendan metabolites by decreasing the dose has also been found in the dog.

DISCUSSION

The pre-column modular HPLC method is used for metabolism studies of new drugs. The method is fast, as solid-phase extraction replaces time-con-

suming solvent extraction and HPLC replaces conventional LC separation. The HPLC procedure can be run automatically by a column-switching technique. The method is sensitive, as only small volumes are necessary for the separation and reproducible quantitation of the whole metabolic pattern. The sensitivity can be improved by enrichment of substance traces from successive injections of the same sample onto the pre-column.

The method can be used as a clean-up procedure for the isolation of hitherto unknown metabolites for structural elucidation. Isolation of the metabolites is necessary for only one animal species if the metabolic pattern is qualitatively identical for all the others.

The method is accurate, as it allows the whole spectrum of non-polar and polar metabolites and metabolite conjugates in various biological matrices to be recorded.

Investigations with pre-column HPLC were also carried out with compounds that are more extensively metabolized than adibendan, for example the antiallergic picumast [9] and the thromboxane antagonist daltroban [10]. For these drugs similarly high percentages of identified drug fractions were obtained. Substances of high polarity and, in the case of plasma, of very high protein binding are not extracted by reversed-phase material. Limitations of the method will be examined systematically in future investigations.

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