Journal of Chromatography, 515 (1990) 597–602
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1814

Direct determination of the antihypertensive agent Cromakalim and its major metabolites in human urine by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method has been developed for the simultaneous determination of Cromakalim, a novel antihypertensive agent, and its urinary metabolites including diastereomeric glucuronides. The HPLC system employed a strong cation-exchange precolumn (Senshu Pak SCX-2051-N) to allow direct injection of urine samples. The unchanged drug and its three major metabolites were simultaneously separated on a reversed-phase column (Develosil ODS-5) and fluorometrically detected (excitation, 254 nm; emission, 306 nm) by the aid of their native fluorescence. The calibration curves for Cromakalim and a metabolite were linear in the range from 10 to 200 ng ml^{-1}, while those for the diastereomeric glucuronides were linear in the range from 20 to 400 ng ml^{-1}. The detection limits (signal-to-noise ratio = 3) of these compounds were 0.3 ng ml^{-1} or less in all cases.

INTRODUCTION

Cromakalim, (±)-*trans*-6-cyano-3,4-dihydro-2,2-dimethyl-4-(2-oxo-1-pyrro-lidinyl)-2*H*-1-benzo[*b*]pyran-3-ol, is a novel antihypertensive agent, discovered by Beecham Pharmaceuticals Research Division (Essex, U.K.), which relaxes vascular smooth muscle by activation of potassium ion channels[1]. In the phase I study, the plasma levels of the parent drug were determined using capillary gas chromatography (GC)[2,3] or high-performance liquid chromatography (HPLC)[4]. Urinary excretion of a drug and its related substances is also an important aspect of a pharmacokinetic study. For this purpose, an HPLC method was developed[5] and used to determine Cromakalim and its three major metabolites, including the diastereomeric glucuronides, in human urine (Fig. 1). However, the method requires a solid-phase extraction

0021-9673/90/$03.50

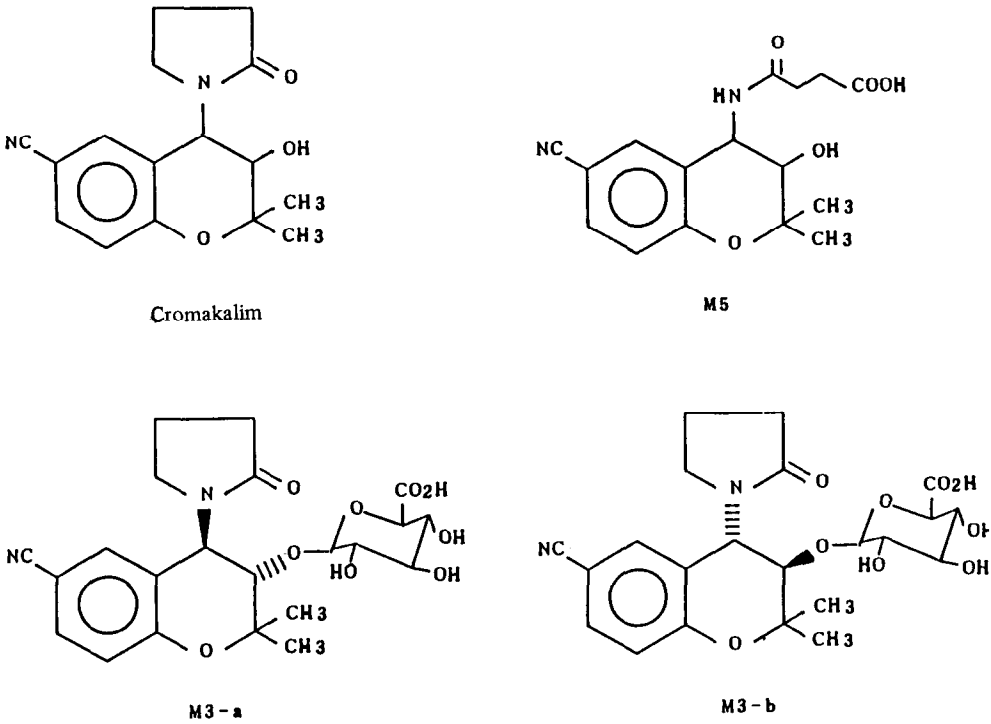

Fig. 1. Structures of Cromakalim and its major metabolites in human urine.

as the pretreatment procedure, which prolongs the time necessary for the assay. It is probable that the number of samples processed would be increased and more information on the metabolism could be obtained if the samples could be analysed without pretreatment.

In this paper, an HPLC method employing two different types of column for the direct determination of Cromakalim and its major metabolites in human urine is described.

EXPERIMENTAL

Reagents

All chemicals and solvents were of analytical-reagent or HPLC grade (Kanto Chemical, Tokyo, Japan), except for physiological saline (Physisalz), purchased from Fuso Yakuhin Kogyo (Osaka, Japan). Distilled water was used to prepare aqueous solutions, and all other chemicals and solvents were used without further purification. Acetate buffers were prepared by mixing the same concentrations of sodium acetate and acetic acid in various proportions. Eluents for HPLC were filtered through an FP-450 membrane filter (0.45 μm) (Gelman Sciences Japan, Tokyo, Japan) before use.

Standard solutions

Standard stock solutions of Cromakalim and its metabolites were prepared by accurately weighing about 10 mg of each reference material. Each sample was dissolved in 5 ml of methanol and then diluted with saline to give concentrations of 40 μg ml^{-1} (M3-a and M3-b) and 20 μg ml^{-1} (Cromakalim and M5). All standard compounds were synthesized and characterized by Beecham Pharmaceuticals Research Division (Essex, U.K.). The solutions were stored in amber-coloured silanized glass bottles at 4°C in the dark until required.

Calibration graphs

Working solutions for construction of the calibration graphs were prepared by diluting each stock solution with saline to give the appropriate concentrations. To 1 ml of blank urine, 100 μl of each working solution were added and a 50-μl aliquot of the spiked urine was subjected to HPLC directly after filtration with an Acro™ LC13 filter assembly (0.2 μm) (Gelman Sciences Japan). Blank urine collected from Japanese male volunteers was pooled and kept at −20°C until used.

HPLC system

The LC-6A HPLC system consisted of two LC-6A pumps, an RF-535 fluorescence detector, a SIL-6A autoinjector, a SCL-6A system controller and a C-R4AX Chromatopac integrator (Shimadzu, Kyoto, Japan). A Develosil ODS-5 (5 μm, 100 Å) column (25 cm × 4.6 mm I.D.) (Nomura Chemical, Seto, Japan) was used as the main analytical column. Either a strong anion-exchange column of Senshu Pak SAX-2051-N (5 μm, 100 Å) or a strong cation-exchange column of Senshu Pak SCX-2051-N (5 μm, 100 Å) (both 50 mm × 6 mm I.D.) (Senshu Scientific, Tokyo, Japan) was used as the precolumn. The columns were maintained at 35°C by an SSC 3520C column oven (Senshu Scientific). Cromakalim and its metabolites were detected by monitoring their inherent fluorescence at excitation and emission wavelengths of 254 and 306 nm, respectively[5].

RESULTS AND DISCUSSION

Selection of precolumn

Initially, a column-switching technique utilizing an ion-exchange column was considered as a possible method for the direct determination of Cromakalim and related substances in human urine. However, experimentation indicated that good separations from urinary endogenous substances could be accomplished even when the urine samples were introduced directly into the system in which an ion-exchange column was connected in series to the C_{18} analytical column. Accordingly, the usefulness of anion-and cation-exchange columns in the separation of urinary substances was investigated. As favourable results were observed when a strong cation-exchange column of Senshu Pak SCX-2051-N was placed before the main C_{18} analytical column as shown in Fig. 2, further experiments were carried out to develop a method involving direct injection via the strong cation-exchange column.

Separation and determination

The separation was optimized based on the analytical conditions of gradient

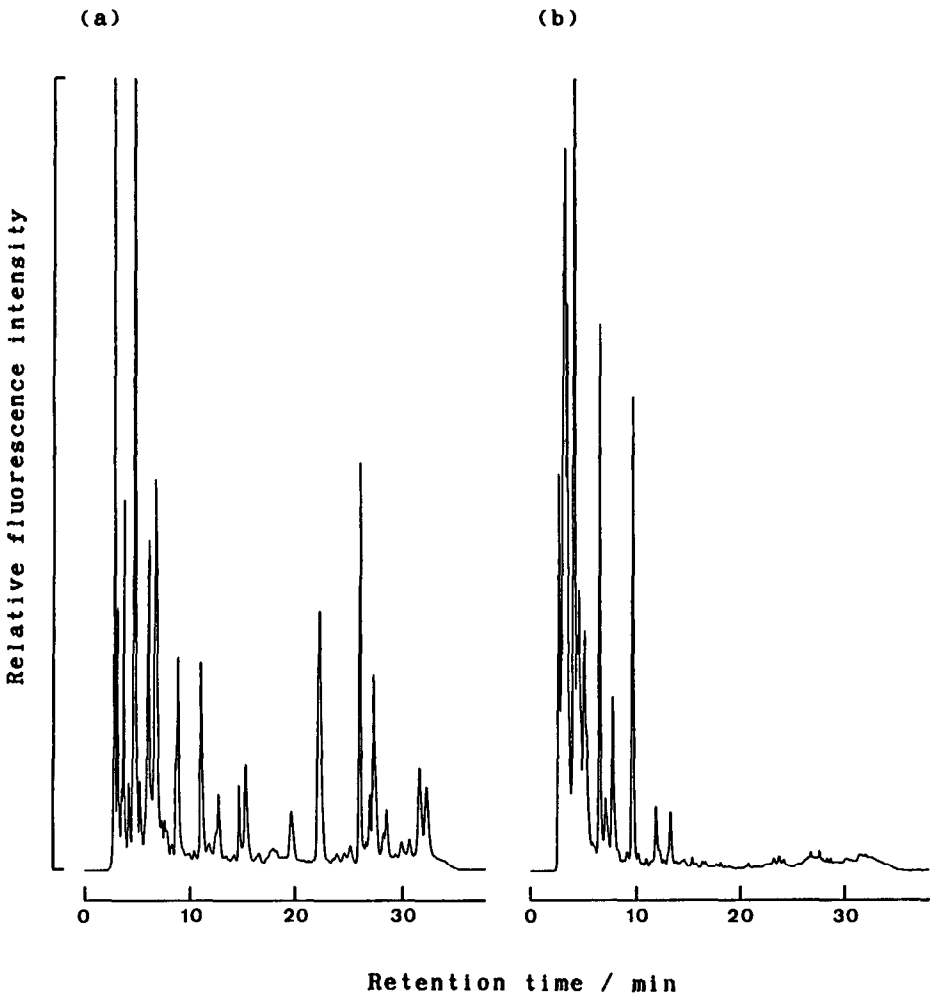

Fig. 2. Effectiveness of Senshu Pak (a) SAX-2051-N and (b) SCX-2051-N precolumn in separation of human blank urine.

elution, on a reversed-phase C_{18} column, with acetate buffer and acetonitrile, established in a previous study[5]. The effects of acetate buffer concentration and its pH on the chromatographic elution were re-examined as the strong cation-exchange column was newly attached to the analytical column being used. Buffer concentrations in the range 50–250 m*M* at pH 3.2 had little effect on the chromatographic patterns. After consideration of this fact and the variation of the pH of the urine samples, the original concentration, 150 m*M*, was employed for the study of the effect of pH in the range 3.2–6.2. The results reconfirmed that a pH of 3.2 was suitable for achieving a baseline resolution between the diastereomeric glucuronides M3-a, M3-b. Moreover, the retention times of M3-a, M3-b and M5 tended to be short and their separation from urinary substances became difficult as the pH of the eluent increased. Therefore,

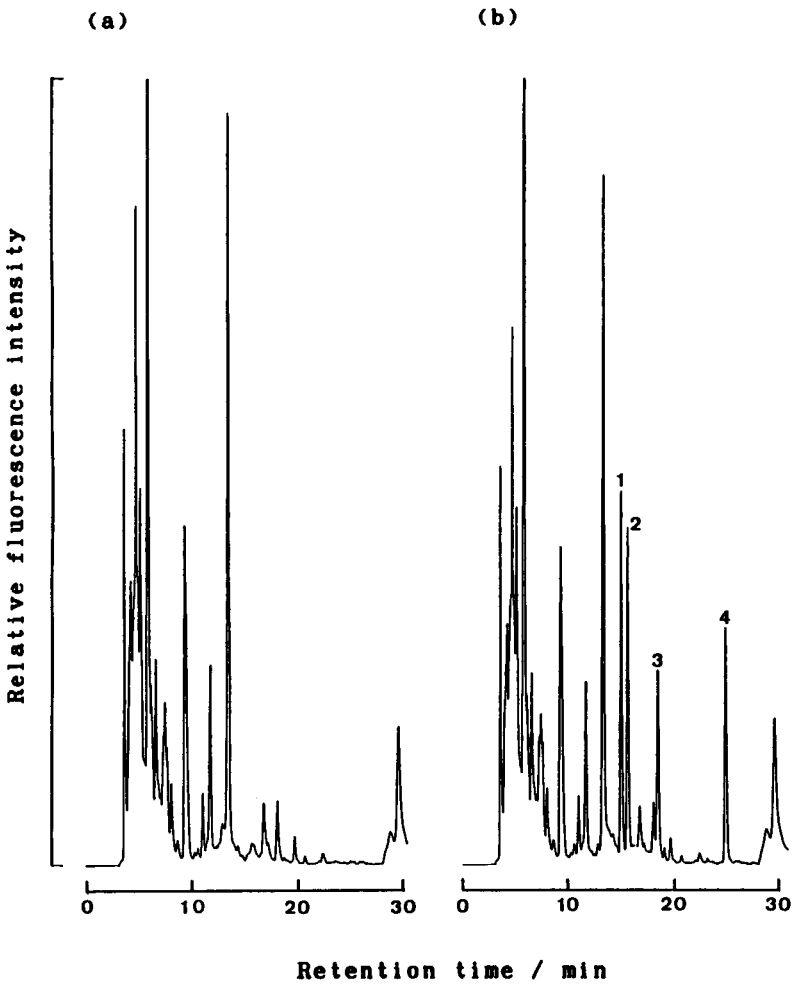

Fig. 3. Typical chromatograms obtained with the proposed HPLC system. (a) Blank urine; (b) working standard solutions added to blank urine. Peaks: 1 = M3-a; 2 = M3-b; 3 = M5; 4 = Cromakalim.

separation was achieved with eluent A [150 m*M* sodium acetate buffer (pH 3.2)] and eluent B [750 m*M* sodium acetate buffer (pH 3.2)–acetonitrile (1:4, v/v)], optimum separation being obtained using a linear gradient from 22 to 40% eluent B over 20 min followed by column washing with eluent B for 8 min. The flow-rate was maintained at 1 ml min^{-1} through this work. Representative chromatograms are depicted in Fig. 3.

The calibration graphs were linear ($r \geqslant 0.9995$ each) in the range 10–200 ng ml^{-1} for Cromakalim and M5 and 20–400 ng ml^{-1} for M3-a and M3-b. The detection limits (signal-to-noise ratio = 3) for Cromakalim, M5, M3-a and M3-b were 0.21, 0.25, 0.29 and 0.26 ng ml^{-1}, respectively. The reproducibility and accuracy were also satisfactory, as shown in Table I. Hence the method can be applied to the assay of real

TABLE I

RECOVERIES OF CROMAKALIM AND ITS URINARY METABOLITES

Compound	*Concentration (ng/ml)*			
	Added	*Determined*	*Recovery (%)*[a]	*Relative standard deviation (%)*[a]
M3-a	50	46.79	93.59	3.73
	200	197.9	98.95	1.91
M3-b	50	50.92	101.8	1.54
	200	203.1	101.5	0.69
M5	25	26.51	106.1	2.51
	100	101.3	101.3	1.26
Cromakalim	25	23.34	93.36	3.38
	100	96.12	96.12	1.38

[a] $n = 3$.

urine samples from preclinical studies. As described above, the method achieved a direct determination of Cromakalim and its major metabolites, including the diastereomeric glucuronides, simultaneously in human urine. As this is a direct measurement, the urine assay can be carried out as a fully automated procedure. The sample throughput can therefore be increased and also more information on the metabolites possibly obtained. The concept of utilizing an ion-exchange column as a precolumn is of value in the development of drug assays in biological fluids. This study has shown that the direct measurement of cationic drugs in urine is possible with the aid of an SCX-2051-N precolumn.

ACKNOWLEDGEMENTS

The authors thank Mr. Tsuyoshi Tanizaki for excellent assistance. They also thank Dr. David R. Summers and Dr. Christine Summers for their kind support throughout this work and valuable advice on the manuscript. Encouragement from Dr. Hisashi Nakazawa, Mr. Kazuo Nakahira, Mr. Yoshitaka Nishioka and Dr. Takao Konishi is acknowledged.

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