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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IONIC DRUGS IN BIOLOGICAL FLUIDS WITH SHORT-WAVELENGTH ULTRAVIOLET DETECTION USING COLUMN SWITCHING COMBINED WITH ION-PAIR CHROMATOGRAPHY: APPLICATION TO BASIC COMPOUNDS

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SUMMARY

A highly sensitive and selective high-performance liquid chromatographic method with short-wavelength UV detection is described for the determination of ionic compounds in biological fluids, which was applied to two basic compounds, 2-{3-[4-(4-fluorophenyl)-1-piperazinyl]propyl}-6,7,8,9-tetrahydro-2*H*-naphtho[2,3-*b*][1,4]oxazin-3(4*H*)-one (I) and methyl 2-(4-diphenylmethyl-1-piperazinyl)ethyl (±)-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate (II), in human serum. The method is based on the combination of the column-switching technique and ion-pair chromatography. In the first ODS column, the analyte is pre-separated from endogenous substances in serum by ion-pair chromatography. After column switching, in the second ODS column the heart-cut fraction containing the analyte is further separated by non-ion-pair chromatography from coeluted endogenous substances from the first ODS column. The proposed method offered high sensitivity and selectivity with UV detection at 215 nm for I and 230 nm for II. The detection limits were 0.2 ng/ml for both I and II using 1 ml of serum. The principle of the proposed method would be applicable to both acidic and basic compounds in biological fluids with a suitable ion-pair reagent.

INTRODUCTION

In the development of a sensitive method for the determination of drugs in biological fluids by high-performance liquid chromatography (HPLC), we often encounter the difficulty that the required sensitivity cannot be obtained because of interferences from endogenous substances. In some instances, selective detection methods such as gas chromatography–mass spectrometry (GC–MS) and HPLC with electrochemical or fluorimetric detection can be utilized. However, GC–MS is not suitable for routine work and only a limited number of compounds

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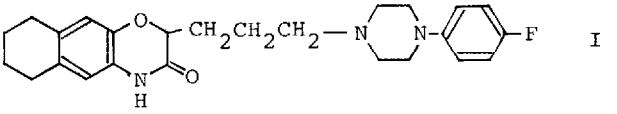
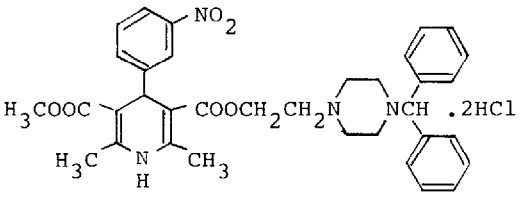


I



II(2HCl)

Fig. 1. Structures of I and II·2HCl.

can be detected sensitively with the other two methods. HPLC with UV detection is the most widely used method for the determination of drugs in biological fluids. Many compounds show strong UV absorption in the short-wavelength region, which cannot usually be used for their sensitive determination in biological fluids because of poor selectivity. However, appropriate clean-up procedures can allow their sensitive determination even with this poorly selective detection method.

Recently, the column-switching technique has been widely used for the direct injection of biological fluids in HPLC [1–11]. In addition to direct injection, another mode of column switching, viz., heart cutting from the first column, has been reported to give an efficient clean-up of biological fluids [12–19]. However, there have been no reports of column switching, to our knowledge, which allows the highly sensitive detection of drugs in biological fluids with UV absorption. Therefore, we have tried to develop a simple method using column switching to offer high selectivity and sensitivity even with short-wavelength UV detection. For ionic compounds whose elution can be delayed by ion-pair formation, we have found that by combining it with ion-pair chromatography, the column-switching technique can offer high selectivity and sensitivity.

2-{3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl}-6,7,8,9-tetrahydro-2*H*-naphtho[2,3-*b*][1,4]oxazin-3(4*H*)-one (I) [20] and methyl 2-(4-diphenylmethyl-1-piperazinyl)ethyl (±)-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate (II) [21] are basic compounds (Fig. 1) whose elution can be delayed by ion-pair formation and they show relatively strong short-wavelength UV absorption (molecular absorption coefficient at 215 nm = 35 500 for I and that at 230 nm = 32 000 for II). In this paper, we describe a column-switching technique combined with ion-pair chromatography for the highly sensitive determination of ionic compounds in biological fluids by HPLC with UV detection, which was applied to the above two basic compounds in human serum.

EXPERIMENTAL

Reagents and materials

Compounds I and II·2HCl were synthesized in the Central Research Division, Takeda Chemical Industries (Osaka, Japan). Acetonitrile, *n*-hexane and ethyl

acetate were of HPLC grade (Wako, Osaka, Japan). All other reagents were of analytical-reagent grade and were used without further purification.

Extraction from serum

Both I and II were extracted from serum with the same simple procedure. To 1.0 ml of human serum was added 1.0 ml of 0.05 *M* sodium hydroxide solution and the mixture was extracted twice with 4 ml of *n*-hexane–ethyl acetate (7:3, v/v). The organic layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 300 μl of the mobile phase for column 1 (MP1), which will be described later. An aliquot of 200 μl was injected into the HPLC system.

Instrument and conditions

The HPLC system consisted of two LC-6A pumps, two SPD-6A UV detectors, an FCV-2AH six-port switching valve and an SIL-6A autosampler, all of which were controlled automatically by an SCL-6A controller (all from Shimadzu, Kyoto, Japan). An C-R3A integrator (Shimadzu) was used for quantitation. Two ODS columns were used: YMC ODS (A type, 5 μm, 70 mm×4.6 mm I.D.) for column 1 and YMC ODS (A type, 5 μm, 150 mm×4.6 mm I.D.) for column 2 (both from Yamamura Chemical, Kyoto, Japan). The mobile phase for column 1 (MP1) was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (58:42 for I, 50:50 for II, v/v) containing 5 m*M* sodium octanesulphonate (SOS) adjusted to pH 3.5 with 10% phosphoric acid. The mobile phase for column 2 (MP2) was the same as for column 1 but without SOS. The temperature and the flow-rate for both columns were 25°C and 1.0 ml/min, respectively. Detection was carried out at UV 215 nm for I and 230 nm for II.

Analytical system and procedure

A schematic diagram of the HPLC system with the column-switching valve is shown in Fig. 2. The retention time of I (II) in column 1 was checked each day before analysis to determine the time programme for the column switching. At time zero, the sample was injected on to column 1 which was eluted with MP1,

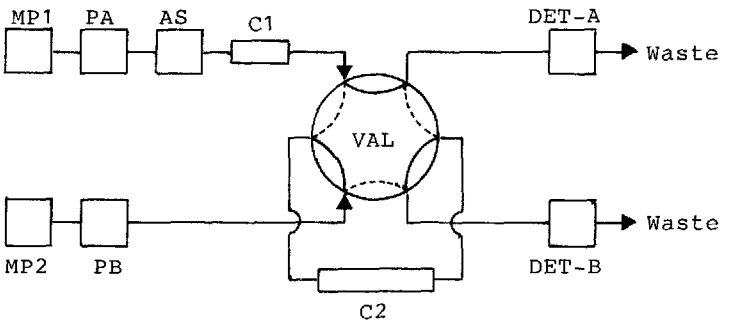


Fig. 2. Schematic diagram of the HPLC system. PA and PB = pumps A and B; AS = autosampler; VAL = six-port valve; C1 and C2 = columns 1 and 2; DET-A and DET-B = UV detectors A and B; MP1 and MP2 = mobile phases 1 and 2. The solid and dotted lines in the six-port valve indicate valve positions A and B, respectively.

while column 2 was eluted with MP2 (valve position A). Just before the elution of I (II) from column 1, the valve was switched to position B and the elutate fraction containing I (II) was introduced into column 2. After the elution of I (II), the valve was switched back to position A. Then the introduced eluate was further separated by column 2 and detected by UV absorption at 215 nm (230 nm), while column 1 was conditioned with MP1 for the next injection. The analysis for each sample was completed within 30 min. These valve operations were all carried out automatically by the SCL-6A controller according to the predetermined time programme.

RESULTS

The HPLC conditions were first investigated for I in serum. Fig. 3 shows a typical chromatogram of blank serum sample with column 1 only, monitored with detector A, i.e., without column switching. The sensitive detection of I was clearly impossible because of interferences. Fig. 4 shows typical chromatograms of serum

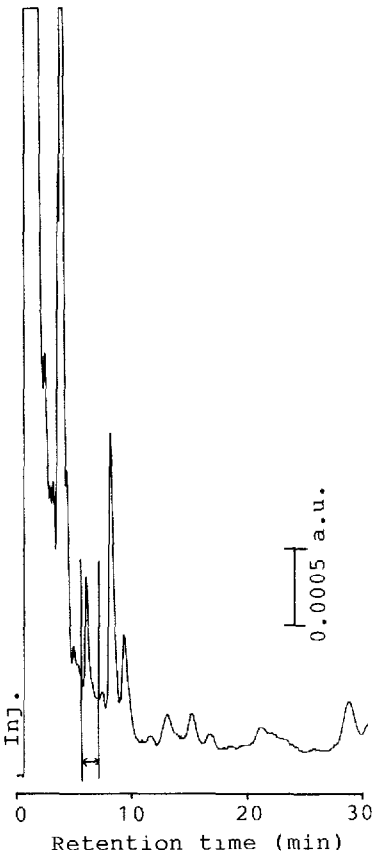


Fig. 3. Typical chromatogram of blank human serum obtained with column 1 only, monitored by detector A, using HPLC conditions for I. The marked zone indicates the heart-cut fraction. HPLC conditions: mobile phase, MP1 for I; detection, UV (215 nm). For other conditions, see text.

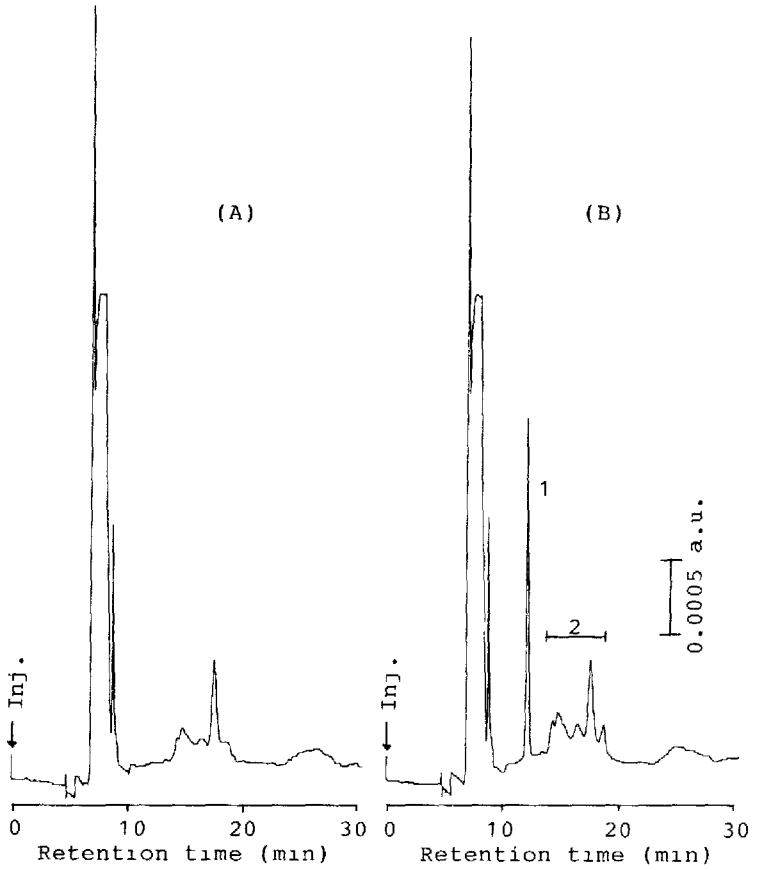


Fig. 4. Typical chromatograms of (A) blank human serum and (B) serum spiked with I (10 ng/ml) obtained with column switching monitored by detector B. Peaks: 1 = I; 2 = endogenous substances in serum. HPLC conditions: mobile phases, MP1 and MP2 for I; detection, UV (215 nm). For other conditions, see text.

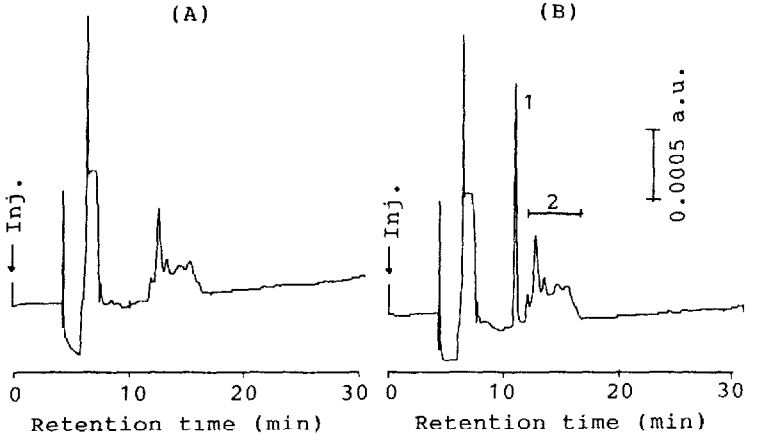


Fig. 5. Typical chromatograms of (A) blank human serum and (B) serum spiked with II (10 ng/ml) obtained with column switching monitored by detector B. Peaks: 1 = II; 2 = endogenous substances in serum. HPLC conditions: mobile phases, MP1 and MP2 for II; detection, UV (230 nm). For other conditions, see text.

samples with column switching monitored with detector B. No interferences were observed at the retention time of I, which demonstrated the satisfactory clean-up efficiency of the proposed method. Similar chromatograms free from interferences were also obtained for II in serum with a slight modification of the HPLC conditions (Fig. 5).

The calibration graph for I or II was obtained by analysing spiked serum samples over the drug concentration range 0.3–20 ng/ml. The least-squares regression fit showed good linearity, passing through the origin for both I and II (correlation coefficient = 0.9999). The recovery of I from spiked serum samples was 91.9% with a coefficient of variation (C.V.) of 2.9% and the recovery of II was 83.7% with C.V. 2.4% (both 5 ng/ml, $n = 5$). The detection limits for I and II were both 0.2 ng/ml in serum at a signal-to-noise ratio of 3.

DISCUSSION

It is usually difficult to develop a highly sensitive method for the determination of a compound in biological fluids because a very selective detection method is not available. We have developed a method that offers high selectivity and sensitivity for ionic compounds in biological fluids, which was applied to two basic compounds, I and II, with poorly selective short-wavelength UV detection by column switching combined with ion-pair chromatography. The proposed method eliminates interferences by column switching and also allows the rapid elution of the analyte and results in high sensitivity.

The column-switching technique, in which an effluent from one column is transferred to another column, usually having a different separation mode, is known to increase separation efficiency [16]. The elution ability of the mobile phase for column 1 is usually weaker than that for column 2 [16]. However, the difference between the two solvents usually causes large baseline disturbances shortly after column switching. This disturbance should be avoided, especially for sensitive analysis which requires fast elution of the analyte. A better baseline stability can be obtained by using the same mobile phase for both columns 1 and 2 [13], but this approach cannot be adopted because of the poor selectivity, i.e., a difference between the elution modes of two solvents is essential but the difference in composition of the two solvents should be minimal.

The elution of ionic ionic compounds can be usually delayed by ion-pair formation and in order to obtain high selectivity we considered utilizing this property. The difference between the two mobile phases used for columns 1 and 2 is only the presence or absence of an ion-pair reagent, SOS, which causes only small baseline disturbances after column switching. In column 1, the analyte is preseparated from endogenous substances in serum by ion-pair chromatography. After column switching, in column 2, the heart-cut fraction containing the analyte is further separated by non-ion-pair chromatography from coeluted endogenous substances from column 1. The existence of interferences in serum that show the same elution profile as the analyte in both ion-pair and non-ion-pair chromatography is considered to be rare. This means that the proposed mode of column switching will offer high selectivity. As predicted, no interferences were observed

at the retention time of the analyte, despite the injection of a treated sample from a large volume of serum and UV detection at 215 and 230 nm (Figs. 4 and 5). Further, this could be achieved with a very simple clean-up procedure before injection. Moreover, it is noteworthy that the analyte was eluted from column 2 faster than all other interferences from serum, which was different from conventional chromatography. This means that the elution of coeluted substances from column 1 was not or was at least less affected by the ion-pair reagent than that of the analyte. The small baseline disturbances and the faster elution of the analyte than the endogenous substances are very advantageous in improving the sensitivity by shortening the retention time of the analyte.

The proposed method, with a suitable alkyl sulphonate or sulphate as an ion-pair reagent, would offer high selectivity and sensitivity for basic compounds in biological fluids even with the injection of simply treated samples from relatively large volumes of biological fluids and a poorly selective detection method. The principle of the proposed method is applicable to both acidic and basic compounds in biological fluids with a suitable ion-pair reagent. Further, the method would have the possibility of utilizing not only UV absorbance but other sensitive detection methods even if they lack selectivity.

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