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DETERMINATION OF URAPIDIL AND ITS METABOLITES IN HUMAN SERUM AND URINE: COMPARISON OF LIQUID–LIQUID AND FULLY AUTOMATED LIQUID–SOLID EXTRACTION

KARL ZECH* and REINHARD HUBER
Research Laboratories of Byk Gulden Lomberg Chemische Fabrik GmbH, Box 6500, D-7750 Konstanz, (F.R.G.)

SUMMARY

Direct injection of biological fluids into an automated pre-column system for high-performance liquid chromatographic quantitation of urapidil and its metabolites was compared with sample preparation by liquid–liquid extraction. On-line sample purification and enrichment proved to be a superior method for quantitation of the parent compound and its metabolites in serum, plasma, and urine. The automatic procedure circumvents the disadvantages of conventional sample work-up by extraction, such as time-consuming and complicated sample preparation, the need for large samples, poor recovery, and formation of artefacts. Injection of 100 μl of serum into the automated pre-column system, followed by high-performance liquid chromatography with electrochemical detection, gave a detection limit for urapidil and metabolites in serum of 5 ng/ml. For urine samples with injection of 50 μl the detection limit for the same compounds was 100 ng/ml using UV detection, which is adequate for evaluating renal excretion. Accuracy as well as precision of the analyses were better than 10% for concentrations above 100 ng/ml. In contrast to the direct injection method, liquid–liquid extraction requires 1–2 ml of serum, plasma, or urine for clean-up. Moreover, the extraction yield for the main human metabolite is significantly lower, and its stability after extraction is inadequate for using an autosampler. The direct injection method was applied in studying the pharmacokinetics and metabolism of urapidil in healthy volunteers and patients. So far, *ca.* 15 000 urapidil samples (both serum or plasma and urine) have been analysed by this technique.

INTRODUCTION

Urapidil (Ebrantil®) (6-{3-[4-(*o*-methoxyphenyl)-1-piperazinyl]propylamino}-1,3-dimethyluracil) has been shown to be an effective, safe, and well-tolerated drug for the oral treatment of hypertension (WHO grade I and II) and the treatment of hypertensive crises by intravenous bolus injection. The semipreparative isolation and structural elucidation of urapidil metabolites by means of high-performance liquid chromatography (HPLC) and off-line mass spectrometry (MS) has been reported

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previously[1]. A comparison of metabolite patterns in serum and urine of rats, dogs, and humans and the biological activity of the metabolites *in vitro* and *in vivo* was reported in a separate paper[2], where HPLC with electrochemical detection (ED) was used for serum, whereas UV detection was applied to urine samples. Quick information on potential metabolites of urapidil in urine could be obtained by using a UV diode-array detection system[3]. In most of the previous investigations sample preparation (serum and urine) prior to analysis was carried out by using liquid–liquid extraction, which is prone to errors in quantitation of metabolites and represents the time-limiting step in the analysis of urapidil and its metabolites. Moreover, 1–2 ml of serum or urine were necessary for each single analysis.

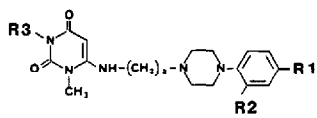
Solid-probe chemical-ionization MS and stable-isotope labelling was the first method described for quantitation of urapidil in human serum[4]. However, this procedure did not allow the determination of metabolites and required a time-consuming sample preparation, which is also the case for other HPLC methods described in literature[2,5,6]. Moreover, the sequence of sample preparation must be followed exactly, otherwise the results are not reproducible[5].

The present paper describes the direct injection of large amounts (50–200 µl) of human serum, plasma, or urine into a pre-column system for automated HPLC determination of urapidil and its metabolites. Sample preparation merely consists of centrifugation after thawing (if necessary) and addition of an internal standard for ED.

EXPERIMENTAL

Materials

Urapidil, the internal standard (6-{-[4-phenyl-1-piperazinyl]propylamino}-1,3-dimethyluracil), and the metabolites M1 (6-{3-[4-(*o*-methoxy-*p*-hydroxyphenyl-1-piperazinyl]propylamino}-1,3-dimethyluracil), M2(6-{-[4-(*o*-hydroxyphenyl)-1-piperazinyl]propylamino}-1,3-dimethyluracil), and M3 (6-{3-[4-(*o*-methoxyphenyl)-1-piperazinyl]propylamino}-1-methyluracil) (Fig. 1) were synthesized in the Research Laboratories of Byk Gulden. Methanol (Lichrosolv®), sodium perchlorate (pro analyse), perchloric acid (pro analyse) and Na_2EDTA (Titriplex® III) were purchased from Merck (Darmstadt, F.G.R.).



Name	R1	R2	R3
Metabolite M1	OH	OCH_3	CH_3
M2	H	OH	CH_3
M3	H	OCH_3	H
Urapidil	H	OCH_3	CH_3
Internal standard	H	H	CH_3

Fig. 1. Structural formulae of urapidil, metabolites and internal standard.

Standard solutions

Stock solutions (100 μg/ml) were prepared by dissolving the samples in methanol (1 ml) and making the final volume 100 ml with water. Spiked serum and urine standards were produced by diluting the stock solutions with water to 5 μg/ml for serum and 80 μg/ml for urine, followed by addition of 50 μg of each solution to 950 μg of either serum or urine. Aqueous standards were similarly prepared by using 950 μg of water instead. Na_2EDTA (0.05 mg/ml) had to be added to urine specimens to prevent degradation of urapidil and metabolite M1. Aliquots (50 μl) of the internal standard were added per 500 μl of serum or urine (standards and samples). Stock solutions were stored at 4°C, whereas spiked standards were prepared daily.

Plasma (serum) and urine specimens

Plasma or serum specimens were obtained from healthy subjects as well as from different kinds of patients (hypertensives, dialysis patients, elderly patients). Blood specimens were centrifuged after keeping them at 4°C for 30 min. Serum and plasma were stored at −18°C until analysed. Aliquots of the thoroughly mixed urine fractions were frozen and stored at −18°C until analysed.

Apparatus

The fully automated HPLC system was similar to the system described by Roth *et al.*[7]. The liquid chromatograph was a Hewlett-Packard Model 1084B or 1090, equipped with a Metrohm (Filderstadt, F.R.G.) 656/641 electrochemical detector, the cell of which was kept at 40°C by using a Haake (Karlsruhe, F.R.G.) FF2 thermostat. The working potential for the electrochemical detector was +1.0 V, the compensation current being 500 nA with a maximum voltage output of 1.0 V. As reference electrode a silver–silver chloride electrode was used.

For column switching, a six-port switching valve (Rheodyne 7010, Latek, Heidelberg) was added to the HPLC system. The six-port valve and the pre-column washing pump (Milton Roy) were controlled by the external contact board of the HP 1084 or 1090 system. Serum (plasma) or urine samples (50–200 μl, for serum typically 100 μl, compared with 10 μl used by Roth *et al.*[7]) or organic solvent extracts (10–20 μl) were introduced into the system via the automatic injection system. Samples were held in 300-μl microvials (Weidmann, Romanshorn, Switzerland).

Columns and mobile phases

Analytical columns were filled with Nucleosil® C_{18}, 5 μm (Macherey & Nagel, Dueren, F.R.G.). Other products (LiChrosorb®, Merck; Hypersil®, Shandon; Spherisorb®, Phase Separations) cause severe tailing, especially with the basic compound urapidil. Tailing can be compensated for only by high buffer concentrations (200 m*M* sodium dihydrogen phosphate, pH 2.0). However, this causes problems with continuous operation. The 125 × 4.6 mm I.D. stainless-steel column was held at 40°C, and a flow-rate of 1.0 ml/min was used. The analytical column was protected by a guard column that also contained Nucleosil C_{18}, 5 μm. Both columns (Bischoff, Leonberg, F.R.G.) were equipped with sieves. Columns were packed by a refill service (Bischoff, or Grom, Tübingen, F.R.G.).

The pre-column was a commercially available cartridge, 5 × 4 mm I.D., held in a double-cartridge holder (Bischoff). Cartridges were dry-filled with Nucleosil C_{18},

30 μm (Macherey & Nagel) or LiChroprep® C_2, 25–40 μm (Merck) and closed with sieves at both ends. It is crucially important to use sieves (althouth with an adequate filter function) instead of frits in order to avoid column blocking. This ensures undisturbed long-term operation with a large number of samples.

The mobile phase for the analytical column consisted of 15% methanol in 20 m*M* sodium perchlorate (pH 2.0, adjusted with perchloric acid). Gradient elution was started 2 min after injection, and the methanol percentage was increased by 1%/min for 25 min. At the end of each analysis, the column was washed with 90% methanol for 2 min and then re-equilibrated for 15 min under starting conditions. The pre-column was washed for 4 min with 5 m*M* sodium dihydrogen phosphate buffer (pH 7.0) at a flow-rate of 1.5 ml.

Column-switching details

After sample injection and washing of the pre-column, the switching valve was actuated and the components retained on the pre-column were transferred to the analytical column in the straight-flush mode by the gradient mobile phase. In order to ensure the complete transfer of urapidil and its metabolites, the pre-column and analytical column remained connected until the last peak had been analysed which is essential in the case of C_{18} pre-columns. More recent investigations showed that if C_2 material is used in the pre-column the transfer of compounds is much faster due to weaker retention. Therefore, it is possible to switch back the pre-column already after 2 min. The combination of C_2 and C_{18} material (pre-column and analytical column) results in a more complete removal of interfering matrix from the pre-column after injection of serum and a faster transfer of the compounds under investigation to the analytical column. Thereafter, the pre-column was switched back and equilibrated with the washing buffer for the next injection. At the same time, the analytical column was briefly washed with the organic modifier and equilibrated again at starting conditions.

Sample preparation by liquid–liquid extraction

Quantitation by direct injection of serum (plasma) or urine was compared with the classical procedure of liquid–liquid extraction for sample work-up. For the latter, a few milligrams of solid Na_2EDTA and 50 μl of the internal standard solution (c = 10.6 μg/ml) were added to 1.0 ml of the serum or plasma sample and extracted by shaking for 30 s with 2 × 5 ml of dichloromethane–2-propanol (9:1). The combined organic solvent layers were dried over anhydrous sodium sulphate and centrifuged. The organic phase was transferred to a tapered flask and evaporated to dryness. After addition of 500 μl of absolute ethanol, an aliquot of the solution was transferred to the sample vial and evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved with 30 μl of a mixture of aqueous 20 m*M* sodium perchlorate (pH 1.0) containing 0.01% Na_2EDTA with methanol (1:1); 10–20 μl were injected. Addition on Na_2EDTA improves the stability of M1, but complete stability is not achieved.

RESULTS AND DISCUSSION

Stability of compounds in serum and urine

Urapidil and its metabolites are stable in serum and plasma at room temperature for at least 24 h. However, in urine, metabolite M1 decomposes very rapidly. Addition of Na_2EDTA to urine (final concentration 50 μg/ml) immediately after thawing was shown to stabilize M1. Na_2EDTA is added together with the internal standard. The influence of Na_2EDTA on the stability of urapidil and metabolites in urine (metabolite M3 data not shown because it is stable) is demonstrated by Fig. 2.

Chromatograms

Typical chromatograms of serum samples containing urapidil and metabolites, obtained after direct injection, are shown in Fig. 3. After a washing period of 4 min, the pre-column was switched to the analytical column in the straight-flush mode. This mode seems to be superior to the back-flush mode for serum samples of patients, which often tend to produce gelatinous precipitates. In this particular case, the pre-column also serves a filter function; because it is short, the overall column efficiency does not seem to deteriorate, which might have been expected in the straight-flush mode.

Retention times recorded on the chromatogram include the washing period of the pre-column. Urapidil and its metabolites can therefore be assayed fully automatically in 17 min. The total analysis time is *ca.* 20 min, including washing of the analytical column and re-equilibration to the starting conditions.

A comparison of chromatograms of a urine sample after direct injection into

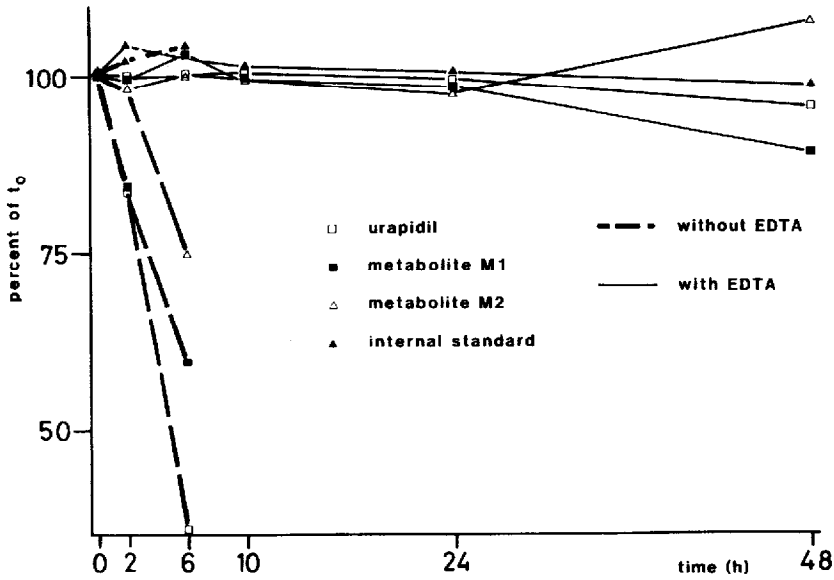


Fig. 2. Decomposition rate of urapidil and metabolites in a human urine sample (urine fraction 0–2 h after oral administration of a solution of 30 mg urapidil fumarate) with and without addition of EDTA. The sample was analysed at the indicated time while standing at room temperature. Results are given as percentage of the initial concentration (t_0).

the automated pre-column system and after injection of a smaller volume directly into the analytical column is shown in Fig. 4. Simultaneous quantitation of polar metabolites and the parent compound from Fig. 4c is not possible. Different conditions have to be used for the determination of metabolites if no pre-column system is used.

Recovery

The recovery of urapidil and its metabolites, M1 and M2, from spiked serum samples was determined by direct injection of 100 µl of serum into the pre-column system and comparison of the peak areas with those obtained after direct injection of an aqueous standard into the analytical column. The results (Table I) were nearly 100% for all three compounds. High recovery rates, even for polar metabolites, are just one of the advantages of the direct injection method[8]. The recovery of M1 tended to fall below 100% when more than *ca.* 50 samples were injected into the pre-column, but replacement of the precolumn restored the recovery to 100%. This phenomenon, which is currently under investigation, is probably caused by the effect on the pre-column of high sample loads. By comparison, when liquid–liquid extraction is used, the recovery of M1 is 73% in the presence of Na_2EDTA[5], whereas without

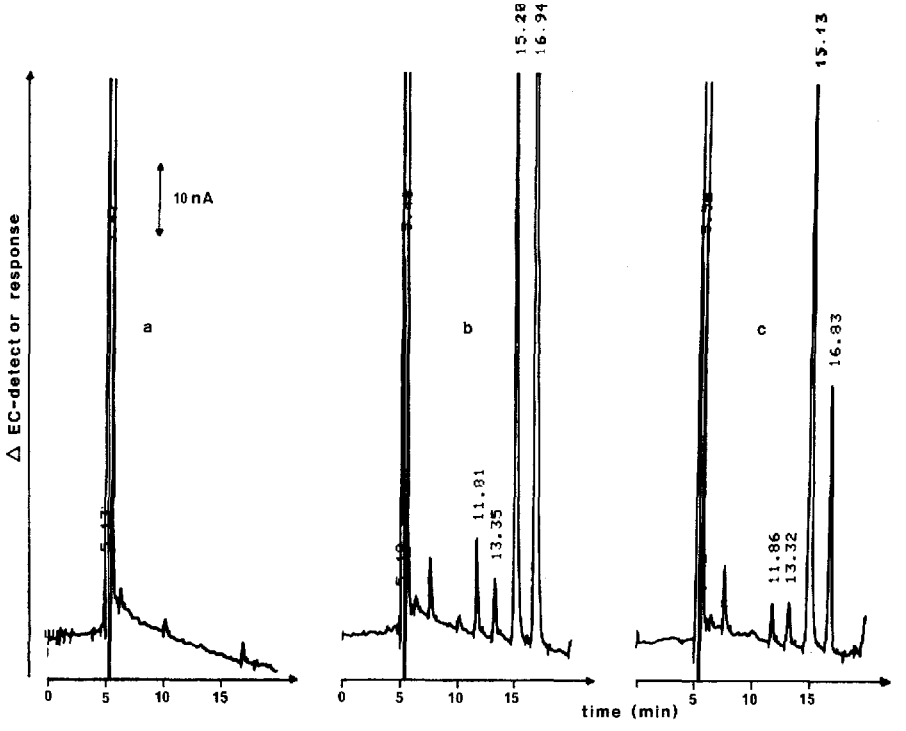


Fig. 3. Chromatograms obtained by direct injection of 100 µl of human serum before (a), 5 h after (b), and 24 h after (c) administration of two capsules (60 mg) of urapidil. Chromatogram (b) peaks: 11.81 min = M1 (58 ng/ml); 13.35 min = M2 (28 ng/ml) 15.20 min = internal standard (252 ng/ml) and 16.94 min = urapidil (713 ng/ml). Chromatogram (c) peaks: 11.86 min = M1 (22 ng/ml); 13.32 min = M2 (27 ng/ml); 15.13 min = internal standard (252 ng/ml); 16.83 min = urapidil (112 ng/ml). For conditions, see text.

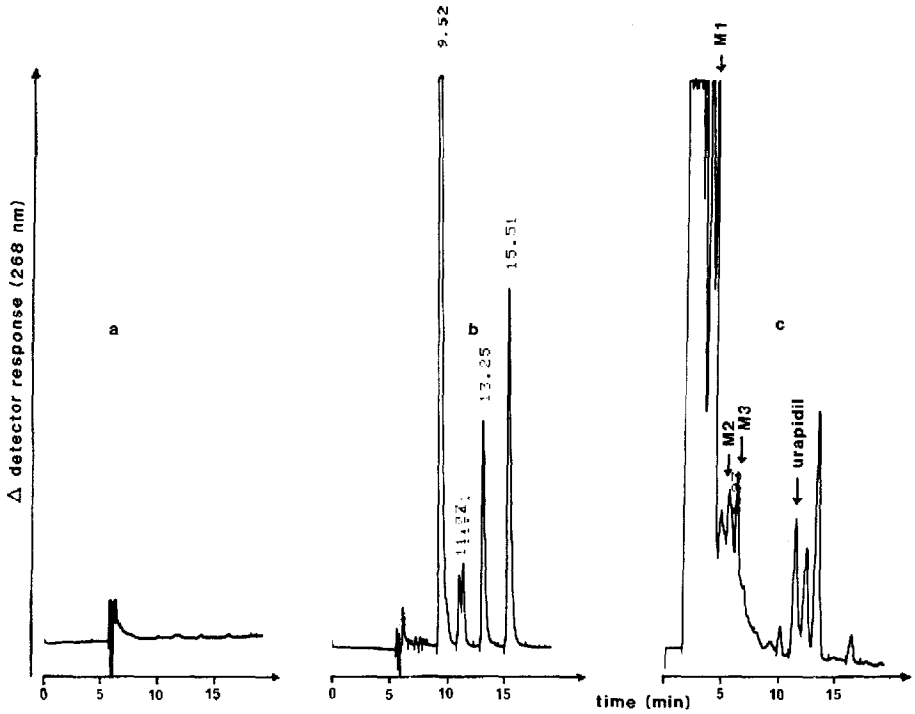


Fig. 4. Chromatograms obtained by direct injection of 50 μl of human urine before (a) and after (b) oral administration of 30 mg of urapidil solution. Urine was collected between 2 and 4 h. Chromatogram (b) peaks: 9.52 min = M1(34.7 μg/ml); 11.07 min = M2 (1.58 μg/ml); 11.41 min = M3 (1.29 μg/ml); 13.25 min = internal standard (20 μg/ml) and 15.51 min = urapidil (7.86 μg/ml). Chromatogram (c) was from the same sample as in (b), but 10 μg were directly injected into the analytical column without using pre-column clean-up and enrichment. For other conditions, see text.

TABLE I

MEAN RECOVERIES OF URAPIDIL AND METABOLITES M1 AND M2 FROM SPIKED SERUM

Values obtained after direct injection of 100 μl of serum (with pre-column), compared with an aqueous standard (without pre-column). Two samples were prepared for each concentration, each sample was determined twice.

Serum conc. (spiked samples) (ng/ml)	*Recovery (%)*		
	Urapidil	*Metabolite M1*	*Metabolite M2*
50	99.4 (1.2)*	110.5 (1.5)	97.8 (2.1)
250	95.5 (1.0)	108.5 (0.2)	105.5 (0.5)

* Standard error of the mean.

TABLE II

ACCURACY AND PRECISION OF DETERMINATION OF URAPIDIL AND METABOLITES FROM SERUM BY DIRECT INJECTION AND LIQUID–LIQUID EXTRACTION

Work-up procedure	*Amount added (ng/ml)*	*Amount found (ng/ml; mean ± S.D.)*	*Accuracy (%)*	*Precision (%)*	*No. of measurements, n*
Urapidil					
Direct injection	11	12.0 ± 3.4	9.1	28.3	7
	26	29.5 ± 5.4	13.5	18.3	8
	52	59.3 ± 7.7	14.0	13.0	8
	105	111.9 ± 10.6	6.6	9.5	8
	262	267.0 ± 16.3	1.9	6.1	8
	524	529.8 ± 23.0	1.1	4.3	8
	1048	1024.1 ± 32.0	−2.3	3.1	8
Liquid–liquid	20	22.6 ± 1.7	12.9	7.6	7
extraction	161	163.1 ± 4.6	1.3	2.8	7
	324	320.4 ± 7.9	−1.1	2.5	7
Metabolite M1					
Direct injection	26	28.0 ± 5.3	7.7	18.9	4
	52	48.2 ± 6.9	−7.3	14.3	6
	104	99.3 ± 10.0	−4.5	10.1	8
	261	269.6 ± 16.4	3.3	6.1	8
	522	549.1 ± 23.4	5.2	4.3	8
	1044	1133.5 ± 33.7	8.6	3.0	8
Liquid–liquid	14	6.1 ± 1.1	−56.1	17.4	7
extraction	117	116.1 ± 3.2	−0.7	2.8	7
	231	225.7 ± 14.1	−2.3	5.6	7
Metabolite M2:					
Direct injection	25	23.4 ± 4.8	−6.4	20.5	7
	50	48.0 ± 6.9	−4.0	14.4	8
	100	99.9 ± 10.0	−0.1	10.0	8
	250	246.0 ± 15.7	−1.6	6.4	8
	500	472.5 ± 21.7	−5.5	4.6	8
	1000	958.8 ± 31.0	−4.1	3.2	8
Metabolite M3:					
Direct injection	11	14.0 ± 4.1	27.3	29.3	4
	27	28.8 ± 4.3	6.7	14.9	4
	53	52.5 ± 4.9	−1.0	9.3	4
	107	107.3 ± 3.0	0.3	2.8	4
	266	255.0 ± 3.4	−4.1	1.3	4

Na_2EDTA only *ca.* 30% is recovered. The parent compound itself is not affected by this problem.

Accuracy and precision

The accuracy and precision of the two methods of sample preparation are compared in Table II. Accuracy, as obtained by the difference between the mean of the calculated and the given concentrations was usually better than 10% for urapidil or metabolites for both procedures, except at the lowest concentrations. The precision

TABLE III

CALIBRATION PARAMETERS FOR URAPIDIL AND METABOLITES IN SERUM AND URINE. SAMPLE WORK-UP BY DIRECT INJECTION AND BY LIQUID–LIQUID EXTRACTION

Sample and work-up procedure	*Compound*	*Slope*	*Intercept (ng/ml)*	*Correlation coefficient*	*Range (ng/ml)*	*n*
Serum; direct injection	Urapidil	0.967	7.6	0.999	11–1048	54
	Metabolite M1	1.091	−11.2	0.998	26–1044	43
	M2	0.955	1.9	0.999	25–1000	48
	M3	1.070	−7.1	0.996	11–266	20
Serum; liquid–liquid extraction	Urapidil	0.971	3.0	0.999	10–644	17
	Metabolite M1	0.918	1.0	0.997	7–468	14
Urine; direct injection	Urapidil	1.013	−77.6*	0.999	108–5400*	20
	Metabolite M1	1.009	−69.9*	0.999	104–5200*	20
	M2	1.013	−52.7*	0.999	100–5000*	20
	M3	1.007	−66.6*	0.999	101–5050*	20

* Values in mg/l, not ng/l.

of the direct injection method at concentrations above 250 ng/ml was typically better than 6%.

Linearity

Table III presents the parameters of the calibration curves of urapidil and its metabolites in serum and urine. The regression coefficients are better than 0.996 for all compounds.

Routine analysis

The present method has been used routinely for the past two years. Approximately 15 000 serum samples have been analysed without major problems. As the recovery of M1 seems to be dependent on the condition of the pre-column, every tenth sample was followed by a serum standard. This allows recalibration for M1 when its recovery falls below 100% after *ca.* 50 injections. The lifetime of the pre-column (extraction column) and of the guard column is reached after a total injection volume of serum of 15–20 ml. After replacement of the guard column (up to three times), the initial conditions of selectivity and efficiency of the analytical system are restored.

CONCLUSIONS

Direct injection of biological fluids on an automated pre-column system offers major advantages over the classical method of sample preparation by liquid–liquid extraction. The time needed for sample preparation is reduced to nearly zero, no organic solvents are required for extraction, the sample volume required for a duplicate analysis is two times 100 μl for serum, compared with at least 1 ml for liquid–liquid extraction, and unstable metabolites present no problems, as sample work-up is performed on-line. Direct injection, in combination with HPLC and ED turned out to be the method of choice for continuous unattended routine quantitation

of urapidil and metabolites in serum (plasma) or urine. Because the extraction and pre-purification of urine samples on the pre-column is very efficient, this technique may also be utilized for the semipreparative isolation of metabolites.

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