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High-performance liquid chromatographic method with electrochemical detection for the determination of idrapril, a novel angiotensin-converting enzyme inhibitor, in biological matrices

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of idrapril in human and rat plasma and urine and in rat tissue homogenates is described. The method is based on the electrochemical detection of idrapril without prior derivatization. Sample preparation simply consists in deproteinization with acetonitrile for plasma and tissue homogenates and in passage through a Sep-Pak C_{18} cartridge for urine. The limit of quantification is 12.5 ng/ml for plasma, 125 ng/g for tissues and 2.5 μg/ml for urine. The method is suitable for monitoring idrapril plasma pharmacokinetics in humans and its tissue distribution and urinary excretion in rats.

1. Introduction

Idrapril, (+)-(1*S*,2*R*)-2-{[N-(2-hydroxyamino-2-oxoethyl)-N-methylamino]carbonyl}cyclohexane-1-carboxylic acid (Fig. 1), is the prototype of a new class of angiotensin-converting enzyme (ACE) inhibitors characterized by a non-amino acid structure and by a hydroxamic group as the zinc binding function [1,2]. Idrapril calcium, a stable salt of idrapril, is currently under clinical evaluation and its pharmacokinetics in experimental animals and in man have been reported [3,4]. The method used for the determination of plasma idrapril levels in these pharmacokinetic studies is an indirect enzymatic assay based on the measurement of the degree of inhibition exerted by extracts of plasma on a standard preparation of rabbit lung ACE [3]. Similar indirect assays have often been used to measure plasma levels of ACE inhibitors because of their high sensitivity, although they are intrinsically not specific, owing to interference from any substance endowed with ACE inhibitory properties (including metabolites of the test compound). Hence they are even less

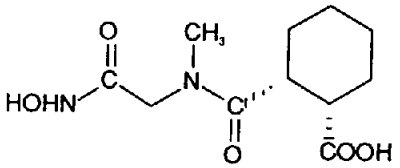

Fig. 1. Structure of idrapril.

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easily applicable to other biological fluids and tissue homogenates, where endogenous inhibitors are known to occur [5–7].

Idrapril lacks a chromophoric group suitable for sensitive UV detection, similarly to most ACE inhibitors, which can be assayed by GLC or HPLC only after appropriate derivatization [8–10]. The high hydrophilic nature and limited stability in acidic environments make idrapril unsuitable for quantitative extraction from biological matrices and ensuing chemical derivatization. However, idrapril's chemical structure, with a hydroxamic group, suggested that a convenient assay method could be developed by coupling HPLC with electrochemical detection. In fact, different groups have reported that alkyl- and arylhydroxamic acids are liable to anodic oxidation, either on glassy carbon electrodes in acetonitrile [11] or on platinum electrodes in acidic media [12], giving the corresponding carboxylic acids and nitric oxide.

In this paper this novel HPLC method is described and its suitability for the determination of idrapril in plasma and urine of rats and humans and in selected rat tissues is assessed.

2. Experimental

2.1. Chemicals

Idrapril (batch CDB178) was synthesized at Laboratori Guidotti (Pisa, Italy). All chemicals were of HPLC or analytical-reagent grade. Sep-Pak C_{18} cartridges were obtained from Waters–Millipore (Milford, MA, USA).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Model 465 autosampler (Kontron Instruments, Milan, Italy) and a Model LC-9A pump (Shimadzu, Kyoto, Japan) connected to a Supersphere 100 RP-18 reversed-phase column (25 cm × 4 mm I.D., particle size 4 μm) (Merck, Darmstadt, Germany) protected by a LiChrosphere 100 RP-18 precolumn (4 cm × 4 mm I.D., particle size 5 μm) (Merck). The detection system consisted of an ESA 5100A electrochemical detector equipped with a Model 5011 dual-electrode analytical cell. It was operated in the "screen mode" with the first (screening) electrode set at +0.4 V and the second (analysis) electrode set at +0.8 V. Signals from the detector were analysed by Class LC-10 software (Shimadzu) on a ProLinea 3/25s Compaq personal computer. The mobile phase for idrapril detection in urine and tissue samples was aqueous 0.1% H_3PO_4 (adjusted to pH 3 with 6 *M* NaOH)–acetonitrile–methanol (83:9:8, v/v/v). The flow-rate was set at 0.8 ml/min. Idrapril showed a retention time of about 19 min, but a total run time of 40 min was necessary in order to allow the complete elution of matrix components. The mobile phase for idrapril detection in plasma was 0.1% H_3PO_4 (pH 3)–methanol (76:24, v/v) for the first 14 min; after this time the methanol concentration was increased in 1 min to give a ratio of 45:55 and kept at that level for 6 min in order to speed up the elution of lower polarity plasma components and shorten the analysis time. The mobile phase composition was then programmed to return to the initial conditions and the system was allowed to re-equilibrate until the next injection. The flow-rate was constant at 0.8 ml/min throughout the analysis. Idrapril eluted with a retention time of about 17 min and each run lasted 40 min.

2.3. Plasma sample processing

Plasma samples were deproteinized with acetonitrile using different procedures depending on the concentration of idrapril to be determined. When the expected drug concentration was between 100 and 10 000 ng/ml, 50 μl of plasma were added to 250 μl of acetonitrile, vortex mixed and centrifuged at 2000 *g* for about 5 min. A 200-μl volume of supernatant was evaporated to dryness under an air stream and the residue was dissolved in 80 μl of 50 m*M* phosphate buffer (pH 7.5) just before HPLC analysis. The injection volume was 25 μl. When the expected drug concentration was below 100 ng/ml, 250 μl of plasma were added to 1 ml of acetonitrile, vortex mixed and centrifuged at 2000 *g* for about 5 min. A 1-ml volume of supernatant was evapo-

rated to dryness under an air stream, the residue was dissolved in 160 μl of phosphate buffer just before HPLC analysis and 50 μl were injected.

Calibration graphs for the two procedures were prepared by spiking drug-free plasma with 100, 250, 500, 1000, 2000 and 4000 ng/ml or with 12.5, 25, 50 and 100 ng/ml of idrapril, respectively.

2.4. Urine sample processing

A 1-ml volume of urine was loaded on to a Sep-Pak C_{18} cartridge, previously conditioned with 2 ml of methanol followed by 1.5 ml of 50 m*M* phosphate buffer (pH 7.5). The cartridge was rinsed with 2.5 ml of the phosphate buffer and idrapril was eluted with 2 ml of 50% methanol in the buffer. A 5-μl volume of the eluate was injected directly if the sample was expected to contain less than 10 μg/ml of idrapril. When the expected drug concentration was more than 10 μg/ml, the eluate from the Sep-Pak C_{18} cartridges was diluted sixfold before HPLC analysis.

Calibration graphs were prepared by spiking drug-free urine with 1.25, 2.5, 5 and 10 μg/ml or with 25, 50, 100 and 200 μg/ml of idrapril for the procedure without or with dilution, respectively.

2.5. Tissue sample processing

All rat tissues were homogenized, using a Polytron homogenizer, in nine volumes of 50 m*M* phosphate buffer (pH 7.5). A 500-μl volume of homogenate was deproteinized with 2.5 ml of acetonitrile mixed and centrifuged at 2000 *g* for about 5 min. A 2-ml volume of supernatant was evaporated to dryness under a stream of air, the residue was dissolved in 100 μl of buffer and 20 μl were injected.

Calibration graphs were prepared by spiking drug-free lung, kidney and heart homogenates with 12.5, 25, 50 and 250 ng/ml of idrapril.

2.6. Data analysis

Linear regression of the idrapril peak area values on standard concentrations was calculated with the Ultrafit program for Macintosh (Elsevier, Amsterdam, Netherlands). Data were weighed case by case in order to optimize residuals.

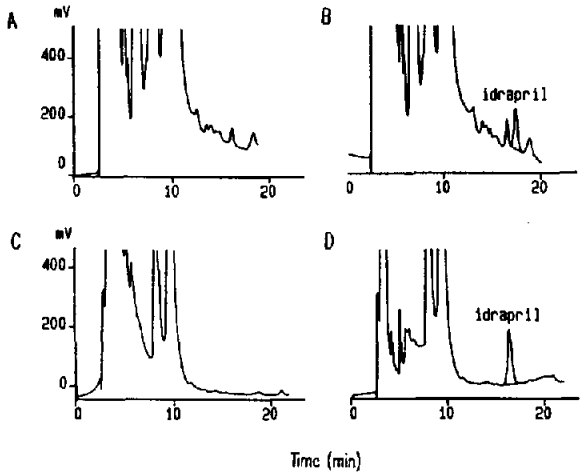

Fig. 2. Representative chromatograms of human plasma samples. (A), (B) Blank plasma before and after spiking with 100 ng/ml of idrapril, respectively; (C), (D) plasma collected from a male healthy volunteer 1 h after oral dosing with placebo and with 200 mg of idrapril calcium, respectively. Samples were processed and analysed as described in the text; 250 μl of plasma were used for (A) and (B) and 50 μl for (B) and (C).

3. Results

3.1. Detection conditions

Detector settings reported under Experimental were the result of the optimization process aimed at obtaining maximum sensitivity with sufficient selectivity. The analysis potential of +0.8 V was a compromise between the true half-wave potential for idrapril in the mobile phase (+0.95 V) and the need to minimize heavy interferences from the matrix. Similarly, the screening potential was set at +0.4 V because higher values would have affected the sensitivity (by lowering the idrapril signal) more than enhancing the selectivity (by suppressing marginally interfering peaks).

3.2. Calibration graphs in buffer

The peak areas obtained for the measurement of idrapril dissolved in buffer were linearly related to the amount of drug injected on to the column between 0 and 50 ng, while the electrochemical signal was saturated with higher amounts. Therefore, dilution of concentrated samples was necessary and more than one calibration graph was used.

3.3. Assay of plasma samples

Fig. 2 shows typical chromatograms for blank and spiked (100 ng/ml) human plasma together with those of samples obtained from a volunteer treated with placebo and 200 mg of idrapril calcium per os. An interfering compound eluting at about the same time as idrapril occurred in the blank plasma of some subjects. The peak area of this compound was never above 20% of that

Table 1
Determination of idrapril in human plasma: precision, accuracy and recovery

Idrapril concentration (ng/ml)	Peak area (mV s)				Precision (C.V., %)	Accuracy (R.E., %)	Recovery (%)
	Sample 1	Sample 2	Sample 3	Mean ± S.D.			
Intra-day determination							
Low-range graph							
12.5	287	288	185	253 ± 59	23	2	64
25	452	530	409	464 ± 61	13	−7	59
50	1135	1231	954	1107 ± 141	13	12	69
100	2655	1732	1342	1910 ± 674	35	−4	60
High-range graph							
100	656	702	620	659 ± 41	6	−2	84
250	1483	1856	1761	1700 ± 194	11	1	106
500	2862	3212	3016	3030 ± 175	6	−10	77
1000	6018	6262	7505	6595 ± 797	12	−2	84
2000	14415	13866	13572	13951 ± 428	3	4	88
4000	28986	25125	26888	27000 ± 1933	7	2	85
	Day 1	Day 2	Day 3	Mean ± S.D.			
Inter-day determination							
Low-range graph							
12.5	253	354	251	286 ± 59	21		
25	464	569	458	497 ± 62	13		
50	1107	1029	1115	1084 ± 47	4		
100	1910	2234	2680	2274 ± 387	17		
High-range graph							
100	659	508	673	613 ± 91	15		
250	1700	1496	1586	1594 ± 102	6		
500	3030	2755	3086	2957 ± 177	6		
1000	6595	5639	6250	6161 ± 484	8		
2000	13951	11322	12801	12691 ± 1318	10		
4000	27000	23549	25714	25421 ± 1744	7		

measured after spiking plasma with 12.5 ng/ml, and the limit of quantification (LOQ) in human plasma was conservatively fixed at 12.5 ng/ml. Similar results were obtained with rat plasma (not shown). Dry deproteinized plasma samples were stable for at least 3 days at room temperature if no solvent was added. However, shortly after addition of phosphate buffer a matrix-related peak, with a retention time slightly longer than that of idrapril, began to appear; after 5 h this unidentified peak was so large that it was impossible to measure idrapril accurately. To avoid this interference, the residue was dissolved in buffer just before HPLC analysis by exploiting the dilution features of the autosampler.

Calibration graphs were obtained in triplicate and the peak area was found to be linearly related ($r > 0.999$) to the concentration of idrapril, after subtraction of the mean value for the interfering peak in blank plasma. In human plasma, straight lines passing through the origin were obtained, having slopes of 6.7 ± 0.1 and 19.8 ± 0.8 (mean ± approximate standard error evaluated by the regression program) for the high and low range of standards, respectively. The calibration graphs obtained with standards in rat plasma (slopes = 3.9 ± 0.03 and 13.7 ± 0.3 for high- and low-range graphs, respectively) showed non-zero intercepts (-218 ± 129 and 23.9 ± 16.1).

In Table 1 precision, accuracy and recovery data are reported for human plasma. The intra-day precision, calculated as the coefficient of variation (C.V.), was always below 15% for points on the high-range graph, and ranged from 13 to 35% for the low-range graph. Accuracy, calculated as percentage deviation from the value computed on the regression line, varied from −3.6 to 11.7%. The recovery was $63 \pm 5\%$ for low-range graphs and $87 \pm 10\%$ for high-range graphs (mean ± S.D.). The inter-day C.V. ranged from 6 to 15% for the high-range graph and from 4 to 21% for the low-range graph. Similar values were obtained for rat plasma: the intra-day C.V. varied from 3 to 12% for high-range concentrations and from 4 to 22% for low-range concentrations, the accuracy was between −8.6 and 8.3% and the recovery was $60 \pm 4\%$ for low-range graphs and $84 \pm 10\%$ for high-range graphs.

3.4. Assay of urine samples

Fig. 3 depicts typical chromatograms of blank and spiked (5 μg/ml) human urine together with those of samples obtained from one volunteer after oral treatment with placebo or with 200 mg of idrapril calcium. As shown in Fig. 3, blank human urine may contain an interfering compound eluting at about the same time as idrapril. Depending on the subject, its peak area can vary between 5 and 50% of that measured after spiking urine with 2.5 μg/ml of idrapril, and this concentration has to be considered as the LOQ in human urine. In rat urine collected from fasted animals no interference was detected, whereas a peak similar to that observed in human samples, but more intense, appeared in urine from fed animals, so that the LOQ must be fixed at 10 μg/ml in fed rats.

Calibration graphs (triplicate samples with

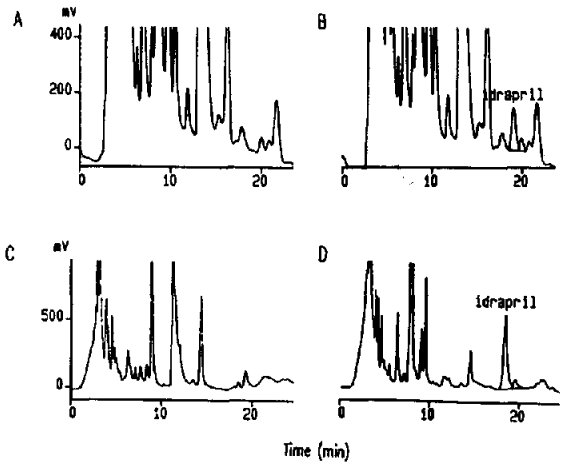

Fig. 3. Representative chromatograms of human urine samples. (A), (B) Blank urine before and after spiking with 5 μg/ml of idrapril, respectively; (C), (D) urine collected from a male healthy volunteer between 0 and 8 h after dosing with placebo and with 200 mg idrapril calcium, respectively. Samples were processed and analysed as described in the text; samples from the treated volunteer were diluted sixfold before chromatography.

human urine, duplicate samples with rat urine) showed that the peak area was linearly related ($r > 0.999$) to the concentration of idrapril after subtraction of the mean blank value. Straight lines passing through the origin were always obtained with slopes of 243 ± 2 and 1265 ± 63 for high- and low-range graphs, respectively, for human urine. For rat urine, the slopes were 398 ± 35 and 2322 ± 291 for the high- and low-range graphs, respectively.

In Table 2, precision, accuracy and recovery data are reported for standards in human urine. The intra-day precision was always below 15% for points on the high-range graphs and ranged from 12 to 25% for the low-range graphs. The accuracy varied between −2.4 and 12%. The recovery was $80 \pm 7\%$ for low-range graphs and $79 \pm 3\%$ for high-range graphs. The inter-day C.V. ranged between 3 and 33% for high-range graphs and was 17–40% for the low-range graphs. When rat urine was used, the accuracy was between −15 and +18% and the recovery was $58 \pm 17\%$ for low-range graphs and $82 \pm 11\%$ for high-range graphs; the inter-day C.V. varied between 6 and 11% for high-range graphs.

3.5. Assay of tissues samples

Fig. 4 shows typical chromatograms relating to

Table 2
Determination of idrapril in human urine: precision, accuracy and recovery

Idrapril concentration (μg/ml)	Peak area (mV s)				Precision (C.V., %)	Accuracy (R.E., %)	Recovery (%)
	Sample 1	Sample 2	Sample 3	Mean ± S.D.			
Intra-day determination							
Low-range graph							
1.25	1275	1390	993	1219 ± 204	17	12.0	71
2.5	3462	3524	2167	3051 ± 766	25	0.7	81
5	6488	6601	5185	6091 ± 787	13	6.5	87
10	13934	14867	11816	13539 ± 1564	12	−1.6	80
High-range graph							
25	5971	6420	5735	6042 ± 348	6	0.5	82
50	10839	13453	12545	12279 ± 1327	11	−1.1	76
100	25697	25555	23382	24878 ± 1298	5	−2.4	81
200	49267	47762	46725	47918 ± 1278	3	1.4	76
	Day 1	Day 2	Day 3	Mean ± S.D.			
Inter-day determination							
Low-range graph							
1.25	1219	1157	2274	1550 ± 628	40		
2.5	3051	2504	3535	3030 ± 516	17		
5	6091	5213	8492	6599 ± 1697	26		
10	13539	10810	18276	14208 ± 3778	27		
High-range graph							
25	6042	5904	6744	6230 ± 450	7		
50	12279	13349	16187	13938 ± 2020	14		
100	24878	26065	30991	27311 ± 3241	12		
200	47918	46498	49813	48076 ± 1663	3		

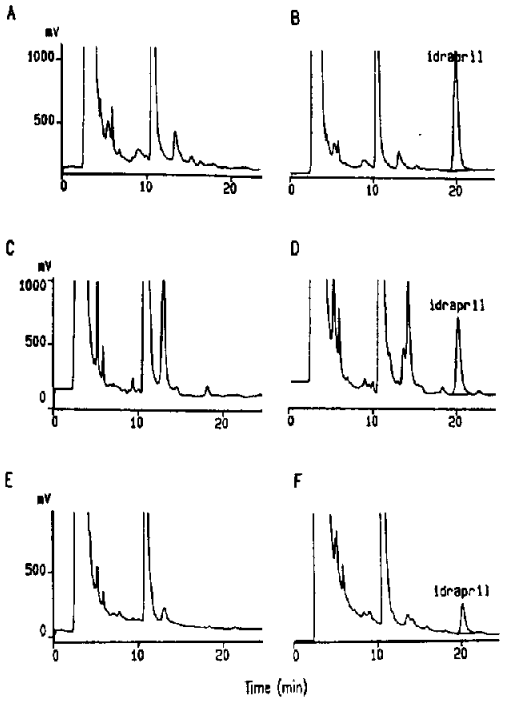

Fig. 4. Representative chromatograms of (A, B) rat kidneys, (C, D) lungs and (E, F) heart samples. Chromatograms in the left-hand panels refer to control rats and those in the right-hand panels refer to rats treated with 3 mg/kg of idrapril calcium intravenously 5 min before killing. Samples were processed and analysed as described in the text; kidneys samples were diluted 1:10 before chromatography.

kidney, lung and heart samples from control rats and from rats injected with 3 mg/kg of idrapril calcium into a tail vein 5 min before killing. When calibration graphs were constructed by adding idrapril to the three different tissues in the concentration range 0–2500 ng/g, almost identical straight lines passing through the origin with a common slope of 5.7 ± 0.3 were obtained. The minimum quantifiable concentration was 125 ng/g.

3.6. *Applicability of the method*

The present method is applicable to pharmacokinetic studies in humans, in addition to the determination of idrapril in rat body fluids and tissues. A characteristic plasma concentration

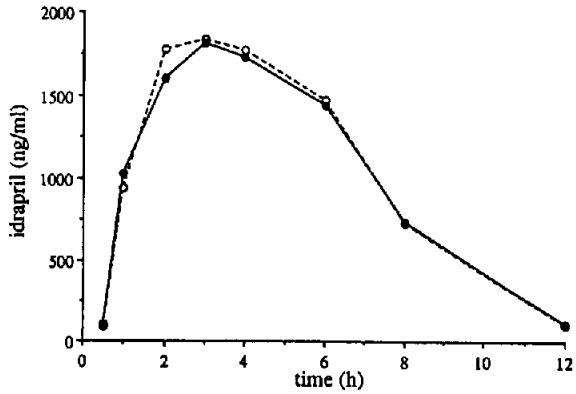

Fig. 5. Plasma kinetics of idrapril obtained with either (●) the HPLC method or (○) the indirect enzymatic assay for a male healthy volunteer administered with a single 200-mg oral dose of idrapril calcium.

versus time profile following a single 200-mg oral dose of idrapril calcium to a male volunteer is shown in Fig. 5; the plasma levels kept well above the LOQ between 0.5 and 12 h after administration. In Fig. 5 HPLC data are compared with those obtained by measuring idrapril concentration by the indirect enzymatic assay; the results are almost coincident.

Concentrations of idrapril measured in different tissues and in plasma and urine from rats treated with relatively low i.v. doses of idrapril calcium are reported in Table 3; in all instances levels 1–3 orders of magnitude higher than the LOQs were observed.

Table 3
Determination of idrapril in rat plasma, urine and tissues

Sample	*n*	Concentration
Plasma	3	13.6 ± 0.9 (μg/ml)
Kidney	4	91.5 ± 38.3 (μg/g)
Lung	4	4.2 ± 0.5 (μg/g)
Heart	4	1.5 ± 0.2 (μg/g)
Urine[a]	5	366 ± 87 (μg)

Idrapril concentration (mean ± S.D.) was measured 5 min after administration of idrapril, 3 mg/kg i.v.
[a] 0–8 h excreted amount after administration of idrapril, 2 mg/kg i.v.

4. Discussion

This paper has described a method for the assay of idrapril in biological matrices that can be conveniently used for pharmacokinetic studies in humans and for ADME (absorption, distribution, metabolism and excretion) studies in animals. Its sensitivity appears to be sufficient to follow plasma levels of idrapril after a therapeutic dose (25–200 mg) for 12 h after administration, with no need for cumbersome manual procedures for preparing samples. Even if the daily number of processable samples cannot exceed 30 per automated chromatographic unit, owing to the lengthy separation step, there appear to be real advantages over the indirect enzymatic method [3] in terms of both saving of operator time and reduced liability to experimental errors. Moreover, the HPLC method is potentially insensitive to any endogenous or idrapril-related inhibitory interference that might be present in the samples and could affect the enzymatic assay: both low-molecular-mass endogenous inhibitors and metabolites or fragments of idrapril are unlikely to co-elute with and to be as electroactive as idrapril itself. Indeed, several compounds, either synthesized as hypothetical metabolites or resulting from chemical hydrolysis of idrapril, were tested and found to show completely different chromatographic behaviour to idrapril itself (data not shown). However, a matrix-linked interference is sometimes present that determines the sensitivity limits of the method to the stated values. When high sensitivity is required, as when studying the terminal plasma half-life in human pharmacokinetics, one can rely on about tenfold more sensitive indirect enzymatic method [3]. In fact, the parallel use of both methods with the same series of samples produced evidence that no major interference affects the data obtained with the indirect method in human plasma. This is certainly not the case with urine and tissues, where low- and high-molecular-mass ACE inhibitors have been documented [5–7, and the authors' unpublished observations] and only the present HPLC method can be used.

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