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Determination of diclofenac and its metabolites in plasma and cerebrospinal fluid by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method with electrochemical detection for the quantitation of diclofenac and metabolites in plasma and cerebrospinal fluid has been developed. Pirprofen is employed as internal standard. Samples are extracted with C_{18} solid-phase extraction columns and eluted with methanol. Oxidation potentials for detection were established by constructing voltammograms for each compound. In the concentration range found in human studies, the intra-day coefficients of variation were always less than 6%. The procedure allows the simultaneous determination of diclofenac and its four major metabolites with very low detection limits (less than 1 ng/ml), which were sufficient even for kinetic studies in cerebrospinal fluid.

INTRODUCTION

The sodium salt of diclofenac, sodium-*o*-(dichlorophenyl)aminophenyl acetate (Voltaren) (D), is a potent anti-inflammatory and analgesic drug, widely employed in rheumatology [1,2]. The molecular structure contains a phenylpropionic acid bound to an aniline group.

This compound was shown to be highly metabolized in animals and humans to mono- and dihydroxylated derivatives, which are excreted in urine as their conjugates. Some of these free metabolites also elicit the activity of parent drug and attain concentrations in plasma and synovial fluid that are much higher than that of D itself several hours after administration [1,3].

A few procedures have been reported for quantitation of D and metabolites in body fluids, employing gas chromatography (GC) or high-performance liquid chromatography (HPLC) [4,5]. Schneider and Degen [4] described a GC method with electron-capture detection for the determination of D and its hydroxylated metabolites in urine. This procedure, based on extractive alkylation, provides a satisfactory separation and quantitation for all five substances in urine. However, the application of this assay to the analysis of plasma and synovial fluid samples did not afford sufficient accuracy, at concentrations below 10 ng/ml [3].

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The method reported by Godbillon *et al.* [5] can measure only D in plasma, and gives a partial separation of metabolites in urine.

Recently another HPLC procedure with UV detection for the quantitation of D and metabolites in plasma and urine, has appeared [6], but it has insufficient sensitivity for kinetic studies of D and its metabolites.

Our study had two aims. First, to find a rapid, simple, sensitive and specific assay for D and its metabolites in plasma. Second, to find a highly sensitive method for analysing these compounds at the lowest concentrations that can be expected in cerebrospinal fluid (CSF). These two purposes were completely achieved by using column extraction, HPLC separation and electrochemical detection.

EXPERIMENTAL

Materials

Diclofenac, its metabolites and pirprofen (internal standard, I.S.) were kindly supplied by Ciba-Geigy (Basel, Switzerland). The metabolites were 4′-hydroxydiclofenac (4′-OH-D), 5-hydroxydiclofenac (5-OH-D), 3′-hydroxydiclofenac (3′-OH-D), 3′-hydroxy-4′-methoxydiclofenac (3′-OH-4′-OMe-D) and 4′,5-dihydroxydiclofenac (4′,5-dOH-D). All chemicals and reagents were of analytical grade (Carlo Erba, Milan, Italy). SPE-Octadecyl (C_{18}) extraction columns were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Stock solutions of D, its metabolites and the I.S. (1 mg/ml) in methanol were prepared monthly and stored at −20°C.

Equipment and chromatographic conditions

A Series 10 Perkin-Elmer (Norwalk, CT, U.S.A.) liquid chromatograph, equipped with an LC-4B/17AT electrochemical detector (BAS, West Lafayette, IN, U.S.A.) with a glassy carbon electrode and an Ag/AgCl reference electrode were employed. A 7125 Rheodyne injection valve (Cotati, CA, U.S.A.) was used. The column was a C_8 (3 μm particle size, 8 cm × 4.6 mm I.D., Perkin-Elmer), operated at room temperature. A potential of +0.95 V was applied *versus* the reference electrode. A mobile phase containing acetonitrile–acetate buffer (35:65, v/v) was applied at a flow-rate of 1.2 ml/min. The buffer was 50 m*M* sodium acetate adjusted to pH 3.00 with H_3PO_4.

Extraction procedure

An appropriate volume of biological fluid (0.5 ml of plasma or CSF) was poured into a glass-stoppered tube with the appropriate amount of I.S. solution (see below) according to the expected concentrations. After shaking, 1.0 ml of 1 *M* phosphoric acid was added. The sample was loaded onto the SPE column, and washed twice with 1 ml of 1 *M* phosphoric acid followed by two 1-ml volumes of water. Finally the compounds were eluted with two aliquots (0.25 ml) of metha-

TABLE I

LIMIT OF DETECTION AND LINEARITY VALUES FOR DETERMINATION OF DICLOFENAC AND ITS METABOLITES IN PLASMA

In the equation $y = a + bx$, x represents the concentration (ng/ml) and y the peak-area ratios (given compound to I.S.).

Compound	a	b	r^2	Limit of detection (ng/ml)
Plasma (range 5–500 ng/ml)				
D	0.01055	0.0273	0.999	0.7
3'-OH-D	0.00620	0.0567	1.000	1
4'-OH-D	0.01487	0.0620	1.000	0.5
3'-OH-4'-OMe-D	0.0593	0.0830	0.992	1.4
4'.5-dOH-D	0.04899	0.0853	0.997	1.2
CSF (range 1–20 ng/ml)				
D	0.05806	0.05104	0.990	0.2
3'-OH-D	0.01917	0.2621	0.986	0.25
4'-OH-D	0.06791	0.1884	0.994	0.1
3'-OH-4'-OMe-D	0.008930	0.2136	0.981	0.4
4',5-dOH-D	0.06774	0.2489	0.992	0.1

nol. The collected methanol solution, containing D and its metabolites, was evaporated at room temperature under a stream of nitrogen. Dry residues were redissolved into 100 μl of the mobile phase, and 10–20 μl were injected onto the chromatograph.

Before use, the extraction columns were primed by passage of two 1-ml volumes of methanol and 1 ml of 1 *M* phosphoric acid. Plasma and CSF calibration standards were prepared for D and its metabolites according to the values given in Table I. The amount of I.S. to be added was 5 ng/ml for CSF and 100 ng/ml for plasma, according to the expected concentration range. Repeated analysis of those standards were performed to determine the recovery, precision and linearity of the assay.

The working potential of the electrode was established by constructing curves of current intensity against the applied potential, operating in the mobile phase for D, its metabolites and the I.S.

Fluid samples

Various blood and CSF samples were obtained from two patients who were given a diagnostic lumbar puncture (myelography) 2 and 12 h before an intramuscular injection of D (75 mg).

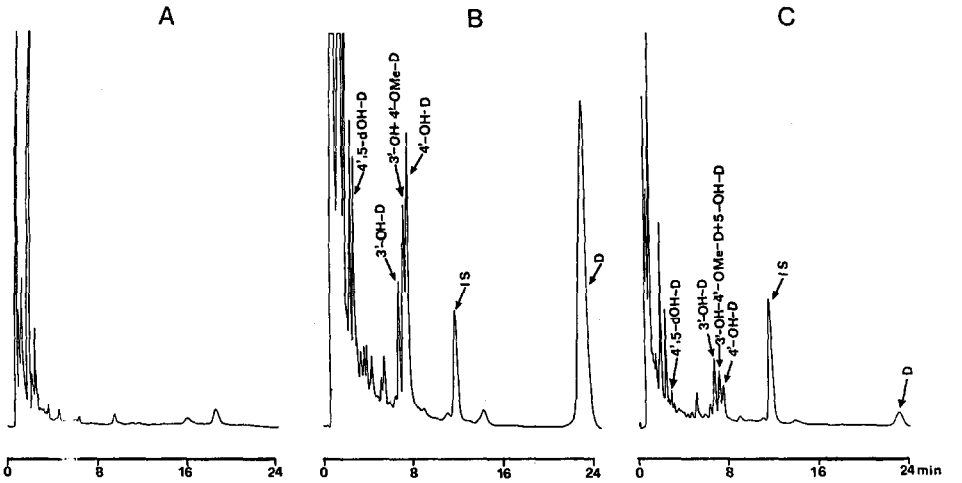

Fig. 1. Chromatograms obtained from plasma extracts. (A) Drug-free plasma; (B) standard spiked with 30 ng/ml each of 4',5-dOH-D and 3'-OH-D, 100 ng/ml each of 3'-OH-4'-OMe-D and 4'-OH-D, 300 ng/ml D and 100 ng/ml I.S.; (C) sample obtained from a patient given 75 mg of D intramuscularly after 6 h and spiked with 100 ng/ml I.S.

RESULTS AND DISCUSSION

The chromatograms obtained from drug-free extracts of plasma and CSF are given in Figs. 1A and 2A. These show no peaks interfering with any of the tested compounds. Problems occurred in the attempt to separate all D metabolites.

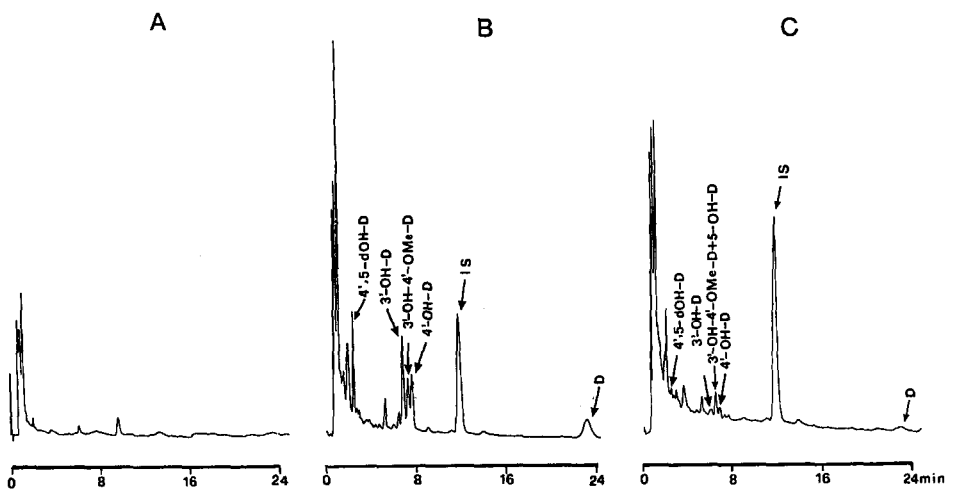

Fig. 2. Chromatograms obtained from CSF extracts. (A) Drug-free CSF; (B) standard spiked with 2 ng/ml of each metabolite, 5 ng/ml of D and I.S.; (C) sample obtained from a patient given 75 mg of D, intramuscularly after 6 h and spiked with 5 ng/ml I.S.

TABLE II

PRECISION AND INTRA-DAY COEFFICIENT OF VARIATION FOR DICLOFENAC AND ITS METABOLITES IN PLASMA CALIBRATION STANDARD

The two concentrations given for each compound are typical values used in kinetic studies. The number of determinations is 5 unless specified.

Compound	Concentration added (ng/ml)	Concentration measured (ng/ml)	Coefficient of variation (%)
D	10	10.17	4.8 (n = 6)
	150	157.83	4.5
3′-OH-D	5	4.62	5.9 (n = 4)
	60	63.58	3.8
4′-OH-D	30	31.26	4.4
	190	198.81	4.0
3′-OH-4′-OMe-D	30	28.64	4.3
	250	261.38	3.6
4′,5-dOH-D	10	11.86	4.2 (n = 4)
	40	43.94	4.1

Several mobile phases and columns were tried, and the best results are given in Figs. 1B and 2B. It is observed that 5-OH-D and 3′-OH-4′-OMe-D coelute in a single peak. From a practical point of view this is a minor disadvantage, because the plasma concentrations reported for 3′-OH-4′-OMe-D are 20–400 times higher than those for 5-OH-D, so this peak can be seen referring to the more abundant metabolite [3]. No baseline separation was obtained for 5-OH-D and 4′-OH-D, but the separation was satisfactory enough for applications of the method, even taking into account the results of accuracy tests (Tables II and III).

The acid extraction, together with the use of SPE columns, provided good sample clean-up and good recovery. The dichloromethane extraction that we adopted in previous work for D analysis [7] was unacceptable here, owing to the large solvent front overlapping the 4′,5-dOH-D peak and to peaks interfering with other metabolites. This effect was particularly serious for plasma samples.

The use of electrochemical detection provided very good sensitivity and specificity for D and its metabolites. High sensitivity was needed, particularly for CSF analysis when levels down to 0.5 ng/ml had to be measured. Specificity was important for plasma samples, when many interfering compounds can be encountered if UV detection is used [5]. The voltammograms for all compounds used were developed and are shown in Fig. 3. A single- or double-step voltammogram was obtained, according to the number of electroactive and electron-donating groups present in the molecule. Owing to the presence of hydroxy and methoxy groups the half-waves potentials of the metabolites are lower than that of D itself.

TABLE III

PRECISION AND INTRA-DAY COEFFICIENT OF VARIATION FOR DICLOFENAC AND ITS METABOLITES IN CSF CALIBRATION STANDARD

The two concentrations given for each compound are typical values used in kinetic studies. The number of determinations is 5 unless specified.

Compound	Concentration added (ng/ml)	Concentration measured (%)	Coefficient of variation (%)
D	2	2.18	4.3
	20	19.77	3.9
3'-OH-D	2	1.83	4.8 (n = 4)
	10	10.59	4.4 (n = 4)
4'-OH-D	4	3.96	3.6
	15	15.64	3.2
3'-OH-4'-OMe-D	5	4.49	4.5
	20	21.07	3.9
4',5-dOH-D	1	1.08	4.3
	5	5.27	3.8 (n = 4)

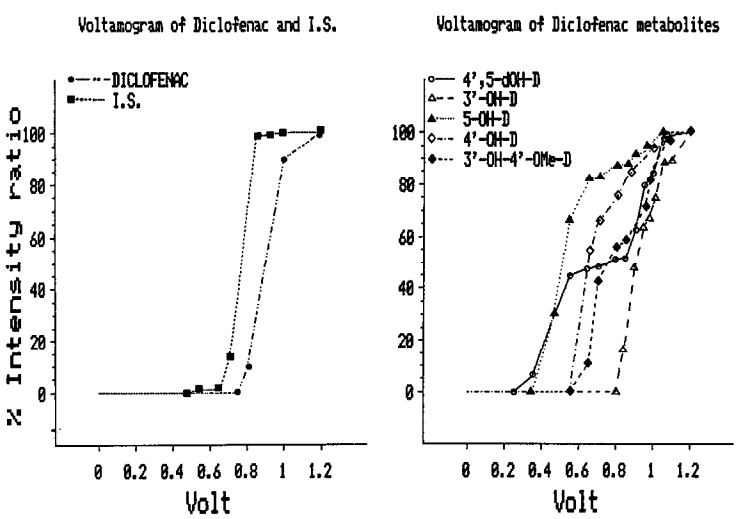

Fig. 3. (Left) Curves of the detector response *versus* applied oxidation voltage for D and I.S. in mobile phase. (Right) The same curves for metabolites of D. Response data are given as ratios of areas relative to the maximum response of each compound.

For this analysis an operating voltage of 0.95 V was adopted in an effort to optimize the signal-to-noise ratio for all the analytes. At potentials greater than 0.95 V the solvent front becomes larger and approaches the metabolite peaks; interfering peaks from the biological sample also start to appear.

Pirprofen was reliable as the I.S., as shown by the reproducibility, recovery and linearity data. This molecule has, like D, an amino group and a carboxyl group in its structure, so behaves in a similar manner to D in the extraction and separation procedures and the electrode reaction.

Tables II and III list intra-day values for precision obtained from replicated analysis of plasma samples and CSF spiked with D and its metabolites. A good reproducibility was obtained in analysing all these compounds, whose values were in any case less than 6% in the fluids tested. Indeed the highest coefficient of variation recorded was 5.9%. The sensitivity and linearity of the method are shown in Table I, and the data show a good relationship between peak-area ratios and the concentrations of all derivatives. The detection limits reported, evaluated for a signal-to-noise ratio of 2, are good enough to allow the quantitation of D and its metabolites in CSF as well as in plasma. All these are in the range 0.5–1.4 ng/ml for plasma and 0.1–0.4 ng/ml for CSF.

Recovery values, for plasma and CSF samples, are listed in Table IV.

Some of the advantages of this method here are worthy of mention. No derivatization is needed, as in the GC method previously cited [4], and the sensitivity is superior to those of all reported GC and HPLC procedures [4,5,7]. The application to the analysis of plasma and CSF samples, obtained according to the conditions previously decribed, is reported in Figs. 1 and 2. At the same sampling time the concentrations of D in CSF are 8.22% of those in plasma. This is to be expected, because this compound does not easily cross the blood–brain barrier. The metabolites, which are more polar than D, are present to an even lesser extent in CSF (4.78% for 4′-OH-D and 0.40% for 3′-OH-4′-OMe-D). Table V lists the concentrations observed for D and its metabolites in plasma and CSF samples obtained according to the conditions described above.

TABLE IV

RECOVERY VALUES OBTAINED FROM REPEATED ANALYSIS OF PLASMA AND CSF SAMPLES, COMPARED WITH PURE STANDARD

Compound	Recovery (%)	
	Plasma (n = 3)	CSF (n = 3)
D	70.8 ± 4.1	87.1 ± 3.5
3′-OH-D	71.8 ± 3.8	80.4 ± 4.1
4′-OH-D	68.4 ± 4.4	73.5 ± 3.9
3′-OH-4′-OMe-D	62.5 ± 4.0	62.1 ± 4.3
4′,5-dOH-D	61.7 ± 4.3	62.0 ± 3.8

TABLE V

CONCENTRATIONS MEASURED AT 2 AND 12 h, FOR DICLOFENAC AND ITS METABOLITES IN PLASMA AND CSF, OBSERVED IN TWO SUBJECTS WHO RECEIVED AN INTRAMUSCULAR INJECTION (75 mg) OF VOLTAREN

N.D. = non-detectable.

Sampling time (h)	Concentration (ng/ml)				
	D	3'-OH-D	4'-OH-D	3'-OH-4'-OMe-D	4',5-dOH-D
Plasma					
2	1140.9	58.4	246.2	148.7	104.1
12	103.4	64.2	156.7	310.9	N.D.
CSF					
2	3.54	N.D.	N.D.	0.62	N.D.
12	8.50	N.D.	7.47	1.26	N.D.

CONCLUSION

On the basis of all these data, this method is the best and simplest yet available, allowing simultaneous quantitation of D and its metabolites in plasma and CSF with high sensitivity and specificity. The application to the analysis of other body fluids, such as urine and synovial fluid, could also be tried.

We believe that this procedure has finally solved, in a simple and elegant manner, the old problem analysing D and its metabolites in body fluids, a problem that has been around for fifteen years of kinetic studies since the early work of Geiger *et al.* [8].

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REFERENCES

1 R. Menassé, P. R. Hedwall, J. Kraetz, C. Pericin, L. Riesterer, A. Sallmann, R. Ziel and R. Jaques, *Scand. J. Rheumatol.*, Suppl., 22 (1978) 2.
2 P. D. Fowler, M. F. Shadforth, P. R. Crook and V. A. John, *Eur. J. Clin. Pharmacol.*, 25 (1983) 389.
3 P. D. Fowler, P. T. Dawes, V. A. John and P. A. Shotton, *Eur. J. Clin. Pharmacol.*, 31 (1986) 469.
4 W. Schneider and P. H. Degen, *J. Chromatogr.*, 217 (1981) 263.
5 J. Godbillon, S. Gauron and S. P. Metayer, *J. Chromatogr.*, 338 (1985) 151.
6 D. Lansdorp, T. J. Janssen, P. J. M. Guelen and T. B. Vree, *J. Chromatogr.*, 528 (1990) 487.
7 L. Zecca and P. Ferrario, *J. Chromatogr.*, 495 (1989) 303.
8 U. P. Geiger, P. H. Degen and A. Sioufi, *J. Chromatogr.*, 111 (1975) 293.