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Electron-Capture GLC Assay of Dichlorphenamide

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Abstract □ GLC with electron-capture detection was applied to the assay of the carbonic anhydrase inhibitor dichlorphenamide and demonstrated a sensitivity of 10 ng in 0.5 ml of rabbit serum or whole aqueous humor (≃0.25 ml) from one rabbit eye. After extraction of the drug and internal standard (monochlorphenamide) from the biological fluid, these compounds were converted to their tetramethyl derivatives by a nucleophilic alkylation method. Dichlorphenamide contents of aqueous humor and serum of rabbits treated with this drug are reported.

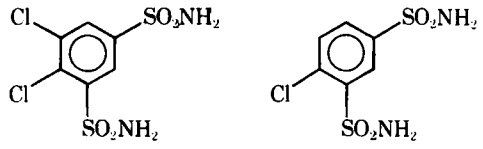
Keyphrases □ Dichlorphenamide—GLC analysis in biological fluids □ GLC—analysis, dichlorphenamide in biological fluids □ Carbonic anhydrase inhibitors—dichlorphenamide, GLC analysis in biological fluids

Dichlorphenamide (I), 4,5-dichloro-1,3-disulfamoylbenzene, is an orally effective carbonic anhydrase [carbonate dehydratase EC 4.2.1.1 (1)] inhibitor[1] (2). This drug possesses diuretic activity (3, 4) and decreases intraocular pressure in rabbits (5) and in normal (2) and glaucomatous (2, 6, 7) humans. Assay of dichlorphenamide in biological fluids, especially aqueous humor, requires a very sensitive method. GLC with electron-capture detection is useful for the determination of submicrogram quantities of compounds in small volumes of body fluids. However, direct GLC of polar compounds is usually not possible; derivatization techniques must be used to convert these compounds to less polar derivatives.

This paper describes a GLC technique combined with electron-capture detection for the assay of dichlorphenamide and its application to the determination of aqueous humor and serum drug levels following dichlorphenamide administration to rabbits by different routes.

EXPERIMENTAL

Reagents and Chemicals—The following were used: dichlorphenamide[2] (I), 4-chloro-1,3-disulfamoylbenzene[2] (II) as the internal standard, toluene[3] (pesticide quality), ethyl acetate[3] (pesticide quality), *N,N*-dimethylacetamide[4] for synthesis, acetic acid[4] for analysis, sodium bicarbonate[4], anhydrous sodium sulfate[4], 10% aqueous tetramethylammonium hydroxide[4], methanol[4], and methyl iodide[5] (purum). A half-saturated solution of sodium bicarbonate and 0.1 *N* NH_4OH were prepared with deionized water. The tetramethylammonium hydroxide was diluted fourfold with methanol.



I II

Instrumentation—The gas chromatograph[6] was equipped with a ^{63}Ni (10-mCi) electron-capture detector. A 1.5-m × 3-mm i.d. glass column containing 3% OV-17 on 100–120-mesh Gas Chrom Q[7] was used. The carrier gas was argon–methane (90:10) with a flow rate of 60 ml/min and <5 ml/min detector scavenge. Temperatures were 265° for the column and 290° for the injector and detector.

GLC–mass spectrometry of the derivative was carried out with a mass spectrometer[8] operated in the electron-impact (70 ev) mode and equipped with a 1.5-m × 3-mm column packed with 2% SE-30 on 80–100-mesh Gas Chrom Q[7] (250°).

Drug Extraction—The extraction method was based on that described by VandenHeuvel *et al.* (8) for hydrochlorothiazide in human plasma. An appropriate, known volume of aqueous humor or serum (containing less than 100 ng of dichlorphenamide) was diluted with water to 0.5 ml in a 7-ml disposable, silanized, glass-stoppered tube to which 75 ng of II in 0.1 ml of ethyl acetate (taken to dryness with a nitrogen

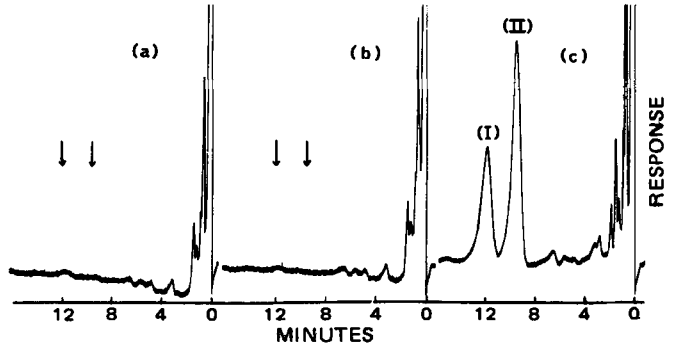


Figure 1—*Chromatograms (1 × 10^{-9} afs at 1 mv) from control aqueous humor (a), control serum (b), and control aqueous humor spiked with 50 ng of dichlorphenamide (I) and 75 ng of the internal standard (II) (c).*

[1] Daranide, Merck Sharp & Dohme.
[2] Merck Sharp & Dohme.
[3] Carlo Erba.
[4] E. Merck.
[5] Fluka.
[6] Girdel 75.
[7] Altech Associates.
[8] Finnigan 3200.

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Table I—Recovery of Dichlorphenamide from Biological Fluids

Amount Added, ng		Amount Recovered[a]								Mean ± *SD*
					From Aqueous Humor					
10	A	4.2	7.7	7.7	—	7.7	3.1	7.7	10.0	6.9 ± 2.4
	B	42	77	77	—	77	31	77	100	69 ± 24
20	A	20.5	21.7	17.00	20.5	20.5	10.00	21.7	25.1	19.6 ± 4.5
	B	103	108	85	103	103	50	108	126	98 ± 22
50	A	43.8	55.4	44.9	34.4	50.7	46.1	49.6	43.8	46.1 ± 6.2
	B	88	111	90	69	101	92	99	88	92 ± 12
100	A	101.9	104.2	71.7	77.5	93.8	84.4	85.6	79.8	87.4 ± 11.6
	B	102	104	72	77	94	84	86	80	87 ± 12
					From Serum					
10	A	6.5	10.0	4.2	5.4	6.5	5.4	8.9	8.9	7.0 ± 2.0
	B	65	100	42	54	65	54	89	89	70 ± 21
20	A	18.2	19.3	25.1	13.5	21.7	27.5	20.6	21.7	21.0 ± 4.3
	B	91	97	126	68	108	137	102	108	105 ± 21
50	A	57.7	61.2	41.4	42.6	48.4	57.7	55.4	49.6	51.8 ± 7.4
	B	115	122	82	85	97	115	111	99	103 ± 15
100	A	120.5	120.50	90.3	85.6	81.0	92.6	—	72.8	94.7 ± 18.7
	B	121	120	90	86	81	93	—	73	95 ± 19

[a] Values in the A rows represent nanograms recovered; values in the B rows represent percent recovery.

stream) had been added. The tube was agitated to ensure dissolution of the internal standard. Toluene (0.5 ml) was added to this sample, and, after agitation[9] and centrifugation, the organic phase was discarded; this step was repeated.

The remaining aqueous phase was extracted twice with 0.5 ml of ethyl acetate, and the organic phases were transferred to another tube. A 0.5-ml aliquot of 0.1 *N* NH_4OH was added to the combined ethyl acetate phases, the tube was agitated (vortex mixer) and centrifuged, and the upper organic phase was discarded. Acetic acid (50 μl) was added to the aqueous phase to give a pH between 3.5 and 4, and this solution was extracted twice with 0.5 ml of ethyl acetate. The extracts were combined and taken to dryness under a nitrogen stream.

Derivatization—Dichlorphenamide methylation was carried out using a procedure similar to that used by Least *et al.* (9) for the butylation of ethosuximide. Purification of the derivative prior to GLC analysis was carried out as described by Greeley (10). *N,N*-Dimethylacetamide (40 μl), 10 μl of methanolic tetramethylammonium hydroxide, and 10 μl of methyl iodide were added to the dry residue. The stoppered tube was allowed to stand for 10 min at room temperature, 0.5 ml of toluene was added, and the tube was agitated[9] and centrifuged.

The upper phase was transferred to another tube, and the extraction was repeated. The combined toluene phases were washed sequentially with 0.5 ml of water, 0.5 ml of bicarbonate, and 0.5 ml of water. After drying over anhydrous sodium sulfate, the organic phase was taken to dryness under a nitrogen stream. The residue was dissolved in 50 μl of toluene, and 2 μl was injected into the gas chromatograph.

Assay—Peak heights for derivatized dichlorphenamide and the internal standard were measured. A standard curve was constructed by derivatization and GLC assay of known amounts of dichlorphenamide added to 75 ng of monochlorphenamide. The ratio obtained by dividing the dichlorphenamide peak height by the internal standard peak height was plotted against the amount of dichlorphenamide used.

Known amounts of dichlorphenamide and 75 ng of the internal standard were added to either 0.2 ml of aqueous humor or 0.5 ml of serum. These spiked samples were extracted, derivatized, and analyzed to obtain standard curves from both biological fluids. Concentrations of dichlorphenamide in biological samples were determined by referring to the corresponding standard curve.

Treatment of Rabbits and Collection of Samples—Dichlorphenamide was used as either a 5% suspension in distilled water or a 5% solution of the potassium salt in saline (all concentrations are expressed as dichlorphenamide). Unanesthetized rabbits in restraining boxes were used throughout this study. Agents were administered as follows: (*a*) a 1-ml/kg dose of the suspension of dichlorphenamide or of the potassium salt solution was given by stomach tube (50 mg/kg); and (*b*) a 1-ml/kg dose of the potassium salt solution was injected intravenously into the marginal ear vein (50 mg/kg).

All rabbits were fasted for 16 hr. At the end of experiments, rabbits were anesthetized by intravenous injection of pentobarbital sodium[10], the aqueous humor of each eye was withdrawn using a needle gun (11), and ≃7 ml of blood was sampled by intracardiac puncture. Blood was allowed to clot for ≃2 hr and then centrifuged, and the serum was pipetted for the assay.

[9] Maxi Mix, Thermolyne.
[10] Nembutal, Abbott.

RESULTS AND DISCUSSION

Chromatograms resulting from assay of control aqueous humor and serum and aqueous humor spiked with dichlorphenamide and the internal standard (50 and 75 ng, respectively) are shown in Fig. 1. The retention times were ≃9 and 12 min for the tetramethyl derivatives of the internal standard and dichlorphenamide, respectively.

A linear response should be obtained with monochlorphenamide to validate its use as an internal standard. By plotting the peak height of the derivative of monochlorphenamide against the quantity of monochlorphenamide used, it was demonstrated that a linear response was obtained between 1.6 and 8 ng injected.

A calibration curve prepared by derivatization of known amounts of dichlorphenamide indicated that a linear relation existed in the 10–100-ng range between the peak height ratio of dichlorphenamide to the internal standard and the amount of derivatized dichlorphenamide. The parameters of this linear relationship, calculated according to Carstensen (12), were: slope, 0.009 ± 0.001; intercept, 0.154 ± 0.034; and coefficient of regression, 0.995. Eight values were obtained for each concentration.

Recovery was calculated by referring to the previous standard curve for samples of serum or aqueous humor spiked with known amounts of dichlorphenamide. The recoveries were 87 ± 13% (*SD*) for aqueous humor and 93 ± 16% for serum (Table I).

By plotting the peak height ratio of dichlorphenamide to the internal standard of these spiked samples against the amount of added dichlorphenamide, it was demonstrated that the relationship was statistically linear in aqueous humor ($r = 0.998$) and serum ($r = 0.997$). The standard curves thus obtained were used for assays.

The electron-impact (70 ev) mass spectrum of the derivative of dichlorphenamide was obtained by GLC–mass spectrometry. The characteristic dichloro molecular ion (*m/e* 360/362) demonstrated the formation of the tetramethyl derivative. Other dichloro ions with their characteristic pattern (13) were: ($M - SO_2NC_2H_5$) (*m/e* 253/255) and $[M - [SO_2N(CH_3)_2]_2]$ (*m/e* 144/146). The base peak (*m/e* 108) was not chlorine containing and could have been $[SO_2N(CH_3)_2]^+$. The mass spectrum of the derivatized internal standard was obtained using similar conditions. The molecular ion (*m/e* 326/328) demonstrated the formation

Table II—Dichlorphenamide Selective Ion Monitoring

Sample	Intensity Ratios *m/e* 255/253	*m/e* 362/360
Standard tetramethyldichlorphenamide	0.61	0.72
Serum spiked with dichlorphenamide	0.62	0.74
Aqueous humor spiked with dichlorphenamide	0.61 0.62	0.74 0.73

Table III—Biolevels of Dichlorphenamide after Administration to Rabbits [a]

Hours	Group A [b] Aqueous Humor Level, ng/100 mg	Group A [b] Serum Level, ng/100 μl	Group B [c] Aqueous Humor Level, ng/100 mg	Group B [c] Serum Level, ng/100 μl	Group C [d] Aqueous Humor Level, ng/100 mg	Group C [d] Serum Level, ng/100 μl
0.5	19.9 ± 31	1.8 ± 1.2	27.8 ± 34.1	468.9 ± 409.7	315.4 ± 145.0	2176.3 ± 732.8
1	14.8 ± 22	54.1 ± 8.9	117.4 ± 43.2	41.7 ± 13.2	344.3 ± 98.9	1915.7 ± 583.8
2	41.8 ± 31.5	118.3 ± 86.3	86.1 ± 58.6	460.0 ± 319.8	277.0 ± 41.7	1580.0 ± 47.2
4	41.6 ± 24.1	148.8 ± 63.9	119.8 ± 75.2	709.7 ± 408.8	192.5 ± 109.3	2370.2 ± 669.2
8	50.1 ± 35.8	164.8 ± 44.6	58.6 ± 39.2	135.6 ± 79.1	58.3 ± 29.2	— [e]
16	28.4 ± 12.1	129.8 ± 60.2	7.3 ± 8.3	31.2 ± 14.6	13.3 ± 3.7	— [e]
24	7.7 ± 5.6	9.3 ± 14	2.1 ± 2.7	24 ± 11.1	— [e]	— [e]

[a] Results are means ± *SD* (12 samples for aqueous humor and six samples for serum). [b] Rabbits received dichlorphenamide suspension orally (50 mg/kg). [c] Rabbits received dichlorphenamide potassium salt orally (50 mg/kg). [d] Rabbits received dichlorphenamide potassium salt intravenously (50 mg/kg). [e] Amount under the limit of detection.

of the tetramethyl derivative; the base peak was ($M - SO_2NC_2H_5$) (*m/e* 219/221).

To confirm that the drug is converted to the tetramethyl derivative in biological extracts at the submicrogram level, selective ion monitoring was carried out after derivatization of extracts from aqueous humor or serum spiked with dichlorphenamide. The ion intensity ratios for *m/e* 253/255 ($M - SO_2NC_2H_5$) and *m/e* 360/362 (M) from these isolates and a sample of tetramethyldichlorphenamide are presented in Table II. Clearly, the values from the isolates were the same as those from the reference standard. The ions of *m/e* 346/348 (M) of the trimethyl derivative also were monitored, but no signals were observed, demonstrating the absence of a detectable amount (1%) of this derivative in the injected sample. Unquestionably, the tetramethyl derivative was formed in each case.

The levels of dichlorphenamide in the aqueous humor and the serum of rabbits receiving this drug are reported in Table III. Usually, the sensitivity of the method was sufficient to determine levels in these two biological fluids. The solubilization of dichlorphenamide as its potassium salt tremendously enhanced the bioavailability in both fluids.

The use of an internal standard eliminated any errors in quantitation as a result of sample manipulation (14). The sensitivity of this method is such that 10 ng of drug/sample (~4 ng/100 μl of aqueous humor or 2 ng/100 μl of serum) can be quantitated.

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